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ISOZYMES AS MARKERS FOR DIFFERENTIATING SUNFLOWER GENOTYPES

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SUMMARY

Sunflower *(Helianthus annuus* L.) being a cross pollinated crop, genetic adulteration is a vested problem and varietal descriptions are cumbersome. In order to identify genotypes and to establish phylogenetic relationships an investigation was carried on sunflower hybrids/parental lines/varieties using isoenzyme variation. The data on six isoenzymes were analyzed using Popgene software (Yeh, 1997). Genotypes which were morphologically closely related were found to be unrelated at the biochemical level. Hybrid KBSH-42 could be distinguished from the rest of the hybrids for the absence of *pgi-2* and *pgi-3*. Alleles g_6*pdh-l*, *me-1* and *skdh-3* served as markers in distinguishing the genotypes. Thus the results obtained could be used in fingerprinting the genotypes.

Key words: sunflower, isozymes, starchgel electrophoresis

INTRODUCTION

Sunflower {*Helianthus annuus* L.) belongs to the family *Asteraceae,* the tribe *Heliantheae,* the subtribe *Helianthinae* and it includes 20 genera with 400 species. As plant breeders we need to describe cultivated varieties because they represent an end product of the investment of time, money and effort. However, sunflower being a cross pollinated crop, genetic adulteration is a vested problem and varietal descriptions are cumbersome; however, they are pre-requisite for the granting of plant variety protection and utility patents. Although morphological descriptions can provide unique identification of cultivated varieties, their ability to provide reliably discriminating identification is at best cumbersome. Increased number of genetically related releases by plant breeders has made unique identification more difficult to achieve. Secondly, morphologies reflect not only the genetic constitution of the cultivars, but also the interaction of the genotype with environment within which it is expressed. Thirdly, genetic control for most morphological traits is unknown. Therefore, in order to establish the genetic identity of sunflower geno-

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types we tried the biochemical method of fingerprinting hybrids, inbred lines and varieties. Since proteins/isoenzymes are inherited in the Mendelian fashion and expresed co-dominantly, they can be used to provide varietal profiles and they can be understood in genetic terms. Hence the present study is an attempt to differentiate hybrids/parental lines/varieties using isoenzyme analysis.

MATERIAL AND METHODS

The genetic material consisted of 7 hybrids, 8 parental lines and 6 varieties. Table 1 lists the studied genotypes.

Table 1: Genetic material

Number	Genotype description		
I	Hybrid	Female parent	Male parent
	KBSH-1	CMS-234A	RHA-6D-1
	PKVSH-27	CMS-2A	AK-IR
	DSH-1	DSF-15A	RHA-857
	APSH-11	CMS-7-1A	RHA-271
II	Experimental Hybrid		
	KBSH-41	-	-
	KBSH-42	-	-
	KBSH-44	-	-
III	Varieties		
	CO-2, CO-3, CO-4, EC-68414, GAU-SUF-15 and Morden		

Ten healthy plants were labeled for each of the 21 genotypes. A single fresh, large fully developed, disease free, undamaged leaf per plant was cut and wrapped in a piece of aluminium foil and maintained at -40°C. The genotypes were assayed by starch gel electrophoresis to identify genotypes polymorphic for at least one of the six enzymes, namely phosphoglucose isomerase (PGI), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), malate dehydrogenase (MDH) and shikimic-acid dehydrogenase (SKDH). Leaves of 220 individual plants were assayed electrophoretically.

The tissue was extracted in chase extraction buffer. The slurry obtained was then centrifuged at 10,000 rpm for 10 minutes at -4°C. The liquid extract was then absorbed into filter paper wicks and then the wicks were placed in grooves cut 4-5 cm from the cathodal end of a l5 × 10 × 2 cm starch gel slab.

Electrophoretic conditions

Buffer system 1: -gel concentration 12% sigma hydrolyzed potato starch, gel buffer pH 8.0, tray buffer pH 8.0, 0.25 mM Tris, 0.075 M citric acid, gel buffer 1:26 dilution of electrode buffer.

Buffer system 2: -gel concentration 12% sigma hydrolyzed potato starch, gel buffer pH 7.0, 0.05 histidin HCl, 1.4 mM, EDTA pH 7.0 (adjusted with 1.0 M Tris). The gel buffer was used at 1:4 dilution, tray buffer pH 7.0, 0.125 M Tris adjusted to pH 7.0 with 1 M citric acid.

