INVESTIGATION OF THE SOMACLONAL AND MUTAGEN INDUCED VARIABILITY IN BARLEY BY THE APPLICATION OF PROTEIN AND DNA MARKERS

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Abstract

Barley, *Hordeum vulgare* L., is one of the most important crop species for Bulgaria. The characterisation of the genetic pool is of great necessity for the Bulgarian barley breeding programme which is directed toward improving quantitative and qualitative traits.

The regenerants from four Bulgarian varieties Ruen, Jubiley, Obzor (two-rowed, brewery) and Karnobat (six-rowed, forage) have been obtained by using a previously developed regeneration procedure [1]. The mutagenic agents, gamma rays and NaN_3, were applied to the initial material from cultivar Ruen. The influence of the mutagens on callus formation and plant regeneration was observed. A dose of 200 Gy was considered as stimulating regeneration. The mutation frequency increased but the spectrum of genetic changes was similar to the control. In order to assess genetic variability among the regenerated plants, multiple analytic tools have been employed. Field evaluation of the individual regenerants and those derived after mutagen treatment was carried out. A number of plants with valuable agronomic performance traits have been selected.

Molecular markers [protein, restriction fragment length polymorphisms (RFLP) and randomly amplified polymorphic DNA (RAPD)] have been applied to characterise the Bulgarian barley cultivars and their regenerants. The changes in DNA loci coding for 26S, 5.8S and 18S rRNA repeats, C hordein locus and mitochondrial DNA organisation have been investigated. The potential for ribosomal DNA length polymorphism in Bulgarian barley cultivars appear to be limited to three different repeat lengths (10.2, 9.5 and 9.0kb) and three plant rDNA phenotypes. Polymorphism was not observed in ribosomal DNA repeat units in somaclonal variants. Variation concerning C hordein electrophoretic pattern was observed in one line from cultivar Jubiley. Analysis of the HorI locus reveals RFLPs in sequences coding for C hordeins in this line. Mitochondrial molecular markers are convenient for detection of DNA polymorphisms in the variant germplasm as well as for the somaclonal variants derived from it. Two lines from Ruen revealed polymorphic bands after hybridisation with mitochondrial DNA probe. RAPD assays have been carried out by using 20 different 10-mer primers. Heritable polymorphism in several tissue culture derived (TCD) lines was observed. RAPD assay is a sensitive and representative approach to distinguish the variability created by tissue culture and mutagenesis.

1. INTRODUCTION

Plant tissue culture instability has been documented in several cereal plant species. Somaclonal variation has been studied intensively also in barley [2-5]. The results obtained till now are contradictory. No significant morphological variation was detected in tested tissue culture derived (TCD) lines [6] or only small variation was observed when TCD lines from four barley cultivars were tested in three locations [7]. On the other hand, in a study of 18 cultivars and breeding lines morphological variation was observed [8].

Mutagen treatment of *in vitro* plants increases variability in cereals [9, 10]. The application of mutagens (gamma rays and NaN_3) influences callus formation and the rate of regeneration [Karadimova, personal communication].

Molecular markers [isozymes, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD)] have been applied to study the genetic changes in nuclear, mitochondrial and chloroplast genomes. Molecular studies on somaclonal variation in wheat and barley [2, 3] have demonstrated that the nucleolus organizer (NOR) loci, coding for rRNA and the electrophoretic pattern of storage proteins could be useful for assessing somaclonal variation.

The plant mitochondrial genome has been proven to be a proper model for investigation of somaclonal variation. Mitochondrial DNA rearrangements in regenerated wheat plants has been reported [11]. A relatively high genetic stability was observed among the 50 regenerated *H. marinum* plants tested [4].

The RAPD technique utilises arbitrary 10 -mer oligonucleotide sequences as primers [12]. Primers hybridise to two nearby sites in the template DNA that are complementary to the primer sequence. Deletions or insertions in the amplified regions or base changes altering primer binding sites will result in polymorphisms. RAPD markers have been applied widely for cultivar identification in barley *H. spontaneum* [13] and for analysing the genetic stability of tissue - cultured plants [14].

In this study we assessed the induced variability in barley tissue culture derived lines and those derived from *in vitro* mutagenesis. The aim was to distinguish reproducibly the level of variation between the donor material and the induced genetic changes at the level of regenerant progeny by utilisation of protein, RFLP and RAPD methods.

