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# GENETIC PURITY OF SUNFLOWER HYBRIDS DETERMINED ON THE BASIS OF ISOZYMES AND SEED STORAGE PROTEINS



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SUMMARY

Maintenance of genetic uniformity of lines and hybrids is a prerequisite for successful production and placement of commercial hybrid seed on the market. Genetic purity of seed as specific seed trait is of great significance for seed science. Protein markers, seed storage proteins and isozymes, which are commonly used for the estimation of genetic purity, were used in this work to estimate genetic purity in sunflower hybrids. Analysis of helianthinin revealed tree zymogram patterns within and between sixteen sunflower hybrids. All of the 6 enzymatic systems analyzed, MDH, PGM, PHI, PGD, IDH and ACP, were polymorphic. A comparative analysis of genetic purity level of the sunflower hybrids was performed using electrophoretic methods. The methods of electrophoretic separation of isozymes and seed storage proteins were in agreement, with differences ranging from 1% to 5% in 81% of the samples. The level of polymorphism obtained by both methods was not distinct enough to be used in genotype identification.

**Key words:** **genetic purity, isozyme, seed storage protein**

## INTRODUCTION

Seed identity and varietal purity testing are essential components of a modern and effective agricultural production system.

Genetic purity of a seed sample defines the percentage of the sample that is not contaminated by seeds or genetic material belonging to other varieties or species. A combination of laboratory and field plot methods may be used to determine the cultivar trueness and genetic purity of the sample. Laboratory control is based on protein markers, isozymes, seed storage proteins and molecular markers.

The development of isozyme and/or DNA databases is a prerequisite to varietal identification and protection. Isozyme marker loci have been available for use in

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quality assurance, variety identification, plant breeding, production and variety protection programs for more than three decades. They are especially useful in solving problems in seed science such as inadvertent mixing of hybrid seeds or lines, uncontrolled pollination, errors during multiplication, *etc*. (Gerić *et al*., 1989; Zlokolica *et al*., 1996).

Electrophoresis of seeds storage proteins (helianthinin) of sunflower shows promising results in genetic purity determination of sunflower hybrids and inbred lines (Anisimova, 1989; Aksyonov, 2005 a,b).

Molecular markers offer a powerful supplement to the morphological and disease resistance data currently used for variety protection, cultivar classification schemes, and estimation of the level of genetic diversity. Molecular markers have many advantages (Lombard *et al*., 2000) compared with morphological markers, resilient to environmental changes, nearly unlimited number and relative ease and rapidity of data collection.

According to the Association of Official Seed Certifying Agencies (AOSCA 2003), maximum limits for seed of other varieties or off-types found in seed lots range from 0 to 0.2% among different species, while the limits for certified seed range from 0.1 to 2% by weight. In practical terms, the maximum number of seeds of other varieties of the same crop permitted in 0.454 kg of certified seed is 6 for sunflower (CCIA 2005).

Minimum genetic purity is 99.5% for female lines, 99.8% for male lines and 95% for sunflower hybrids (OECD Seed Scheme, 2006).

According to the Official Gazette of SFRY (No. 47, 1987), genetic purity can be tested for scientific research purposes or for controversial cases. Hybrid seed is considered as satisfying if it contains no more than 5% of self-pollinated individuals.

The aim of the present study was to compare two methods which are commonly used for estimation of genetic purity in sunflower hybrids, electrophoresis of isozymes and seed storage protein – helianthinin, and to estimate the applicability of a modified method for preparation of storage proteins from sunflower seed.

## MATERIALS AND METHODS

Sixteen sunflower hybrids were analyzed in this work. Preparation of helianthinin solution was modified from Samarah *et al*. (2006). Each seed was finely ground and protein extracted from flour by adding 400 $\mu$l 0.03 M Tris-HCl buffer pH 8 containing 0.01% 2-mercaptoethanol for four hours. After centrifugation at 11,000 rpm for 15 min, the supernatant was used for electrophoresis. Proteins were dissociated by heating to 90ºC for 3 min in the presence of a denaturing buffer (0.15 M Tris-HCl pH 6.8 containing 3% SDS, 5% 2-mercaptoethanol and 7% glycerol). Individual seeds were tested from each sample. Polypeptides were resolved by

electrophoresis of proteins under denaturing (SDS) and reducing (2-mercaptoethanol) conditions in 12.5% PAGE using the method of Laemmli (1970).

