The efficiency of different molecular indices in sunflower breeding

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ABSTRACT

The sunflower heterosis breeding is justified by the possibility of obtaining increased yield. In order to obtain a yield increase in first generation hybrids, it is necessary to determine the genetic diversity of selected parental lines. Genetic distances between sunflower genotypes employed in breeding processes could improve the inbred line selection efficiency for obtaining higher yielding hybrids.

Key words: genetic diversity – hybrid vigor– sunflower – UPGMA.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is one of the main oil crops in the Republic of Moldova, and it is planted every year on about 180-200 thousands ha. Varieties characterized by their high content in edible oil, elaborated by V.S. Pustovoit in 1976-1978, were gradually substituted by hybrids, due to their higher productivity and technological effectiveness.

The hybrid vigor phenomenon refers to the capacity of first generation hybrids to exceed by certain traits the best parents. Heterosis has been applied to obtain a high yield and represents one of the most important phenomena of plant morphogenesis. Hybrid vigor assures an optimal viability and increased yield of crops (Anashenko, 1974; Gundaev, 1971).

Many hypotheses have been postulated with the purpose of explaining the hybrid vigor process, but, up to today, its nature has not been elucidated. Usually, the determination of a heterosis effect is based on certain morpho-genetical indices such as morphological, functional and biochemical ones (Konarev, 1993). In addition, the quantitative value of heterosis can be established both by comparison of the average value of homozygous breeding genotypes (parents) and by comparison of first hybrid generation with homozygous breeding lines (Vrânceanu, 2000). The increasing practical implementation of heterosis requires the accomplishment of new fundamental studies at the level of molecular and supramolecular systems of the cell (Boppenmaier et. al., 1993; Konarev, 1993). Hence, it is necessary to study comparatively the breeding sunflower genepool in order to disclose different molecular indices related to a heterosis effect.

Our objective was to determine the efficiency of different molecular indices, such as total protein, hydro-soluble protein, salt-soluble protein, and RAPD amplicons in an estimation of their association with heterosis effect.

MATERIALS AND METHODS

Sunflower hybrids (Performer, Valentino, Xenia, Oxana) and their parental forms are annual, diploid sunflower genotypes (2n=34), used within a commercial scope. The male sterile genotypes analyzed (Performer, Valentino, Xenia, Oxana) possess cytoplasmic male sterility (CMS) PET1. The male fertile parental lines (Performer, Valentino, Xenia, Oxana) carry a fertility restorer gene (*Rf*).

Total soluble proteins from peeled seeds were isolated in buffer: 0.628 mM Tris-HCl, pH=8.0 (1g tissue: 5 ml), 0.03% ascorbic acid, 1 mM EDTA. Water-soluble proteins were extracted in: 0.01 % Trilon B, 0.5 % ascorbic acid, 1 % mercaptoethanol. Salt-soluble proteins were isolated in buffer: 50 mM Tris, pH 8.0, followed by 10% NaCl (1:15). Electrophoresis was carried out according to Laemmli, in 1mm gel of polyacrylamide (GPAA), in denaturant conditions (Laemmli, 1978; Duca et.al., 2001). Post-electrophoresis processing was carried out according to the standard method (Duca et.al., 2001). The relative molecular mass of the polypeptide fractions was established by stepladder - SDS Protein Standards for Capillary Electrophoresis (USA).

Genomic DNA was isolated from sunflower plants, at the stage of 2-leaves, in buffer: 133 mM Tris-HCl, pH 7.8; 6.7 mM Na_2EDTA; 0.95 M NaCl; 1.33% Na sarcosyl; 1.33% mercaptoethanol.

RAPD (Random Amplified Polymorphic DNA) analyses were performed with five arbitrary primers (P2, P6, P8, P37, P39). The PCR was carried out using the following profile: 95ºC for 5 min; 45 cycles: 95ºC for 1 min, 37-42ºC for 1-2 min, 72ºC for 2 min, and the final extension was at 72ºC for 7 min.