2. MATERIALS AND METHODS

2.1. Plant material

The regenerants from four Bulgarian varieties Ruen, Jubiley, Obzor (two-rowed, brewery) and Karnobat (six-rowed, forage) have been obtained by using a previously developed regeneration procedure [1]. Mutagenic agents, gamma rays (100 and 200Gy) and NaN_3 ($x_1 = 5 \times 10^{-4}$M and $x_2 = 5 \times 10^{-3}$M) were applied to the initial seed material from cultivar (cv.) Ruen. Vigorous, green R_0 regenerants were propagated and advanced to the R_5 generation. Plants were evaluated in R_3-R_5 generations. A number of TCD lines originating from individual regenerants have been evaluated for their agronomically valuable characteristics:12 lines from cv. Karnobat, 21 lines from cv. Jubiley, 21 lines from cv. Ruen and 5 lines from cv. Obzor. The following number of plants were evaluated in the *in vitro* mutagenesis experiment: regenerants (R)-25; regenerants after treatment with 100 Gy (R-10)-36; regenerants after treatment 200 Gy (R-20)-30; regenerants after treatment with NaN_3x_1 concentration (R-x_1)-29; and regenerants treated with NaN_3x_2 concentration (R-x_2)-26.

2.2. Hordein assay

Single seeds (20) from ten independent spikes per line were used for hordein extraction. Fractionation of hordeins was achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [15].

2.3. DNA extraction, endonuclease digestion and Southern blot hybridisation

DNA was extracted according to Saghai-Maroof et al. [16], with some modifications. The DNA was digested with the following restriction enzymes: *Eco*RI, *Eco*RI and *Bam*HI, *Sac*I, *Taq*I, and *Hind*III.

2.4. RAPD assay

Twenty different 10-mer primers (kit A, OPERON) were used for the amplification of genomic DNA from 23 plants which originated from single seeds of each line. PCRs were carried out in a 20µl reaction solution containing 15 ng of genomic DNA template, 7.5 pmol of each primer, 200mM of each of dATP, dCTP, dGTP and dTTP;1x PCR buffer (Pharmacia) and 0.8 units Taq DNA polymerase (Boehringer Mannheim). The amplification was performed in a Hybaid Omnigene Thermal Cycler.

The PCR conditions for RAPD analysis were as follows: for pre-denaturation 1 min at 94°C followed by 35 cycles of polymerisation reaction each consisting of a denaturation step for 5 sec at 94°C, an annealing step for 30 sec at 36°C and an extension step for 1 min at 72°C. The last cycle was followed by a pause of 10 min at 72°C to ensure that primer extension reaction proceeded to completion. Amplified DNA fragments were run in 2.0% agarose gels (in 1xTAE running buffer). Each gel was stained with ethidium bromide (0.5 mg/ml), visualised under illumination with UV light and photographed.

3. RESULTS

3.1. Field evaluation

Most of the observed morphological abnormalities in the primary regenerants are likely to be epigenetic and disappear in the progeny generations. Sterile, chlorotic and very weak plants were eliminated in the R_0 generation. Fasciation, shortened internodes, and seed malformations were already reported [1]. Plants were advanced and propagated to R_3 generation. A range of deviations has been observed. They concern agronomically important traits such as: grain yield, lodging, height, days to heading, percentage of plump kernels, test weight and cold tolerance.

TABLE I. CLONES USED FOR ASSESSMENT OF THE NUCLEAR AND MITOCHONDRIAL GENOME ORGANISATION OF *H. vulgare* REGENERATED PLANTS

Probe	Plasmid designation	Contents of plasmid	Vector	Cloning site	Sequence origin
Nuclear	pTA71	18S, 5.8S and 26S and nontranscribed spacer of rDNA	pUC18	EcoRI	wheat
	pTA 630	5S rRNA	pBR322	BamHI	wheat
	pCp387	cDNA of C hordein	pUC18	HindIII	barley
Mitochondrial	ATP A	subunit A of ATPase	pBN121	HindIII	sunflower

The variation between TCD lines is significant and strongly genotype dependent. Final evaluation shows that three TCD lines from Jubiley have increased grain yield, one possesses a shorter stem and one heads two days earlier. Four TCD lines from cv. Ruen show better yield, one is earlier, one has a shorter stem and the other is taller (Table II).

