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Molecular analysis of Bulgarian common bean genotypes and their characterization after growing under rainfed and irrigated conditions

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Abstract
Experiments were conducted in the field of Agricultural University in Plovdiv, Bulgaria. A standard method for cultivation in 5 replicates was applied. Biometric evaluation of common beans (*Phaseolus vulgaris* L.) - 10 mutant lines and 10 varieties, grown under rainfed and irrigated conditions was conducted. Main traits, associated with productivity in common beans: plant height, mass of plants with pods, number of branches, height of betting on the first pod, number of fruit branches, number of pods per plant, weight of pods with seeds, number of seeds per plant, weight of seeds and average length per 10 pods, were haracterized. Mutant line D_2-0.0125 M EMS (6) has the best manifestation of the studied traits among other mutant lines and it may be included in breeding schemes for evaluation as a new cultivar. BAT 477 (20) differs significantly by its traits from other genotypes, irrespectively of the cultivation mode. RAPD and ISSR analyses were done to the studied genotypes. On the basis of molecular characterization clear allocation of genotypes was found on dendrogrames bilt by NTSYS programe. It was demonstrated that the studied Bulgarian varieties are promising germoplasme for their introduction in hybridisation breeding schemes, as well as in application of mutagenesis and biotechnological aproaches.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the grain legume of greatest volume for direct human consumption in the world and is an important staple crop for small farmers (Broughton et al., 2003; Blair et al., 2009).

Yet abiotic stress tolerance may be the key to improving yields of common bean in

both stressed and unstressed environments (Beebe et al., 2004).

It should be noted that in recent years, drought is increasingly becoming a major problem and compromising factor for common bean production worldwide (Beebe et al., 2008; Tera´n and Singh, 2002; Ramirez-Vallejo and Kelly, 1998).

It is known (Ishitani et al., 2004) that if soil fertility is limiting, expression of drought tolerance requires that plants have to possess tolerance to low soil fertility, expressed as early vigor and good root development. Thus, multiple stresses represent a particularly complex challenge for crop improvement, but the common denominator for tolerance to both low soil fertility and drought is the vigor of the root system of the plant.

Quantification of stress response to identify QTL for resistance is often based on the comparison of crop yield in stressed and unstressed treatments. Local adaptation is an important component of drought resistance, as evidenced by a common set of genotypes evaluated in several countries (White, 1987).

The potential to select drought tolerance with QTL analysis and MAS was investigated by Schneider et al. (1997a, b). Using RAPD, four markers for QTL were identified in one population and five in a second population. Additional preliminary drought QTL have been identified for the BAT 477 source under non-irrigated conditions at CIAT (Blair et al., 2002).

Ishitani et al. (2004) considered that phenotypic selection has led to significant advances in abiotic stress resistance, especially in drought resistance, but for sustained development of improved common bean cultivars with resistance to abiotic stress, researchers need to continue to gain knowledge about the stresses; to identify, share, and preserve sources of resistance to the important stresses; to conduct molecular genetics and genomic studies relevant to gaining a better understanding of the genetics and physiology of resistance.

The purpose of our investigation was to conduct molecular analysis of Bulgarian common bean genotypes and to characterize them on the basis of their biological traits associated with the formation of productivity in this crop and its manifestation in rainfed and irrigated conditions of cultivation.

2. Materials and Methods

Experiments were conducted in the field of Agricultural University in Plovdiv, Bulgaria. A standard method for cultivation in 5 replicates was applied.

2.1. Plant Material

10 common bean (*Phaseolus vulgaris* L.) mutant lines and 10 cultivars, grown under rainfed and irrigated conditions (Table 1) were tested. BAT 477 is obtained by exchanging germoplasme between Dobrudja Agricultural Institute, General Toshevo, Bulgaria and CIAT, Colombia.