Gels were allowed to run initially with 25-30 mA for 30 min. The current was later increased to 45-50 mA for 6-7 hours.

The following enzymes were used:

Phosphoglucose isomerase (PGI): 5 ml Tris HCl/pH 8.0, 10 mg fructose-6-phosphate, 2 ml glucose-6-phosphate dehydrogenase 0.5 ml $MgCl_2$, 0.5 ml NADP, 0.5 ml MTT and 0.5 ml PMS.

Glucose-6-phosphate dehydrogenase (G_6PDH): 5 ml 0.2 m Tris HCL pH 8.0, 100 mg glucose-6-phosphate, 0.5 ml $MgCl_2$, 0.5 ml NAD, 0.5 ml MTT and 0.5 ml PMS.

Malate dehydrogenase (MDH); 5 ml 0.2 m Tris HCl pH 8.0, 5 ml 0.5 M malic acid/pH 7.0, 0.5 ml NAD, 0.5 ml NBT and .5 ml PMS.

Isocitrate dehydrogenase (IDH): 5 ml 0.2 M Tris HCl pH 8.0, 100 mg isocitric acid, 0.5 ml $MgCl_2$, 0.5 ml NADP, 0.5 ml MTT and 0.5 ml PMS.

Malic enzyme (ME): 5 ml 0.5 m malic acid/pH 7.0, 5 ml electrode buffer H, 0.5 ml NADP, 0.5 ml $MgCl_2$, 0.5 ml MTT and 0.5 ML pms.

Shikimic acid dehydrogenase (SKDH): 5 ml 0.2 M Tris HCl/pH 8.0, 16 mg shikimic acid, 0.5 ml $MgCl_2$, 0.5 ml NADP, 0.5 ml MTT and 0.5 ml PMS.

Analysis of binary codes

The scored data pertaining to the six enzyme systems were analyzed following the Popgen software (Yeh, 1997). The analysis was aimed at estimating average genetic relatedness, frequency of alleles and differentiating genotypes based on principal component analysis.

RESULTS AND DISCUSSION

Table 2 shows mean frequencies of alleles from each of the 21 genotypes.

PGI: A total of 6 electromorphs with 13 different zymotypes were obtained by the enzyme. The possible number of putative loci was 2 (Reisbergand Soltis, 1989). Electromorphs *pgi-1*, *pgi-3* and *pgi-5* were most common while *pgi-4* and *pgi-6* were highly variable. The allele *pgi-1* present in hybrid PKVSH-27 had been contributed by the restorer line AK-1R. Hybrid KBSH-42 could be distinguished from the rest of the hybrids for the absence of *pgi-2* and *pgi-3*. Similar results were reported by Loskutov *et al.*, (1990). Among the varieties, all of them lacked *pgi-1* except for EC-68414. CO-2 was the only variety that did not show *pgi-2* while all the remaining varieties had *pgi-2*.