TABLE II. FIELD TESTING OF SC_3 PROGENY OF SELECTED BARLEY TISSUE CULTURE DERIVED LINES

Cultivar	Selected lines No.	Grain yield, t/ha	Heading date	Height cm.	Protein content mg/g	Weight /1000 g	Plump kernels %
Ruen	Control	6.51	13.05	85	12.80	46.0	85.6
	19	6.28	11.05	80	12.62	48.5**	93.0***
	34	6.95**	09.05	80	12.88	45.0	89.5**
	78	7.01	08.05	78	11.93*	40.5	82.6
	103	7.23***	10.04	75***	13.30	45.5	90.9***
	81	6.39*	12.05	75***	13.38	46	87.5*
	89	6.81*	13.05	70*	12.31	46.5	88.6**
Obzor	Control	6.92	16.05	75	12.30	38.5	77.8
	309	6.30	13.05	95***	12.50	40.3*	82.8**
	313	6.80	15.05	98***	12.88	39.8	80.5**
Karnobat	Control	6.72	06.05	104	12.30	33.4	
	39	6.11	08.05	96	13.40	35.0	
	44	6.05	07.05	98	13.50	36.0**	
Jubiley	Control	5.30	08.05	100	12.50	50.0	80.2
	60	5.77**	05.05	94	11.40	52.0*	85.4***
	70	5.90**	08.08	104	12.50	54.0*	91.2***
	57	5.95***	04.05	95	10.30*	52.0*	83.2**

*, **, *** = significant at the 0.05, 0.01 and 0.001 levels, respectively.

3.2. Analysis of ribosomal DNA spacer length polymorphism

The *Eco*RI restriction enzyme defines two main rDNA repeat unit lengths, 9.0 and 10.2 kb, detected in the both control material (cv. Ruen, cv. Jubiley) and their TCD lines and 9.5 and 10.2 kb in cv. Obzor, cv. Karnobat and their TCD lines (Fig. 1).

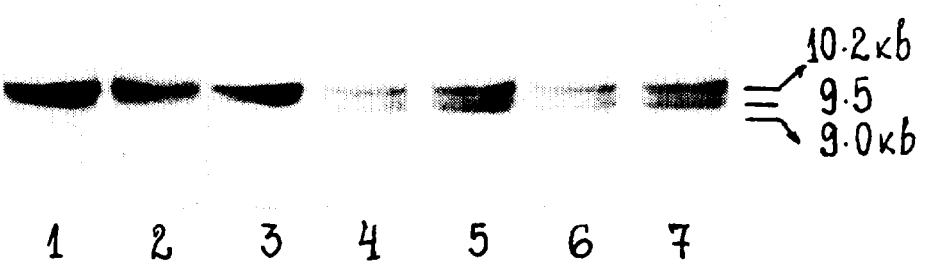

Fig. 1. Southern blot hybridisation analysis of EcoRI digested barley genomic DNA with probe pTA 71. Lane 1 - cv. Obsor; lane 2 - cv. Karnobat; lane 3 - som. 44; lane 4 - cv. Ruen; lane 5 - som. 89; lane 6 - cv. Jubiley; lane 7 - som. 70.

Double *Eco*RI-*Bam*HI digestion of barley DNA results in a range of fragments hybridising with clone pTA 71. No variation in the fragment length or relative intensity of hybridisation bands of *Bam*HI sites in rDNA units was observed among the regenerated plants from cv. Karnobat, cv. Jubiley, cv. Ruen and cv. Obzor (Fig. 2).