Table 1. *Investigated common bean genotypes*

№	Mutant lines	Selection	№	Cultivars	Selection
1.	D_2-0,0062 M EMS	1, BG	**11.**	Plovdiv 11 M	1, BG
2.	D_2-0,0031 M NEU	1, BG	**12.**	Plovdiv 10	1, BG
3.	D_2-0,0062 M EMS	1, BG	**13.**	Abritus	2, BG
4.	D_2-0,0125 M EMS	1, BG	**14.**	Plovdiv 2	1, BG
5.	D_2-0,0062 M EMS	1, BG	**15.**	Doubrudjanski ran	2, BG
6.	D_2-0,0125 M EMS	1, BG	**16.**	Doubrudjanski 7	2, BG
7.	D_2-0,0062 M EMS	1, BG	**17.**	Plovdiv 15 M	1, BG
8.	D_7-0,0125 M EMS	1, BG	**18.**	Plovdiv 564	1, BG
9.	D_2-0,0125 M EMS	1, BG	**19.**	Doubrudjanski 2	2, BG
10.	D_2-0,0031 M NEU	1, BG	**20.**	BAT 477	CIAT, Colombia

Note: *The mutant lines and cultivars are selected in: 1 - Agricultural University, Plovdiv, 2 - Dobrudja Agricultural Institute, near the town General Toshevo, Bulgaria.

**The numbers in parentheses after each genotype, as described in the text, are taken from Table 1.

At the beginning, mutagenic factors ethylmethan sulfonate (EMS) and N-nitroso-N'-ethyl urea (NEU) were used to treat seeds of output varieties. We used, in our study, stable mutant lines (M_{19}-generation). They are mainly derived from a cultivar Dobroudjanski 2. Exception line D_7-0.0125 M EMS (8), which is obtained from a cultivar Dobrudjanski 7. Concentrations are listed at the end of the name of the mutant line.

All studied genotypes are with Mesoamerican origin.

2.2. Molecular Investigations

2.2.1. DNA Extraction

After grinding the young threefoliate leaves in a mortar with a pestle in the presence of liquid nitrogen, the resulting

fine powder was resuspended in buffer containing: 200 mM Tris–HCl pH 8.5; 25 mM EDTA; 255 mM NaCl; 0.5% SDS and 2% PVP. The extract was further purified by a RNA-se treatment (20 mg/ml) performed at 37 ^{0}C for 30 min and followed by a "classical"- phenol: chloroform extraction (Sambrook et al., 1989). After precipitation with isopropanol the DNA was washed with cold ethanol (75%), dried, and resuspended in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA). The DNA concentration was assessed spectrophotometrically and by 1% agarose gel electrophoresis.

2.2.2. RAPD Analysis

RAPD analysis was conducted in Biometra T-Gradient PCR-machine at an initial stage of 90s at 94 ^{o}C, followed by 35 cycles at 94 ^{o}C for 30s, 36 ^{o}C for 30s, 72 degrees ^{o}C for 60s, and finally the last stage at 72 degrees ^{o}C for 10min.

2.2.3. ISSR Analysis

ISSR markers were synthesized using 18 bp primers (GIBCO BRL Custom Primers). ISSR analyses were performed using a Biometra T-Gradient thermal cycler programmed for an initial step of 4 min at 94 ^{o}C followed by 40 cycles at 94 ^{o}C for 30 s, 46 ^{o}C for 45 s, 72 ^{o}C for 2 min and finally a 7 min extension step at 72 ^{0}C.

Reactions for RAPD and ISSR analyses were performed in a 20 µl volume, using 40 ng of template DNA, 1 mM of primer, 1 U of Taq DNA polymerase (Pharmacia, Biotech), and 0.2 mM of each dNTP (100 mM dNTP Set, Life Technologies) in reaction buffer containing 10 mM Tris–HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$. The amplification products were separated by electrophoresis in 2% agarose gels and visualised by ethidium bromide staining. A molecular weight marker ladder of 100 bp scales was used in electrophoresis. Gels were photographed using a Kodak EDAS 120 system.

2.2.4. Morphological Studies

Main traits, associated with productivity in common beans: plant height (A), weight of plants with pods (B), number of branches per plant (C), high betting on the first pod (D), number of fruit branches (E), number of pods per plant (F), weight of pods with seeds (G), number of seeds per plant (H), weight of seeds (I) and average length of 10 pods (J) were studied.