Electrophoresis was performed at an initial voltage of 50 mV, reduced to 25 V when the tracking dye reached the gel mold; analysis time was 16 h. The Marker Wide Molecular Weight Range (205-6.5kD, Sigma) was used for determination of protein molecular weight in electrophoretograms.

Proteins were simultaneously fixed and stained using a solution containing 0.24 g Coomassie Brilliant Blue R250 in 90 ml of a 1:1 (V/V) methanol : water and 10 ml of glacial acetic acid.

Stem tissues of 5-day-old seedlings homogenized in 50 mM TrisHCl, pH 6.8, in which 1% mercaptoetanol was added, were used for isozyme analysis. Isozyme systems phosphohexose isomerase (PHI), phosphogluconate dehydrogenase (PGD), phosphoglucomutase (PGM), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH) and acid phosphatase (ACP) were analyzed according to Stuber *et al.* (1988).

## RESULTS AND DISSCUSION

The main storage proteins in sunflower make up about 85% of the total protein content. Helianthinin has been reported to be present as a globular oligomeric protein with a molecular weight of 300–350 kDa (Sabir *et al.*, 1973; Schwenke *et al.*, 1979). It belongs to the cupin superfamily, which is comprised of 11S and 7S seed storage proteins. The members of the 11S family include pea and broadbean legumins, rape cruciferin, rice glutelins, cotton $\beta$-globulins, soybean glycinins, sunflower helianthinin, etc. Comparison of the results of Lakemond *et al.* (2001, 2000) and Gonzalez-Perez *et al.* (2004) clearly reveals many structural similarities of the soy 11S fraction (glycinin) and helianthinin. Starting from this fact, we used in our work a modified method for extraction of storage proteins from soybean (Samarah *et al.*, 2006). This method efficiently extracted proteins from sunflower seeds (Figure 1).

Helianthinin showed subunit heterogeneity: in addition to the presence of multiple subunits within a single genotype, there were also differences in the SDS-PAGE patterns of helianthinin components between different cultivars (Raymond *et al.*, 1994, 1995). The number of polymorphic zones corresponds to the number of subunits composed of the helianthinin molecule, controlled by *Hel1, Hel2, Hel3*, *Hel4*, *Hel5* and *Hel6* genes. The analysis of helianthinin revealed three zymogram patterns within and between the hybrids (Figures 1 and 2). Differences were found in the electrophoretic spectrum *Hel4*, which was in agreement with the results of Aksyonov (2005b) who claimed that *Hel4* made specific allelic variants (Figure 1). The gene controlling *Hel4* is inherited as a single codominant gene with independent alleles of *Hel4* in inbred lines and hybrids.

In Figure 1, there are two evident features: only the low molecular weight proteins (lower than 66,000 Da) produced high intensity bands, while the high molecular weight proteins were present in relatively low concentrations, as observed by Rodriguez *et al*. (2002).