Gel electrophoresis was performed using 2% agarose gel. Determination of products of amplification molecular masses was done by Smart stepladder 200-3000 pb.

The starting point of the analysis was the presence of amplified fragments at a given level designated as "1", and its absence, "0". The amplified fragments were quantified from the printed images.

Based on scored data, the following indices (Nei et.al., 1983; Lynch, 1990; Sivolap et.al., 1998) were calculated:

* Similarity coefficient: $GS = \frac{2 \times N_{ij}}{N_i + N_j}$, where N_{ij} – the number of common bands for two samples, N_i and N_j - the number of bands in samples *I* and *J*.

* Genetic distance: $GD = -\ln(GS)$, where GS – similarity coefficient (Gentzbittel et.al., 1992).

Matrices of genetic distances were constructed. Furthermore, these matrices were subjected to cluster analysis (unweighted pairwise method with arithmetic mean - UPGMA) (Michener et.al., 1957).

RESULTS

The heterogeneity of the breeding material was demonstrated by cluster analysis (UPGMA), based on the values of the genetic distances and the genetic similarity calculated from protein and genomic DNA polymorphisms.

Total protein. Analysis of total sunflower proteins from seed revealed a high level of polymorphism (Duca et.al., 2005a). The analyzed genotypes were grouped in two clusters: homozygous (with similarity level of 84-88%) and heterozygous (83-96% of genetic similarity). Only the fertile line Valentino was characterized by an average similarity of 77% in comparison with hybrids and 73% in comparison with analyzed lines (Fig. 1A).

Water-soluble protein. The genetic distances (GD) of sunflower lines, based on albumin pattern analysis, showed values of between 0.05 and 0.12 (Duca et.al., 2005b). The greatest genetic distances were revealed in the homozygous genotypes Performer, while the smallest GD was typical for Valentino lines. Homozygous lines Xenia and Oxana were characterized by similar distances (GD=0.07).

A dendrogram of albumin was made based on the genetic distance analysis of homozygous and heterozygous sunflower genotypes. Three main clusters represented are A, B and C (Fig. 1B). The A cluster included five sunflower genotypes: male fertile line Performer, Performer F_1, Oxana F_1, Valentino Rf line and Valentino F_1, with genetic similarity of 0.91-1.

Male sterile lines Xenia, Valentino and Oxana, characterized by a 99% similarity, represented the B cluster. The CMS line Xenia was very similar to male sterile genotype Valentino (94%). Grouped in the last cluster were male sterile line Performer, Rf line Xenia and Xenia F_1, which were characterized by a genetic similarity of about 0.90.

Elaboration of an albumin dendrogram of sunflower homozygous and heterozygous genotypes based on genetic distances analysis revealed the separation of sterile lines from Rf genotypes.

Salt-soluble proteins. The study of the relations of salt-soluble proteins in sunflower homozygous and heterozygous genotypes (Duca et.al., 2005c) revealed the presence of three main clusters: A, B and C, which, in general, sum up the genotypes belonging to the breeding combinations analyzed - Performer, Valentino, Oxana, Xenia (Fig. 1C).

The cluster A was characterized by the presence of the following genotypes: male sterile line Performer, Rf line Performer, Performer F_1 and CMS-line Oxana, which shared values of genetic similarities between 0.91-1.00. The first generation hybrid Performer (100%) proved to have high similarities with its parental lines. Cluster B was represented by male sterile genotypes Oxana, Xenia and Xenia F_1, with similarities of 85%. In C cluster, the Valentino genotypes and the fertile line Xenia, GS=0.78-0.84 (Fig. 1C) were grouped.