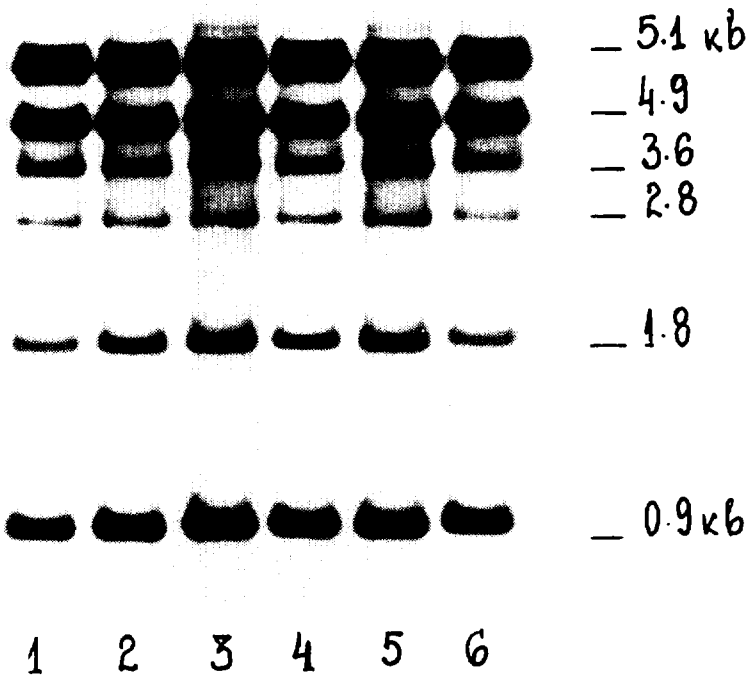

Fig. 2. EcoRI-BamHI pattern of genomic DNA hybridised with 9.0 kb. fragment of clone pTA 71. Line 1 - cv. Obzor; line 2 - som. 309; line 3 - som. 313; line 4 - cv. Karnobat; line 5 - som. 44; line 6 - som. 39.

In vitro mutagen treatment influences the efficiency of callus formation and plant regeneration (data not shown). Mutation frequency was increased but the spectrum of genetic changes was similar to the control (data not shown). Several mutant lines have been selected in R_3 progeny, showing valuable agronomic performance (Table III).

TABLE III. SELECTED LINES AFTER TISSUE CULTURE AND MUTAGEN TREATMENT

Selected lines	Stem height cm	Tillers No.	Internode No.	Spike length cm	Seed number/ spike	Seed weight/ spike	Yield t/ha
Control	98	15.0	4.8	8.9	23.0	1.19	5.86
126 R	95.7	19.0	5.6	9.8	26.2	1.29	5.80
157 R	93.5	18.5	5.9*	10.3**	25.9	1.68**	6.10
139-R-10	90.0**	30.0***	5.5	10.9**	30.9**	1.71***	6.40
152-R-20	92.0*	19.8	5.6	9.8	25.8	1.76***	5.86
153-R-20	90.0**	25.5**	5.7*	9.0	25.2	1.49	5.89
145-R-20	94.0	30.0***	5.5	10.9**	26.3	1.48	5.73
12-5-(-X1	87,0***	12.3	5.1	10.1**	26.0	1.5	6.90
166-2-R-x1	90.0**	19.75	5.2	10.4**	27.7	1.31	5.53
307-R-x2	92.0*	26.9**	5.3	9.8	26.8	1.5	6.70

*, **, *** = significant at the 0.05, 0.01 and 0.001 levels, respectively.

3.3. Eletrophoretic pattern of storage protein by SDS-PAGE and organisation of C hordein coding sequences

The tested B C hordein patterns of somaclones originating from cvs. Ruen, Karnobat and Obzor were similar to those shown by the donor plants. Additional bands in C and B hordein patterns, respectively, in clones 70-3 and 70-4 derived from cv. Jubiley were observed.

The rearrangements in gene sequences coding for C hordeins have been analysed by using DNA probe pCp387. Uniformity in hybridisation of *Hind*III DNA patterns of all investigated TCD plants, except plants showing polymorphism at the protein level, was obtained. (Fig. 3). An additional band (2.8kb) was detected in comparison with cv. Jubiley's profile. The results obtained imply that the polymorphism in C hordein pattern in the investigated plants of TCD line 70R (cv. Jubiley) is probably due to alteration in the gene sequences coding for C hordein.