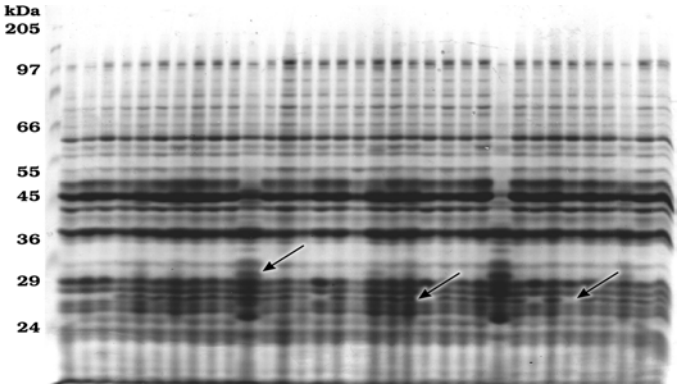


*Figure 1: Electrophoretogram of seed storage proteins of sunflower hybrid H1*

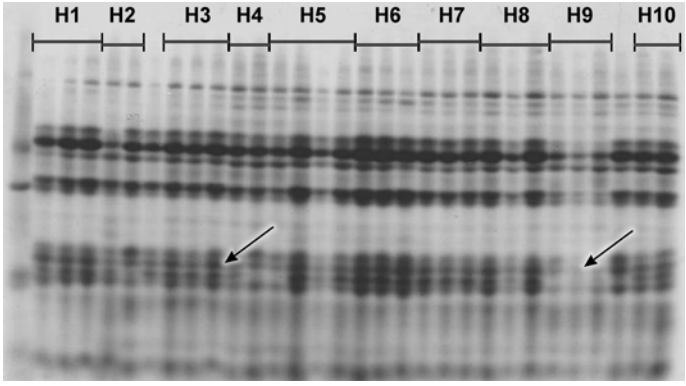


*Figure 2: Electrophoretogram of seed storage proteins of sunflower hybrids H1-H10*

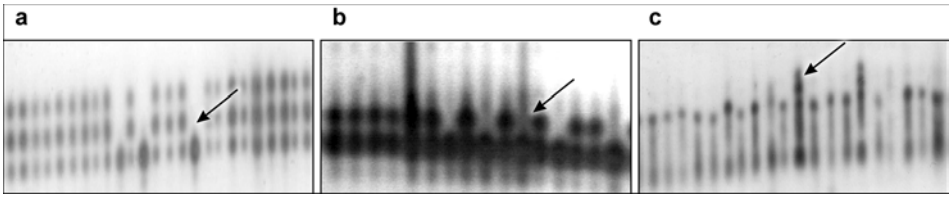


*Figure 3: PHI (a), PGM (b) and PGD (c) isozyme patters of sunflower hybrids*

All 6 analyzed enzymatic systems (MDH, PGM, PHI, PGD, IDH, ACP) were polymorphic, with two- or three-allele variants. The obtained differences in zymograms of the enzymes are useful for estimation of the genetic purity of sunflower hybrids,

especially distinct for PHI, PGM and PGD (Figure 3). Similar results of polymorphism were reported by Chikkadevaiah and Nandini (2003).

The comparative analysis of genetic purity level of the sunflower hybrids showed that the methods of electrophoresis of isozymes and seed storage proteins were in agreement in most cases. Differences in genetic purity level ranged from 1% to 5% in 81% of the samples. However, in the case of hybrid 1, the isozyme and seed storage protein analyses showed the values of 98% and 87%, respectively, while in the case of hybrid 11, the respective values were 89% and 97% (Table 1).

The main sources of error in the analytical methods used were sampling and reduction of samples in the laboratory. Both the sample size and sampling procedure dramatically impact the conclusions that may be drawn from any of these testing methods. Analytical results can also be influenced by type of sample, seedling or seed. In the isozyme analysis, seedlings of germinated seed were tested, while in the analysis of seed storage proteins any form of seed sample was applicable. Reproducibility, sensitivity and specificity of results was critical for validation of the methods used.

Table 1: Comparative data of genetic purity level in sunflower hybrids measured on the basis if isozyme and seed storage protein analyses

| Sample number | Genetic purity (%) | | Sample number | Genetic purity (%) | |
|---|---|---|---|---|---|
| | EI | ESSP | | EI | ESSP |
| 1 | 98 | 87 | 9 | 94 | 92 |
| 2 | 90 | 95 | 10 | 97 | 98 |
| 3 | 91 | 89 | 11 | 89 | 97 |
| 4 | 98 | 98 | 12 | 88 | 92 |
| 5 | 92 | 94 | 13 | 91 | 95 |
| 6 | 99 | 96 | 14 | 96 | 96 |
| 7 | 98 | 95 | 15 | 97 | 92 |
| 8 | 93 | 89 | 16 | 95 | 98 |

EI - electrophoresis of isoenzymes
ESSP- electrophoresis of seed storage proteins

In a study of sunflower genetic purity using field trials and electrophoresis of storage proteins (helianthinins), Aksyonov (2005a) concluded that albumin markers allowed to define the genetic homogenity level of hybrids at 80%. Our results showed that the level of genetic purity of sunflower hybrids could be accepted as satisfactory in most cases (OECD, 2006). Seed storage proteins and isozymes cannot be used for genetic identification of sunflower hybrids due to their low polymorphism levels.