2.2.5. Statistical Analyses

Band profiles generated by RAPD or ISSR were completed onto a data matrix on the basis of the presence (1) or absence (0) of selected bands. Data were statistically analysed by the software program NTSYSpc 2.01b (Numerical Taxonomy and Multivariate Analysis System, Applied Biostatistic Inc., 1986–1997.) (Rolf, 1989). Dendrograms were constructed by UPGMA (Unweighted Pair Group Method Arithmetic Averages) cluster analysis using DICE coefficient.

Two- and three-dimensional graphics for the studied genotypes were designed, but the traits were represented by vectors (Cruz and Viana, 1994; Sneath and Sokal, 1973). To determine the relative weight of studied morphological traits an analysis of the main components (Principal component Analysis) was conducted (Philippeau, 1990).

3. Results

3.1. RAPD Analysis

Amplifications were first performed with all 29 primers using bulked DNA samples from four different cultivars. All tested primers amplified informative and reliable PCR products and were selected for subsequent RAPD and ISSR analyses. In general, the size of amplified DNA fragments ranged from 200 to 1900 bp.

A total 29 RAPD primers were tested (Table 2). The number of scored bands for each primer varied from 3 (primers - F 16 and Q 03) to 11 (primer - N 12), with a mean number of 6.45 markers per primer.

Totally 33 polymorphic bands were scored and they range from 0 (primers – AA 10; AD 18; D 04; OPA 09; OPB 15; OPC 14; OPF 10; Q 03 and U 12) to 5 (S 11). Each primer generated, in average, 1.14 polymorphisms.

Genetic relationships among the genotypes, based on RAPD genetic similarities, were represented in a UPGMA dendrogram, which cluster the 20 genotypes into five main groups and three subgroups (Fig. 1). Correlation coefficient between the cophenetic matrix computed from the dendrogram and the original similarity matrix was 0.973 (t = 5.945, p = 1.00) suggesting a very good fit of the tree representation to the rough data values.

The similarity coefficient between all samples was 0.963 and varied from 0.942 (mutant lines 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M, 9 M and 10 M versus 11-Plovdiv 11 M) to 1.0 (mutant lines 1 M, 2 M, 3 M, 4 M, 5 M, 6 M, 7 M, 9 M and 10 M versus 19-Dobroudjanski 2, as well as 16-Dobroudjanski 7 versus mutant line 8M), with an average of 0.969.

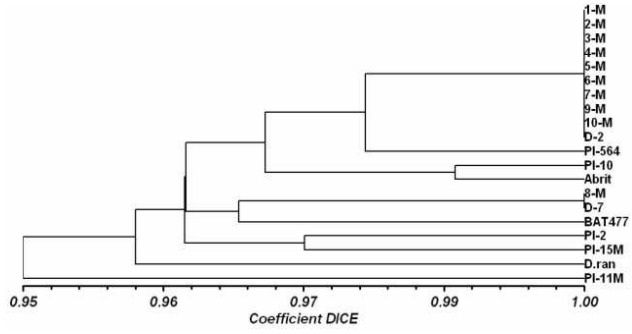

Fig. 1. *A UPGMA dendrogram of genetic relationships of the 20 genotypes based on DICE similarity coefficient values from 187 RAPD products.*