Table 2: Mean frequency of alleles from each of 22 genotypes of sunflower

No.	Allele	A	B	C	D	E	F	G	H	I	J	K	L	M	N	o	P	Q	R	S	T	U	V
1	*pgi-1*	0.9	0.0	0.03	0.0	0.0	0.1	0.1	0.4	0.2	0.0	0.1	0.3	0.4	0.4	0.2	0.1	0.0	0.5	0.0	0.0	0.1	0.0
2	*pgi-2*	0.0	0.9	0.6	0.9	1.0	0.9	0.9	0.4	0.4	0.4	0.1	0.3	0.5	0.4	0.6	0.8	0.9	0.3	0.8	0.8	0.4	0.0
3	*pgi-3*	0.0	0.1	O.i	0.1	0.0	0.0	0.3	0.2	0.5	0.6	0.8	0.3	0.1	0.1	0.2	0.1	0.1	0.4	0.3	0.3	0.4	0.9
4	*pgm*	0.9	0.9	0.9	0.9	0.1	0.0	0.0	0.0	0.1	0.0	0.3	0.1	0.3	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.3
5	*pgi-5*	0.2	0.1	0.1	0.1	0.9	1.0	0.7	0.6	0.4	0.2	0.2	0.6	0.5	0.5	0.4	0.9	1.0	0.5	0.7	0.7	0.3	0.7
6	*pgi-6*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	0.8	0.6	0.3	0.2	0.2	0.5	0.1	0.0	0.3	0.2	0.2	0.7	0.1
7	*idh-1*	0.8	0.6	'0.6	0.3	0.2	0.4	0.3	0.0	0.3	0.3	0.0	0.0	0.3	0.5	0.0	0.0	04	0.2	0.0	0.0	0.0	0.5
8	*idh-2*	0.1	0.4	0.4	0.7	0.8	0.6	0.5	1.0	0.6	0.7	0.7	0.5	0.7	0.5	0.5	0.3	0.5	0.8	0.7	0.7	0.7	0.4
9	*idh-3*	0.1	00	0.0	0.0	0.0	0.0	0.2	0.0	0.1	0.0	0.3	0.5	0.0	0.0	0.5	0.3	0.0	0.0	0.3	0.3	0.3	0.1
10	g_6*pdh-1*	0.4	0.2	0.4	0.4	0.3	0.4	0.5	0.5	0.0	0.3	0.0	0.0	0.0	0.0	0.0	00	0.0	0.2	0.0	0.0	0.6	0.0
11	g_6*pdh-2*	0.6	0.8	0.5	0.5	0.5	0.4	0.5	0.0	0.0	0.6	0.0	0.0	0.2	0.3	0.3	0.0	0.6	0.0	0.0	0.0	0.0	0.2
12	g_6*pdh-3*	0.0	0.0	0.1	0.1	0.2	0.2	0.0	0.0	0.1	0.1	0.5	0.5	0.0	0.6	0.0	0.3	0.4	0.8	1.0	1.0	0.4	0.8
13	g_6*pdh-4*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	g_6*pdh-5*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.5	0.1	0.5	0.0	0.7	0.7	0.0	0.0	0.0	0.0	0.0	0.0
15	g_6*pdh-6*	0.6	0.1	0.5	0.0	0.0	0.0	0.0	0.4	0.4	0.0	0.0	0.4	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	*me-1*	0.6	0.3	0.5	0.0	0.0	0.0	0.4	0.4	0.2	0.0	0.0	0.0	0.2	0.0	1.0	0.3	0.5	0.1	0.3	0.3	0.1	0.0
17	*me-2*	0.2	0.0	0.0	0.3	0.0	0.3	0.6	0.6	0.5	0.4	0.2	0.2	0.2	0.9	0.3	0.0	0.4	0.5	0.6	0.6	0.6	0.3
18	*me-3*	0.2	0.6	0.5	0.7	1.0	0.4	0.0	0.0	0.4	0.6	0.8	0.5	0.6	0.1	0.7	0.6	0.1	0.4	0.0	0.0	0.2	0.7
19	*me-4*	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.1	0.0
21	*mdh-1*	0.3	0.3	0.2	0.3	0.0	0.3	0.4	0.3	0.3	0.0	0.3	0.0	0.4	0.0	0.0	0.5	0.0	0.2	0.3	0.3	0.4	0.2
20	*mdh2*	0.7	0.7	0.8	0.5	1.0	0.7	0.6	0.6	0.3	0.5	0.7	0.4	0.6	0.2	0.0	0.5	0.7	0.6	0.7	0.7	0.5	0.0
22	*mdh-3*	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.5	0.4	0.5	0.7	0.6	0.0	0.7	1.0	0.0	0.3	0.2	0.0	0.0	0.1	0.8
23	*skdh-1*	0.7	0.8	0.4	0.4	0.3	0.4	0.6	0.6	0.0	0.0	0.4	0.9	0.4	0.0	0.0	0.2	0.3	0.1	0.0	0.0	0.2	0.2
24	*skdh-2*	0.3	0.2	0.6	0.6	0.7	0.6	0.4	0.4	0.9	1.00	0.3	0.1	0.5	1.0	0.9	0.6	0.7	0.0	0.0	0.0	0.5	0.8
25	*skdh-3*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.1	0.0	0.8	0.9	0.9	0.3	0.0
26	*skdh-4*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.9	0.5	0.9	0.9	0.4	0.0	0.1	0.1	0.1	0.0	0.0

A=KBSH-41	E=APSH-11	I=CMS-2A	M=RHA857	Q=CO-4	T=Morden
B=KBSH-42	F=PKVSH-27	J=DSF-15A	N=RHA271]	R=EC68414	U=CO-3
C=KBSH-44	G=DSH-1	K=CMS71-A	O=RHA6D-1	S=GAU-SUF-15	V=CO-2
D=KBSH-	H=CMS-234A	L=RHA-27	P=AK-1R		