Loss of genetic purity or varietal changes can occur due to various reasons: handling methods, storage facilities, natural crosses, genetic mutations, random genetic drift and other selection factors. Therefore, no commercial seed is one hundred percent genetically or mechanically pure. The results on the genetic purity of maize, sunflower, barley and wheat seed obtained by Nikolić *et al*. (2007) showed

that increased attention should be paid to seed genetic purity and systematic control of genetic uniformity of parent components, lines, hybrids and varieties.

## CONCLUSION

Seed certification programs and seed production companies are required to achieve various levels of seed purity for different species. The results of genetic purity level of the sunflower hybrids produced by the two widely used methods, seed storage proteins and isozyme, were in agreement in most cases. The differences ranged from 1% to 5% in 81% of the samples. The obtained results validate the two methods and could be useful for further work. The methods for genetic purity testing that are presently in use need to be standardized.

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## PUREZA GENÉTICA DE HÍBRIDOS DE GIRASOL DETERMINADA SOBRE LA BASE DE ISOENZIMAS Y PROTEÍNAS DE RESERVA EN SEMILLA

RESUMEN

El mantenimiento de la uniformidad genética de líneas e híbridos es uno de los prerrequisitos para la producción exitosa y la ubicación de semilla híbrida comercial en el mercado. La pureza genética como un carácter específico de las semillas es de gran importancia para la ciencia de las semillas. Los marcadores de proteínas, proteínas de reserva e isoenzimas, que comúnmente se utilizan para la estimación de pureza genética, se utilizaron en este estudio para estimar pureza genética en híbridos de girasol. Los análisis de *heliantina* revelaron tres patrones de zimogramas dentro y entre 16 híbridos de girasol. Los seis sistemas enzimáticos analizados: MDH, PGM, PHI, PGD, IDH, ACP, fueron polimórficos. El análisis comparativo del nivel de pureza genética de los híbridos de girasol demostró que los métodos de electroforesis de isoenzimas y proteínas de reserva de semilla fueron coincidentes, con diferencias entre el 1% al 5% en el 81% de las muestras. Los niveles de polimorfismos obtenidos por ambos métodos no fueron lo suficientemente diferentes para ser utilizados en identificación genotípica.

## PURETÉ GÉNÉTIQUE D'HYBRIDES DE TOURNESOL DÉTERMINÉE SUR LA BASE DES ISOZYMES ET DES PROTÉINES DE RÉSERVE DANS LA GRAINE

RÉSUMÉ

La maintenance de l'uniformité génétique des lignées et des hybrides est une des nécessités préalables au succès de la production et au positionnement sur le marché de de semences commerciales hybride. La pureté génétique de la semence comme trait spécifique est d'une grande importance pour le métier de la semence. Les marqueurs de protéines, les protéines de réserve et les iso-

zymes, qui sont communément utilisés pour l'estimation de la pureté génétique, ont été utilisé dans ces travaux pour estimer la pureté génétique dans les hybrides de tournesol. L'analyse d'helianthin a révélé trois modèles zimogram entre 16 hybrides de tournesol. La totalité des 6 systèmes d'enzymes analysés : MDH, PGM, PHI, PGD, IDH, ACP étaient polymorphiques. L'analyse comparative du niveau de pureté génétique des hybrides de tournesol a montré que les méthodes d'électrophorèse des isozymes et les protéines de réserve étaient en accord, avec divers niveaux allant de 1% à 5% pour 81% des échantillons. Le niveau de polymorphisme obtenu par les deux méthodes n'était pas assez distinct pour être utilisé dans l'identification du génotype.