Table 2. *Number of RAPD bands scored with the 29 primers used*

№	Primers	Sequence (5'-3')	Total number of bands	Number of bands	
				Polymorphic	Monomorphic
1.	AA 04	AGGACTGCTC	7	1	6
2.	AA 10	TGGTCGGGTG	8	-	8
3.	AA 18	TGGTCCAGCC	7	3	4
4.	AB 09	GGGCGACTAC	4	1	3
5.	AC 01	TCCCAGCAGA	6	1	5
6.	AC 09	AGAGCGTACC	7	2	5
7.	AD 18	ACGAGAGGCA	5	-	5
8.	D 04	TCTGGTGAGG	8	-	8
9.	F 16	GGAGTACTGG	3	1	2
10.	I 07	CAGCGACAAG	9	1	8
11.	N 12	CACAGACACC	11	2	9
12.	OPA 09	GGGTAACGCC	6	-	6
13.	OPB 15	GGAGGGTGTT	4	-	4
14.	OPC 14	TGCGTGCTTG	4	-	4
15.	OPC 16	CACACTCCAG	7	4	3
16.	OPF 10	GGAAGCTTGG	4	-	4
17.	OPG 14	GGATGAGACC	8	2	6
18.	OPI 16	TCTCCGCCCT	7	1	6
19.	OPN 02	ACCAGGGGCA	5	1	4
20.	OPQ 02	GGTCACCTCA	7	1	6
21.	OPR 15	GGACAACGAG	6	1	5
22.	OPZ 20	ACTTTGGCGG	6	1	5
23.	P 16	TCGGCGGTTC	7	1	6
24.	Q 03	GGTCACCTCA	3	-	3
25.	S 11	AGTCGGGTGG	8	5	3
26.	S 12	CTGGGTGAGT	5	2	3
27.	U 03	CTATGCCGAC	7	1	6
28.	U 12	TCACCAGCCA	8	-	8
29.	U 19	GTCAGTGCGG	10	1	9
Total			187	33	154
Average			6.45	1.14	5.31
% Polimorphisms				17.65	

3.2. ISSR Analysis

A total of 63 markers were analysed (Table 3). The number of scored bands for each primer varied from 2 [(GT)8YG] to 11 [(AG)8YG], with a mean number of 6.30 markers per primer.

Eight polymorphic bands were observed, and ranged from 0 [$(ACTG)_3$RG]; [$(GACA)_3$YR]; [$(AG)_8$YT]; [$(AG)_8$YC] and [$(AC)_8$YT] to 3 [$(GACA)_3$RT], per primer. Each primer generated, in average, 0.80 polymorphisms.

Genetic relationships among the cultivars, based on ISSR genetic similarities, are represented in a UPGMA dendrogram, which cluster the 20 genotypes into six groups (Fig. 2). Correlation coefficient between the cophenetic matrix computed from the dendrogram and the original similarity matrix was 0.925 ($t = 4.658$, $p = 1.00$) suggesting a very good fit of the tree representation to the rough data values.

Table 3. *Number of ISSR bands scored with the 10 primers used*

№	Primers	Sequence (5'-3')	Total number of bands	Number of bands	
				Polymorphic	Monomorphic
1.	ISSR 1	$(GACA)_3$RT	8	3	5
2.	ISSR 3	$(ACTG)_3$RG	8	-	8
3.	ISSR 5	$(GACA)_3$YR	6	-	6
4.	ISSR 7	$(GA)_8$RG	7	1	6
5.	ISSR 10	$(AG)_8$YT	6	-	6
6.	ISSR 11	$(AG)_8$YC	6	-	6
7.	ISSR 12	$(AC)_8$YA	4	1	3
8.	ISSR 15	$(GT)_8$YG	2	1	1
9.	ISSR 16	$(AG)_8$YG	11	2	9
10.	ISSR 17	$(AC)_8$YT	5	-	5
Total			63	8	55
Average			6.30	0.80	5.50
% Polymorphisms				12.70	

Note: R, Purine; Y, Pyrimidine.

The genetic similarity (S) between cultivars was assessed on the basis of DICE's similarity coefficient and complemented with UPGMA cluster analysis. Pairwise comparisons of all ISSR profiles resulted in a similarity matrix (not shown).

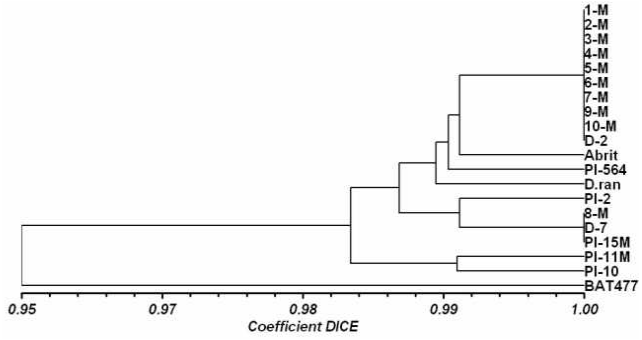

Fig. 2. *A UPGMA dendrogram of genetic relationships of the 20 genotypes based on DICE similarity coefficient values from 63 ISSR products.*

The similarity coefficient between all samples is 0.891 and varied from 0.942 Genotype BAT 477 formed separate cluster and showed completely different morphological traits than other Bulgarian genotypes.