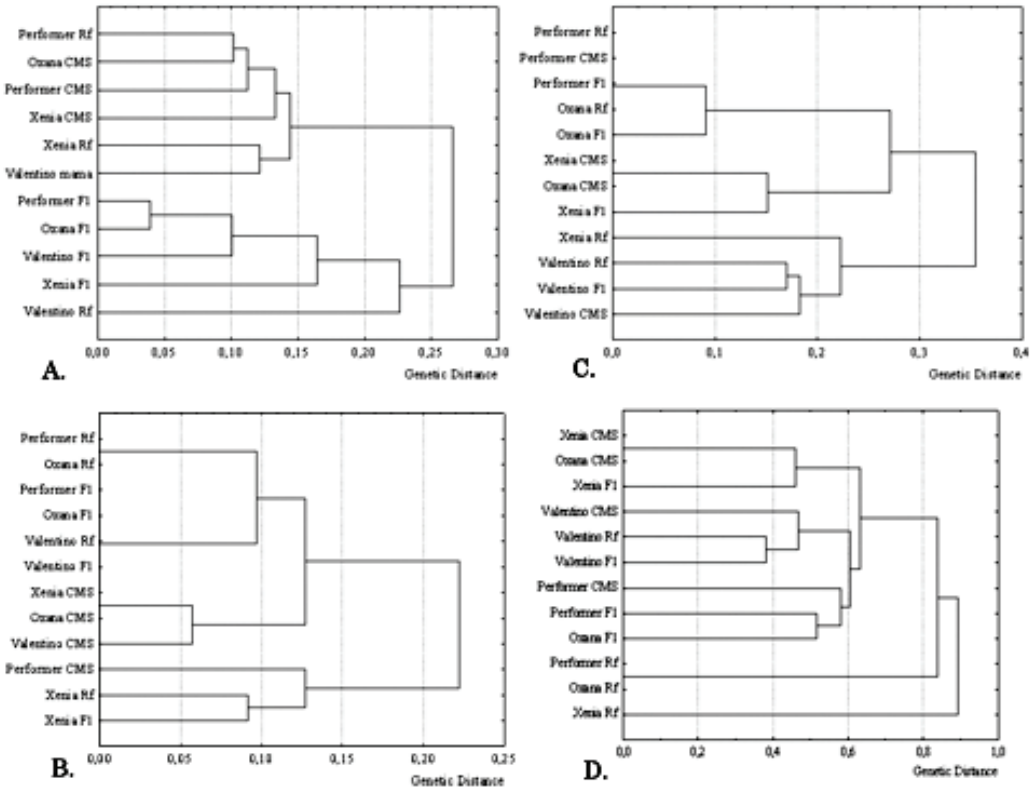

Fig. 1. Cluster analysis of sunflower genotypes based on:
A. total proteins, B. water-soluble proteins, C. salt-soluble proteins, D. RAPD sequences.

The study of genotypic relations based on electophoretic mobility of salt-soluble proteins revealed the presence of three main clusters, which, in general, sum up the genotype belonging to analyzed breeding groups - Performer, Valentino, Oxana, Xenia. Therefore, grouping of genotypes in this way was according to breeding groups.

RAPD analysis. Random amplified polymorphic DNA analysis on different sunflower inbred genotypes and on their hybrids (Duca et.al., 2005d), revealed the presence of 69 loci. PCR amplified sequences were represented by amplicons with lengths of 90-3000 bp. Number of amplified fragments differed depending on the primer used, and varied between 3 and 11 bands. The number of polymorphic loci varied within the experiments, by between 5 and 20 loci depending on the primer and the genotype (Table 1). Genotype polymorphism within analyzed families varied in the range of 5-46 %, while the average value of polymorphism was about 31%.

Table 1. Genetic polymorphism in the framework of different sunflower genotypes.