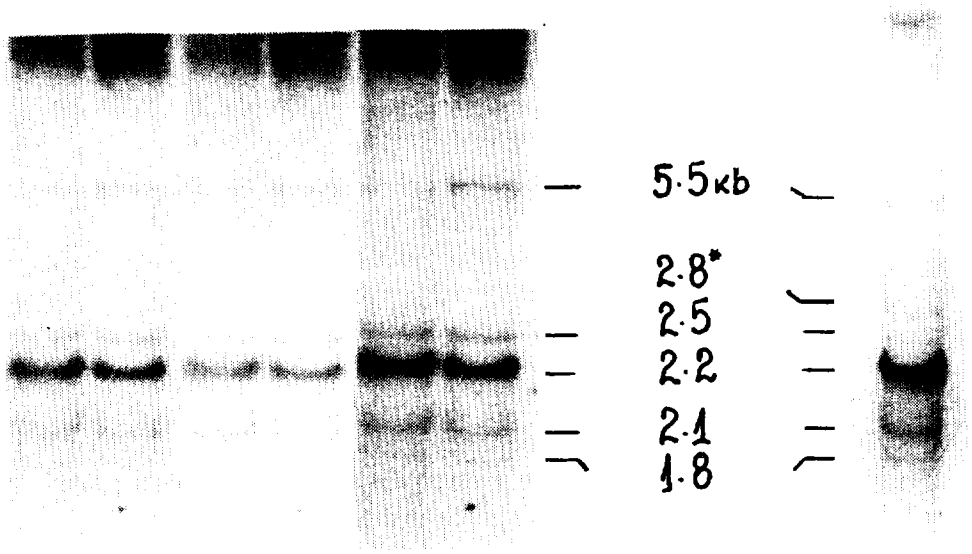

Fig. 3. *Southern blot hybridisation analysis of HindIII digested DNA probed with C hordein Coding sequences (pCp387). Lines 1,2 - som. 57; lines 3,4 - cv. Jubiley; lines 5,6 - som. 70-1, som. 70-2; line 7 - som. 70-3.*

3.4. Mitochondrial DNA organisation

The mitochondrial coding sequences among cereals are highly conserved. Therefore we have used the sunflower mitochondrial gene coding for the α subunit of the ATP-ase complex as a heterologous probe to characterise the organisation of some mtDNA sequences on Southern blots of total DNA. This allows screening of regenerated plants without the need to purify mitochondrial DNA. Hybridisation of *Hind*III digested DNA with the sunflower ATP-α probe revealed two main (12kb and 9.7kb) bands and a few fragments with low intensity in both the cultivars and somaclones. Variant bands (5.1kb, 4.6kb, and 3.2kb) with low intensity were obtained only in *Hind*III DNA patterns of somaclones 81 and 89 of cv. Ruen (Fig. 4).

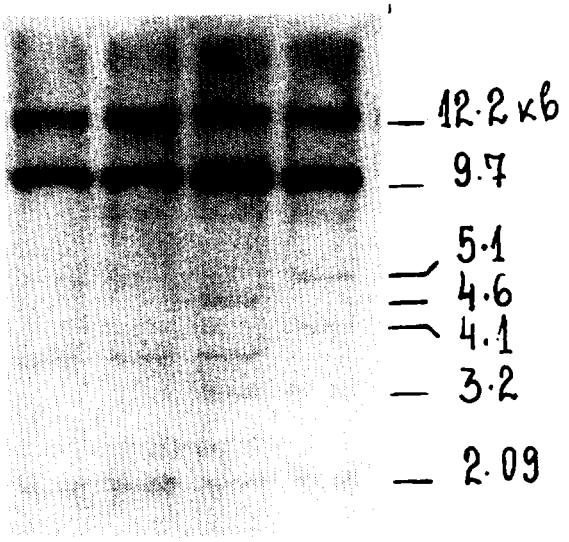

Fig. 4. Southern blot hybridisation analysis of HindIII digested barley genomic DNA with mitochondrial probe ATP-A. Lane 1 - cv. Ruen; lane 2 - som. 78; lane 3 - som. 81; lane 4 - som. 89.

3.5. RAPD analysis of tissue culture derived lines

A few primers providing DNA polymorphism in somaclones were selected. The amplification was repeated twice by using the DNA templates from the same lines of the next generation (RC_5) in order to investigate the reproducibility of the generated polymorphisms in RAPD profiles of these somaclones. Our study shows that primers OPA 08 and OPA 17 produce reproducible polymorphic bands in RAPD profiles (Figs 5 and 6).