3.3. Combining RAPD and ISSR Data

In order to obtain more consistent and balanced results, the 250 bands scored (187 RAPDs and 63 ISSRs) were pooled together and a common genetic similarity matrix was calculated (not shown). The average similarity coefficient between all samples was 0.977 and the interval of the smallest to highest values was between 0.949 (all mutant lines 1 – 10 M without 8 M and variety Dobroudjanski 2 versus Plovdiv 11M) and 1.0 (mutant lines versus their output varieties Doubrudjanski 2 and Doubrudjanski 7).

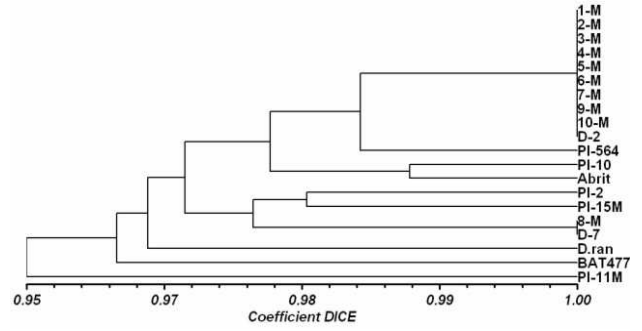

Fig. 3. *A UPGMA dendrogram of genetic relationships of the 20 genotypes based on DICE similarity coefficient values from combined RAPD and ISSR products.*

The dendrogram built on the basis of combined data from RAPD and ISSR analysis (Fig. 3) showed also a high correlation (r = 0.977; t = 5.84; p = 1.00) to the original similarity matrix. This dendrogram compiles the main characteristics of both independent trees (Figs. 1 and 2). The genotypes Plovdiv 11M, BAT 477 and Dobroudjanski ran formed independent clusters.

We conducted field experiments to investigate plant reactions, from different genotypes, to different environmental conditions.

Figure 4 shows the distribution of the genotypes grown under rainfed conditions in two-dimensional and three-dimensional space.

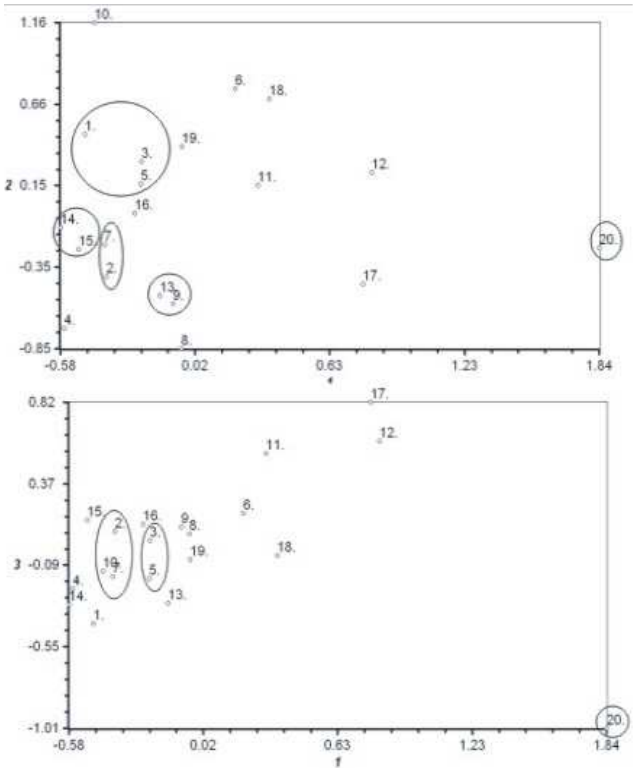

Fig. 4. *Analysis of major components for 20 genotypes grown under rainfed conditions*

In two-dimensional projection it is very well visible the proximity between cultivars - Plovdiv 2 (14) and Dobroudjanski ran (15); mutant lines D_2-0.0062 M EMS (1), D_2-0.0062 M EMS (3) and D_2-0.0062 M EMS (5), D_2-0.0062 M EMS (7) and D_2-0.0031 M NEU (2), as well as the proximity between cultivar Abritus (13) and mutant line D_2-0.0125 M EMS (9) on the base of cultivar's complex traits. BAT 477 (20) differs substantially and stays far away from the other genotypes.