Primer	Nucleotide sequence	GC composition	Loci	Polymorphism, %
P2	gAc AgA cAg AcA	50	20	45.65
P6	gAg cAA gTT cAg ccT g	56	13	28.57
P8	cAg gAA AcA gcT gAc	53	9	37.5
P37	cTg Acc Agg Agc	67	12	5.26
P39	ccA ggT cgc c	80	15	39.47
Total			69	31.29

The dendrogram of RAPD loci on sunflower genotypes highlighted five main clusters (Duca et.al., 2008): A, B, C, D and E (Fig. 1D). Cluster A is composed of three sunflower genotypes: male sterile Xenia, Xenia F_1, and male sterile Oxana, which are characterized by genetic similarity values of 0.57-0.81, with an average of 0.69. B cluster was represented by the genotypes of Valentino breeding group, with a similarity of about 60%. The hybrid Valentino was similar to Rf line Valentino (62%) and to its sterile line (55%). The cluster C was represented by male sterile line Performer, Performer F_1 and Oxana

F_1, which were characterized by genetic similarity values of 0.41-0.44. The male fertile genotype Performer represented cluster D, which differed considerably from the other genotypes from clusters A, B and C. Cluster E was represented by the Rf line Xenia and it showed a resemblance of 12% with all the genotypes analyzed.

The analysis of RAPD amplicons (GD=0.41) disclosed that the genetic distance within homozygous lines (Rf and CMS) of hybrid Valentino was reduced, a fact that could contribute to explain its reduced productivity (2500 kg/ha) in comparison with the yield of the hybrid Oxana (2900 kg/ha). RAPD sequence pattern clusterization separated the heterozygous genotype Oxana from both parent genotypes with a great distance between them ($DG_{F1-♀}$=0.62 and $DG_{F1-♂}$=0.82).

DISCUSSION

Heterosis estimation. Increasing crop yields based on the heterosis phenomenon is a never-ending problem and permanently in force, and contributes to the realization of society's food program. One of the main problems in crop breeding consists of the evaluation and classification of the material implicated and in the selection of parental lines with the aim of obtaining high yield hybrids. The unravelling of the heterosis mechanisms and their estimation will open up new and important prospects in crop breeding.

Development of crop varieties and hybrids is based on traditional breeding methods (Siminel, 1998; Vitalis and Couvet, 2001) or on the use of genetic engineering (Tracy, 2003; Duca et.al., 2007). Both of these require the application of some efficient techniques that ensure the monitoring of breeding material used in breeding programs.

The specific biological peculiarities of plants can be determined by molecular markers such as DNA markers (Mohan et.al., 1997; Vrânceanu, 2000) and protein markers (Anisimova et.al., 2004; Durante et.al., 1989; Konarev, 1993). These can be applied for the evaluation of genetic diversity during the selection of quantitative and qualitative traits, for the choice of parental genotypes, for determination of hybridization, etc. Heterosis effect can be appreciated by the increase in the amount of DNA in somatic cells, DNA replication rate (Fedin, 1980) and cell division rate (Capatana, 2004).

Molecular markers, such as proteins, have contributed to the elucidation of the nature and origin of different crop genomes. This allowed the prognosis of certain cross breeding and the analysis of uniform populations with respect to their morphological traits. In addition, protein markers allowed the direct appreciation of hybridization level (Konarev, 1993).

In our experiments, the total sunflower seed proteins proved to have a high protein polymorphism (Duca et.al., 2005a; Capatana, 2006), the analyzed genotypes being grouped into two clusters: homozygous (with similarity level of 84-88%) and heterozygous (83-96% of genetic similarity). Therefore, from these results we can confirm that total proteins cannot be associated with crop productivity, and cannot be used in heterosis prognosis.

The total hydro-extractable proteins from seeds are represented by different protein fractions such as enzymes, enzyme inhibitors etc. (Anisimova, 1992; Norton, 1989). The seed albumins were used for electrophoretic patterns analysis (Duca et.al., 2005c; Capatana, 2006). The elaboration of an albumin dendrogram based on genetic distance analysis of sunflower genotypes revealed the separation of cytoplasmic male sterile from Rf genotypes. Thus, the absence of any association with the heterosis effect was verified.