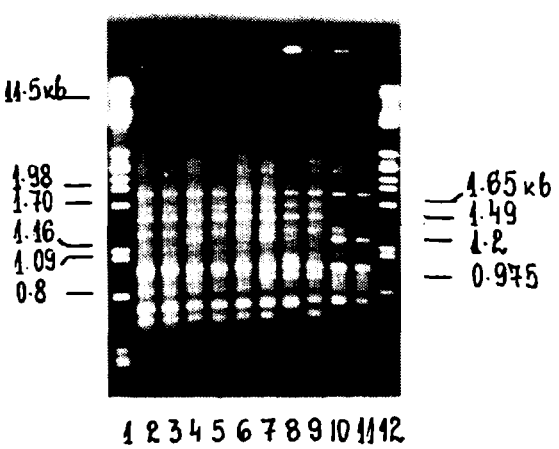

Fig. 5. RAPD patterns generated with primer OPA 08. Lane 1 - marker λ^{PstI}; lane 2 - cv. Ruen; lane 3 - som. 103; lane 4 - som. 78; lane 5 - som. 34; lane 6 - som. 19; lane 7 - cv. Obzor; lane 8 - cv. 468; lane 9 - cv. Kamobat; lane 10 - som. 44; lane 11 - som. 39; lane 12 - marker λ^{PstI}.

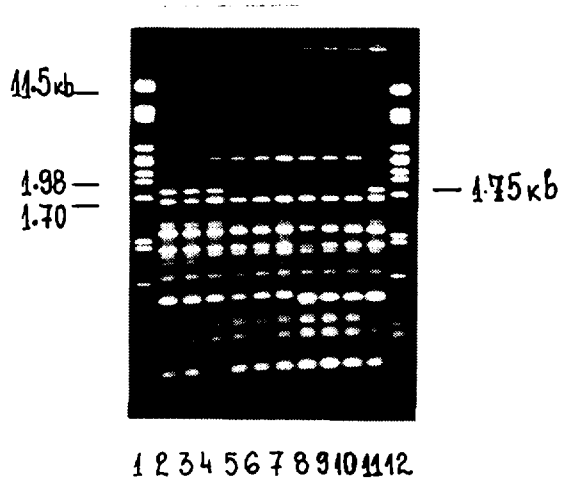

Fig. 6. RAPD patterns generated with primer OPA 17. Lane 1 - marker λ^{PstI}; lane 2 - cv. Ruen; lane 3 - som. 103; lane 4 - som. 78; lane 5 - som. 34; lane 6 - som. 19; lane 7 - cv. Obzor; lane 8 - cv. 468; lane 9 - cv. Karnobat; lane 10 - som. 44; lane 11 - som. 39; lane 12 - marker λ^{PstI}.

Somaclones 39 and 44 derived from cv. Karnobat are characterised with an additional band (1.2 kb) and three missing fragments (1.65 kb, 1.49 kb, and 0.975 kb) in RAPD profiles generated with OPA O8 when compared to the control material. Two additional bands (1.2 kb and 0.975 kb) were observed in somaclone 78 comparing to cv. Ruen and the other somaclones 103, 34, 19.

Primer OPA 17 produced polymorphism in somaclones 34 and 19 (a 1.75 kb fragment) which is not observed in the other two somaclones 103 and 78. An additional band with the same length was present in the RAPD pattern of somaclone 39 in comparison to the control (cv. Karnobat).

Few out of 10 tested primers (OPERON KitA) showed polymorphism in RAPD profiles in several mutant lines. Lines 95 (*in vivo* NaN_3 10^{-3}M) and 307 (*in vitro* NaN_3 10^{-3}M) reveal the absence of DNA fragments in RAPD profiles generated with primer OPA 01 in comparison to the control material and other mutant lines (Fig. 7).

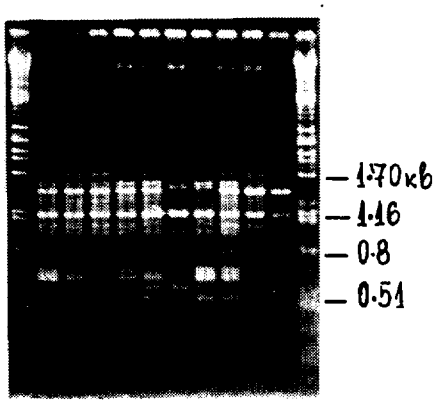

Fig. 7. RAPD patterns generated with primer OPA 01. Line 1 - marker λ^{PstI}; line 2 - cv. Ruen; line 3 - som. 90 (in vivo 10 kRad); line 4 - som. 139 (in vitro 10 kRad); line 5 - som. 78 (in vivo 20 kRad); line 6 - som. 152 (in vitro 20 kRad); line 7 - 87 (in vivo NaN3 105); line 8 -12-5 (in vitro Na N3 105); line 9 - 215 (in vivo NaN3 103); line 11 - 307 (in vitro Na N3 103); line 12 - marker λ^{PstI}.