Line D2-0.0125 M EMS (6) differs from other mutant lines and could be considered as a good breeding material because some very promising traits - average length of pods (J) and weight of pods with seeds (G) in rainfed conditions (Fig. 6).

A similar picture of the distribution of the genotypes, which were grown under irrigated conditions is presented on Fig. 2. Genotypes BAT 477 (20); Abritus (13); Dobroudjanski ran (15), mutant lines D2-0.0031 M NEU (2) and D2-0.0062 M EMS (5) are clearly distinguishable.

Mutant line D2-0.0125 M EMS also differs very clearly from the rest genotypes, having multiple branches per plant. (C), (Fig. 7). That is why, that mutant line can be included in breeding schemes for evaluation as a new cultivar. This is a

good reason that mutant line to be considered as a promising candidate for involvement in breeding schemes.

To assess the strength of influence of the traits, at both modes of cultivation, analysis of main components was carried out.

The strength of influence of traits in genotypes, grown under rainfed conditions are listed in Table 4. The analysis is conducted to the third main component and they explained 93.20% of the total variation.

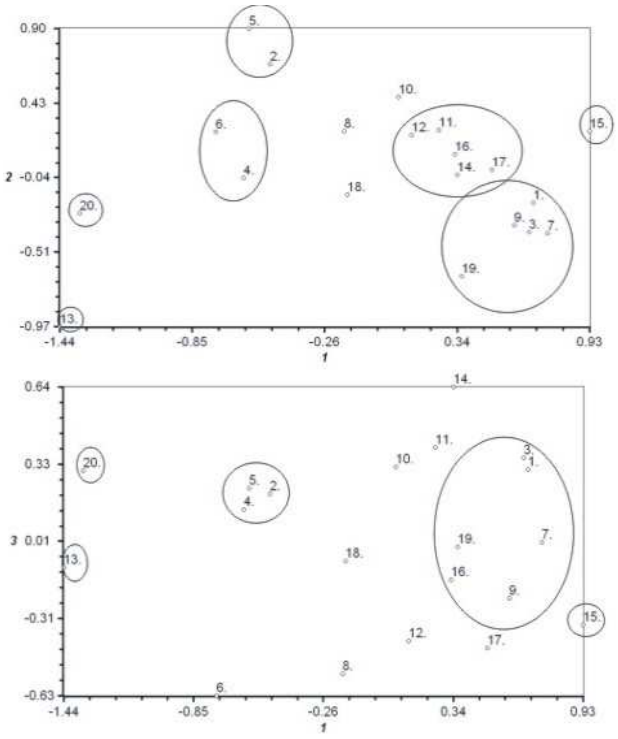

Fig. 5. *Analysis of major components for 20 genotypes grown under irrigated conditions*

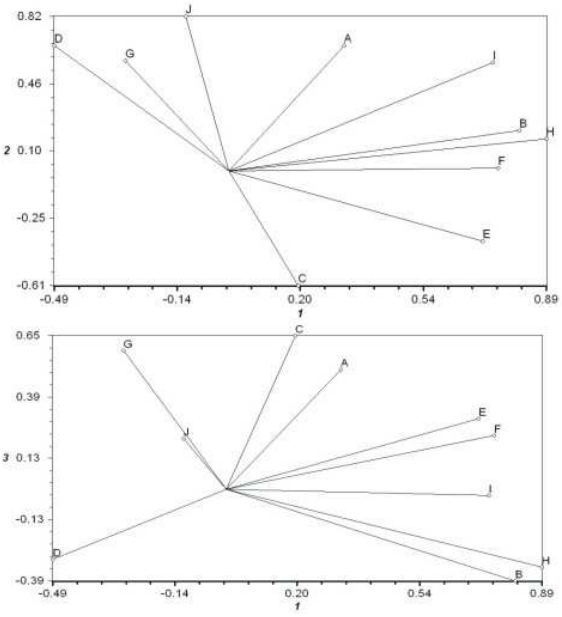

Fig. 6. *Analysis of major components for 20 genotypes grown under rainfed conditions and projections of studied 10 traits on axis 1 u 2; 1 u 3.*