In sunflower seeds, the main storage proteins are represented by globulins, especially by the major globulin fraction heliantinin (Anisimova et.al., 2004; Schwenke et.al., 1975; Vonder Haar et.al., 1988). Our results related to salt-soluble proteins (Duca et.al., 2005c) showed three main groups: A (Performer group of genotypes), B and C (Valentino group of genotypes), which in general sum up the genotypes representing the breeding groups studied (Fig. 1C). In this way, the association of less productive hybrid genotypes (Valentino and Performer) with small genetic distance between their parental lines and their location in the same cluster was shown, while hybrids that are more productive (Oxana, Xenia) were located apart from their parental lines.

The cluster analysis of RAPD products of amplification on sunflower genotypes revealed five main clusters (Capatana, 2006; Duca et.al., 2008): A, B, C, D and E (fig. 1D), with the separation being made on the basis of their genetic distance. Analyzing data of the breeding group Valentino by RAPD sequences (DG=0.41) it was disclosed that the genetic distance within homozygous lines (Rf and CMS) of hybrid Valentino was low, a fact that could contribute to explain the lower productivity (2500 kg/ha) in comparison with the yield of hybrid Oxana (2900 kg/ha). Clusterization of RAPD amplified sequences separated the heterozygous genotype Oxana from both parent genotypes, and these results were demonstrated by the large distance between them ($DG_{F1-♀}$=0.62 and $DG_{F1-♂}$=0.82).

Thus, UPGMA clusterization method of proteins and nucleic acids polymorphic spectra proves that salt-soluble proteins and RAPD markers could be used as a basis for the prognosis and selection of lines with different combining abilities. The methods of heterosis evaluation against genome peculiarities can serve as a basis for the elaboration of a heterosis prognosis method and the selection of breeding material.

ACKNOWLEDGEMENTS

The authors are grateful to Dr., Rotaru T. (SRC ``Magroselect``, Soroca, Republic of Moldova) for the provision of sunflower seeds and discussion during the investigation and to Dr. N. Barbacar for detailed discussions on RAPD analysis. The paper was realized with the support of National Grant Nr.32006C and MRDA/CRDF Travel Project MTFP-1017A.

REFERENCES

Anaschenko, A.V. 1974. Method of genetically regulated heterosis in sunflower's breeding (in russian). Publ. of VNII Crop Production 40:39-48.

Anisimova, I.N. 1992. Sunflower seed 11S globulin (helianthinin) as genetic marker. p 501-512. In: Proc.13th Int. Sunflower Conf., Pisa, Italy.

Anisimova, I.N., V.A. Gavrilova, A.V. Loskutov, V.T. Rozhkova, and V.V. Tolmachev. 2004. Polymorphism and inheritance of seed storage protein in sunflower. Russian J. Genet. 40:995-1002. (Translated from Genetika 40:1215-1223).

Boppenmaier, J., A. Melchinger, and G. Seitz. 1993. Genetic diversity for RFLPs in european maize inbreds 3. Performance of crosses within versus between heterotic groups for grain traits. Plant Breed. 111:217-226.

Capatana, A. 2004. The cytogenetic study of different sunflower's genotypes, Romanian Biotechnol. Letters 9:1763-1770.

Capatana, A., 2006. Genetical and molecular aspects of heterosis at sunflower (*Helianthus annuus* L.). Abstract of PhD thesis in biology. Chisinau, p.1- 28.

Duca, M., E. Savca, and A. Port. 2001. Fiziologia vegetala. Tehnici speciale de laborator, Chisinau.

Duca, M., A. Capatana, A. Port, and A. Glijin. 2005a. Protein polymorphism of different sunflower seed genotypes. Romanian Biotechnol. Letters 10:2087-2094.

Duca, M., A. Capatana, A. Port, A. Glijin, and G. Articov. 2005b. Albumins electrophoretic analisis at different sunflower genotypes, Actual problems of Genetics, Biotechnologies and Breeding, Chisinau, p.115-119.