IDH: This enzyme exhibited 3 electromorphs with 3 different zymotypes, *idh-1* and *idh-2* were highly conserved while *idh-3* was highly variable. Based on the zones of banding a single putative locus was observed. *Idh-1*, although present in all the hybrids, was completely absent in CMS-234A, CMS-7-1A and AK-1R, one of parents of KBSH-1, APSH011 and PKVSH-27. Varieties GAU-SUF-15, Morden and CO-3 lacked this band. Allele *idh-3* occurred in DSH-1, but was absent in both parents. This particular band was also absent in APSH-11 and RHA-271 but present in CMS-7-1A.

G_6PDH: Only one detectable staining zone was detected. Six alleles were recorded exhibiting 6 different zymotypes. This enzyme was highly polymorphic compared with the remaining 5 enzymes. *G_6pdh-2* and *$g_6$pdh-3* were highly conserved occurring in almost the populations while *$g_6$pdh-4*, *$g_6$pdh-5* and *$g_6$pdh-6* were highly variable. The fastest allele was present in all the hybrids except RHA-6D-1 and RHA-857, the restorers of KBSH-1 and PKVSH-27, indicating that the allele *$g_6$pdh* present in the two hybrids is contributed by their respective female parents. The same was seen with respect to hybrid APSH-11. While all the varieties lacked this electromorph, EC-68414 and CO-3 were fixed for this allele and also for *$g_6$pdh-3*. The allele *$g_6$pdh-2* was present only in CO-4 and CO-2 and absent in all the remaining varieties.

ME: A single staining zone was observed for the tetramer malic enzyme with 4 distinct alleles exhibiting 4 different phenotypes. *Me-2* and *me-3* were highly conserved while *me-1* was slightly conserved and *me-4* was highly variable. The fastest allele was noticed in all the hybrids except KBSH-1, APSH-11 and PKVSH-27. Hybrid DSH-1 is said to have inhinted this band from RHA-857. The absence of *me-2* and *me-4* can also serve as isozyme marker. APSH-11, KBSH-42 and KBSH-44 could be differentiated from the other hybrids in lacking *me-2* allele. Allele *me-3* is likely to have been contributed to KBSH-1 by RHA-6D-1 since its female parent, CMS-234 A, does not have *me-3*.

However, all the varieties had *me-1* except CO-2. The second allele was most common. GAU-SUF-15 and Morden varieties lacked the third allele while the others had it.

MDH: The dimeric enzyme was characterised by a single zone of activity. Three alleles were distinguishable among the genotypes studied. Each of the genotypes exhibited single allele thereby producing there different zymotypes. However, an additional fourth zymotype was recorded in the genotype DSF-15A wherein two bands were observed simultaneously. *Mdh-1* and *mdh-3* were highly variable. Hybrid APSH-11 could be differentiated from the rest of the hybrids for the absence of *mdh-1* while KBSH-1 could be differentiated from the rest for having *mdh-3* which was absent in the remaining hybrids. This allele is said to be contributed to the hybrid KBSH-1 by RHA-6D-1. Zlokolica *et al.* (1996) reported similar inferences.

SKDH: Although a single zone of activity was recorded for this enzyme, a total of seven different zymotypes were exhibited by four distinct alleles of SKDH. A few genotypes, namely CMS-234A, CMS-2A, CMS-7-1A, RHA-274, RHA-0857, RHA-271, RHA-6D-1 and AK-1R, exhibited two alleles at a time. The allele *skdh-2* was highly conserved while *skdh-3* and *skdh-4* were highly variable. DSF-15A was fixed for the second allele. The first allele was present in all hybrids but it might have been contributed by any of the parents. Allele *skdh-3* was rare and was found only in CMS-7-1A and AK-1R. Allele *skdh-4* was present only in the restorers. Among the varieties, EC-68414 lacked *skdh-2*, but the varieties GAU-SUF-15 and Morden lacked both *skdh-1* and *skdh-2*. The variety CO-2 was fixed with the first and second allele but CO-3 had an extra third allele. Reisberg and Soltis (1989) and Carrera and Poverene (1995) reported similar results.