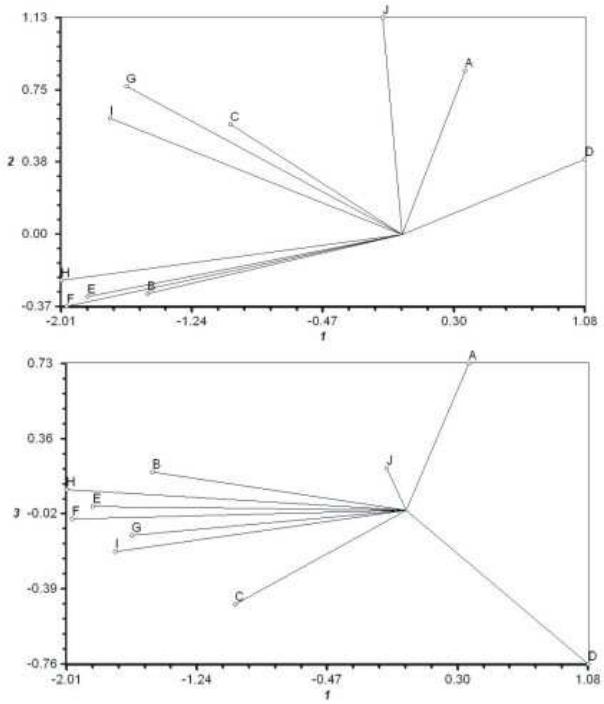

Fig. 7. *Analysis of major components for 20 genotypes grown under irrigated conditions and projections of studied 10 traits on axis 1 u 2; 1 u 3.*

The first principal component explains 53,45% of the variation in the studied population. Determining for the shown distributions is the impact of the traits - weight of pods with seeds (G), weight of seeds (I), number of seeds per plant (H), number of branches per plant (B) and number of pods per plant. Regarding to the second main component stands the influence of the traits - hight of betting on the first pod (D), number of branches per plant (B), average length of 10 pods (J) and number of fruit branches (E). That component explained 26.22% of the total variation.

The third main component, explaining 13.53% of the total variation, it is essential the impact of the traits plant height (A), average length of 10 pods (J) and number of branches per plant (C).

The distribution of the traits, which depends on the strength of their correlation with the axes in two- and three-dimensional space, is presented in Figure 3.

Table 4. *Principal component analysis, applied to study some biological traits in plants grown under rainfed conditions*

TRAITS		Main components		
		1	2	3
Plant height, cm	**A**	0.514	0.346	0.722
Number of branches per plant. g	**B**	0.838	- 0.024	- 0.269
Number of branches per plant	**C**	- 0.098	- 0.778	0.460
High of betting on the first pod. cm	**D**	- 0.172	0.853	- 0.088
Number of fruit branches	**E**	0.468	- 0.674	0.264
Number of pods per plant	**F**	0.689	- 0.359	0.088
Weight of pods with seeds. g	**G**	0.940	0.265	- 0.104
Number of seeds per plant	**H**	0.901	- 0.127	- 0.341
Weight of seeds. g	**I**	0.922	0.210	0.005
Average length of 10 pods. cm	**J**	0.199	0.741	0.498
Explained % of total variance:		53.45	26.22	13.53

Table 5. *Principal component analysis. applied to study some biological traits in plants grown under irrigated conditions*

TRAITS		Main components		
		1	2	3
Plant height. cm	**A**	- 0.172	0.610	- 0.661
Number of branches per plant. g	**B**	0.698	- 0.219	- 0.173
Number of branches per plant	**C**	0.469	0.409	0.423
High of betting on the first pod. cm	**D**	- 0.499	0.280	0.690
Number of fruit branches	**E**	0.861	- 0.229	- 0.018
Number of pods per plant	**F**	0.917	- 0.265	0.040
Weight of pods with seeds. g	**G**	0.753	0.551	0.111
Number of seeds per plant	**H**	0.933	- 0.170	- 0.095
Weight of seeds. g	**I**	0.798	0.434	0.186
Average length of 10 pods. cm	**J**	0.054	0.806	- 0.189
Explained % of total variance:		57.34	20.81	12.17