Duca, M., A. Capatana, A. Port, A. Glijin, and G. Articov. 2005c. Globulins electrophoretic analisis at different sunflower genotypes, Actual problems of Genetics, Biotechnologies and Breeding, Chisinau, p.125-130.

Duca, M., A. Capatana, A. Port, and N. Barbacar. 2005d. The estimation of heterosis effect based on molecular analysis (RAPD) in sunflower/Genetic polymorphism in homo- and heterozygpote genotypes of sunflower. Romanian J. Genet. 1:22-31.

Duca, M., A. Levitschii, and T. Rotari. 2007. Posibilitati de estimare a puritatii genetice la floarea-soarelui, Chisinau, USM, Studia universitatis 7:54-61.

Duca, M., A. Capatana, and N. Barbacar. 2008. The estimation of heterosis effect based on some molecular indexes (RAPD) at sunflower. The genetic distance – breeding factor in obtaining of productiv hybrids. Romanian Biotechnol. Letters 13.

Durante, M., R. Bernardi, M.C. Lupi, and P. Sabelli. 1989. Characterization of *Helianthus annuus* L. storage proteins. J. Agric. Food Chem. 37:852-855.

Fedin, M.A., D.Ia Silis, and A.V. Smireaev. 1980. The statistical methods of genetical analysis (in russian). Tutorial, Kolos, Moscow, Russia. 207 p.

Gentzbittel, L., A. Perrault, and P. Nicolas. 1992. Molecular phylogeny of the *Helianthus* genus, based on nuclear Restriction Fragment Length Polymorphism (RFLP). Mol. Biol. Evol. 9:872-892.

Gundaev, A.I. 1971. The basic principle in sunflower breeding. p. 417-465. In: The genetical basis of plant breeding. Nauka, Moscow, Russia (in russian).

Konarev, V.G. 1993. Molecular-biological aspects of heterosis in applicative botany, genetics and breeding Kolos, Moscow, Russia (in russian).

Laemmli, U.K. 1978. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lynch, M. 1990. The similarity index and DNA fingerprinting. Mol Biol. Evol. 7:478-484.

Michener, C.D., and R.R. Sokal. 1957. A quantitative approach to a problem in classification. Evolution 11:130-162.
Mohan, M., S. Nair, A. Bhagwat, T.G Krishna, M. Yano, C.R. Bhatia, and T. Sasaki. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. Mol. Breed. 3:87-103.
Nei, M., and R. Tajima. 1983. Maximum likelihood estimation of the number of nucleotide substitutions from restriction sites data. Genetics 105:207-217.
Norton, G. 1989. Nature and biosynthesis of storage proteins. p. 105-191. In: G. Robbelen, R.K. Downey, and A. Ashri. (eds), Oil Crops of the World. McGraw-Hill, New York, USA.
Schwenke, K.D., M. Schultz, and K.J. Linow. 1975. Isolierung und Characterisierung des 11S Globulins aus Sonnenblumensamen (*Helianthus annuus* L.). Nahrung 19:817-822.
Siminel, V.D. 1998. Ameliorarea generala a plantelor de camp. Chisinau. Tipografia Centrala.
Sivolap, Yu.M., A.E. Solodenko, and V.V. Burlov. 1998. RAPD Analysis of molecular-genetic polymorphism in sunflower *Helianthus annuus*. Russian J. Genet. 34:199-203.
Tracy, W.F. 2003. What is plant breeding? p .51-60. In: Proc. Conf. on Seeds and Breeds for 21st Century Agriculture. Washington, DC, USA.
Vitalis, R., and D. Couvet. 2001. Estimation of effective population size and migration rate from one-and two-locus identity measures. Genetics 157:911-925.
Vonder Haar, R.A., R.D. Allen, Cohen E.A., C.L. Nessler, and T.L. Thomas. 1988. Organization of the sunflower 11S storage protein gene family. Gene 74:433-443.
Vrânceanu, A.V. 2000. Floarea-soarelui hibrida, CERES, Bucarest, Romania.