4. DISCUSSION

The presented data suggest that *in vitro* cultivation induces variability and that the majority of barley plants recovered by callus culture possess both negative and positive changes. While a lower degree of heritable variation among regenerants was reported by Karp et al. [17] our results confirm previous findings in barley [5, 8]. The application of mutagenic agents affects the rate of regeneration. A stimulating effect of 200 Gy gamma ray irradiation was observed. Gross genetic changes were not found in the regenerants. Probably a strong diplontic selection takes place during plant regeneration.

Field tests show that some of the deviations disappear in later progenies. The yield differs over years and it is difficult to use it as a test for detecting somaclonal variation. Probably *in vitro* regeneration influences some characteristics associated with stress factors which are reflected in productivity. Somaclonal variation is not the result of gross genetic changes and field evaluation is not precise enough to detect deviations.

Molecular markers have been used for the estimation of genetic variability induced after regeneration via callus culture in barley [3, 4]. Our data suggest that by using clone pTA71 carrying genes for 26S, 18S and 5.8S rRNA any qualitative or quantitative differences were not detected in the non-transcribed spacer of rDNA of parental and regenerated barley plants. Similar results were reported for wheat by Rode et al. [18]. The results demonstrate relatively high genetic stability in respect to highly repetitive domain of the ribosomal genes.

The *in vitro* culture manipulations may induce qualitative and quantitative variation in the mitochondrial DNA of calli and cell suspensions as described by Shirzadegan et al. [19]. In our investigation two TCD lines had some changes in mtDNA. Those variations in hybridisation patterns could be attributed to somaclonal variation. It is assumed that the alterations occurred in noncoding sequences of the mitochondrial genome [4] and the rearrangements which occur in barley mitochondrial DNA during the tissue culture are rarely transferred to the progenies of the regenerants [3]. In our case the variation was detected in RC_3 progeny which indicates the stable heritable nature of the changes in the barley genome as a result of tissue culture regeneration.

RFLP analysis of the Hor1 locus was performed as a complement to the protein analysis. The observed differences in mobility and number of fragments corresponding to C and B hordeins in some somaclones were not dependent on the changes in the organisation of the coding sequences [2]. Breiman et al. [3] interpreted the lack of relationship between protein and RFLP as a result of post-transcriptional events affecting the level of expression of hordeins or of the rearrangements occurring outside the coding sequences. In our case the observed variation in C hordein SDS-PAGE patterns in one TCD line of cv. Jubiley was confirmed by using RFLP analysis.

Recently, Song and Henry [13] applied RAPD markers to assess the genetic variation within and between wild barley populations (*H. spontaneum*). To our knowledge, RAPD analysis has not yet been reported for the detection of somaclonal variation in barley. We succeeded in finding several polymorphisms in TCD barley lines by using 10-mer primers. They were stable and were inherited in the progeny.

In our studies, 15 families out of 64 TCD lines have been analysed by molecular markers for differentiating the genetic variation induced by tissue culture. Selection of the lines was made on the basis of field evaluation. This could be one of the reasons for finding

only limited variation on the basis of DNA analyses. The second reason could be that the number of the molecular markers used may not be sufficient to screen for somaclonal variation unless sufficient probes are used to saturate the entire genome [20].

The results described here show that somaclonal variation is induced by tissue culture in barley by using a particular regeneration procedure (mutagenic effect of 2,4D - data not shown) and genotypes which may affect the genetic stability in both positive and negative directions. The selected TCD lines show valuable agronomic changes. Tissue culture may induce rearrangements in any part of the entire plant genome. In this respect RAPD markers showing heritable polymorphism are more representative than the limited number of the applied RFLP probes use for detection of somaclonal variation in barley.

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