Results obtained for genotypes grown under irrigated conditions are presented in Table 5. Among 10 possible components corresponding to the test traits, the analysis was also carried out only to the third, as may be explained 90.32% of the total variance. Traits number of seeds per plant (H), number of pods per plant (F), number of fruit branches (E), weight of seeds (I), weight of pods with seeds (G) and number of branches per plant (B) are with higher degree of influence on the distribushon of genotypes, since their relative degree of variation correlated most strongly with the first principal component. The first principal component explains 57.34% of the total variation in the studied group of genotypes.

The second main component explains 20.81% of total variation, and here determining is the importance of traits: average length of 10 pods (J), plant height (A), weight of pods with seeds (G) and number of branches per plant (G).

The influence of the third main component is 12.17% and is mainly due to traits: of high of betting on the first pod (D), plant height (A) and number of branches per plant (C).

A visible view of the severity of the studied traits and their responsibility for the resulting distribushion of genotypes grown under irrigation could be obtained from Figure 4. Traits with the highest correlation coefficients to the respective axes in two-dimensional and three-dimensional space have the largest share on total variation in the studied population.

It fully corresponds to found quantitative values, shown in Tables 4 and 5.

4. Discussion

Apart from the interest of molecular discrimination and characterization of studied genotypes, they represent a good model for investigation of DNA polymorphism in *P. vulgaris* L. The diverse level of genetic proximity of the studied genotypes, allowed RAPD and ISSR techniques to be tested and compared in their suitability for genome discrimination.

The fact that either RAPD or ISSR markers divided the samples in similar major clusters, the fact that the correlation between RAPD and ISSR similarity matrices was significant ($r = 0.977$; $t = 5.84$; $p = 1.00$), and the fact that a similar level of polymorphisms was observed with both techniques (17.65% for RAPD and 12.70% for ISSR), demonstrated the reliability of these two techniques for assessing the genetic relationships within common bean (*P.vulgaris* L.) genotypes. The RAPD technique, with a multiplex index (6.45) is nearly the same of ISSR technique (6.30), showed the same advantage to be efficient as ISSR.

Beebe et al. (2004) concluded that one advantage in the breeding of *Phaseolus* beans is the wide genetic variability, both within the species, and in sister species with which it can be crossed.

Concerning our previous investigations (Svetleva et al., 2006), applying ISSR and AFLP analyses, we found that Bulgarian germoplasma of common bean represent good source for common bean breeding programes.

Molecular diversity between studied common bean mutant lines and cultivars is not very large. The distance between them varied from 0.95 till 1.00 (Fig. 1, 2 and 3). That is because their pedigrees are closely related. By dendrogram can be seen that our mutant lines are very close to their output cultivars and the distance between them is 1.00. The reason for that is the lack of not very big changes in mutant line's DNA.

Genotype BAT 477 is more different and it is allocated on different places of built dendrogrames and two- or three-demention projections.

Sponchiado et al. (1989) found that the line BAT 477, among others, showed intermediate tolerance to drought stress. It was shown that this tolerance was due to greater root growth under water deficit conditions and further showed that genetic control of this trait was expressed in roots, not shoots (White and Castillo, 1992). However, deep rooting alone does not assure drought resistance (Beebe et al., 2004).

5. Conclusions

1. Stronger degree of variation, in studied traits, was observed in genotypes grown under irrigated conditions.
2. In the survey the variety BAT 477 (20) differs significantly by its traits in comparison to other genotypes.
3. Both analyses (RAPD and ISSR) gave sufficient and reliable information for successful genetic characterization of common bean genotypes, as well as for variety identification.
4. Studied Bulgarian cultivars are promising germoplasme for inclusion in hybridization schemes and the application of mutagenesis and biotechnological practices.

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