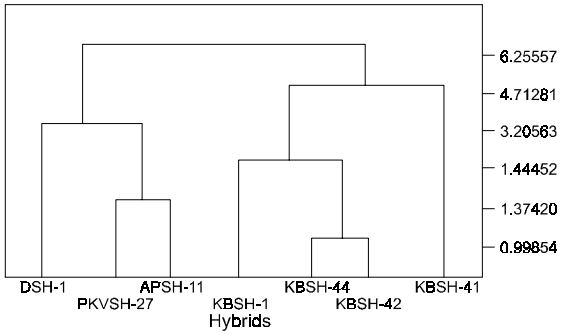

Figure l: Dendrogram showing clustering of hybrids based on isoenzyme data

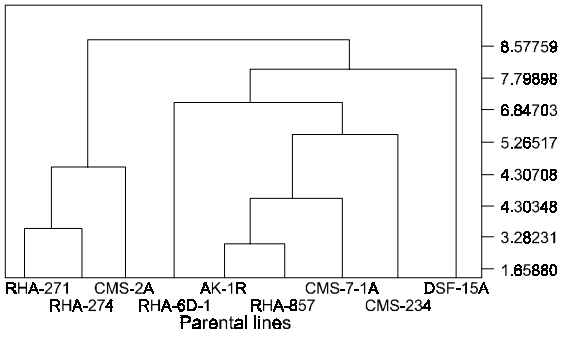

Figure 2: Dendrogram showing clustering of parental lines based on isoenzyme data

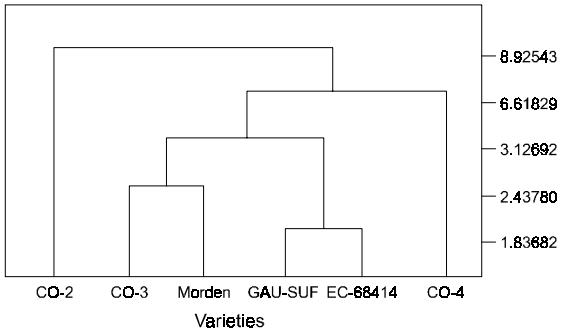

Figure 3: Dendrogram showing clustering of varieties based on isoenzyme data

Based on the Nei's genetic distances obtained using the scored data for all isoenzymes, dendrograms were constructed separately for hybrids (Figure l), parental lines (Figure 2) and varieties (Figure 3). As seen in Figure 1, all the seven hybrids fall into 2 major clusters. These hybrids may share some common background as their parentage. The second cluster includes APSH-11, PKVSH-27 and DSH-1. Among the varieties, the closely related ones are EC648414 with GAU-SUF-15 and CO-3 with Morden. Varieties CO-2 and CO-4 formed single genotype clusters.

CONCLUSION

Following conclusions were drawn from the study.

Isoenzymes PGI, IDH, G_6PDH, MDH, ME and SKDH which were studied systematically to differentiate sunflower genotypes, indicated that hybrid KBSH-42 could be distinguished from the rest of the hybrids for the absence of *pgi-2* and *pgi-3*, while among the varieties EC-68414 was the only one to lack *pgi-1*.

The allele g_6*pdh-1* present in hybrids KBSH-1 and PKVSH-27 was contributed by their respective female parents. Among the varieties, the allele was present only in EC68414 and CO-3.

Restorer RHA-857 is the contributor of *me-1* to the hybrid DSH-1 while RHA-6D-1 is the contributor of *me-3* to KBSH-1. Except for CO-2, all the varieties had *me-1* allele.

Skdh-3, a rare allele cited only in inbred lines CMS-7-1A and AK-1R, aided in easy identification of these two inbred lines from the others.

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ISOENZIMAS COMO MARCADORES PARA DIFERENCIACIÓN DE GENOTIPOS DE GIRASOL

RESUMEN

Debido a que el girasol *(Helianthus annuus* L.) es una planta de polinización abierta, la contaminación genética representa un problema constante, y la descripción de variedad resulta complicada. Para identificar los genotipos y establecer las relaciones filogenéticas entre ellos, se ha investigado la variabilidad de isoenzimas en los híbridos, los linajes parentales y las variedades de

girasol. Los datos sobre seis isoenzimas fueron analizados por medio del software Popgene (Yeh, 1997). Se estableció que los genotipos que eran estrechamente vinculados en el nivel morfológico, no estaban vinculados en el nivel bioquímico. El híbrido KBSH-42 se diferenciaba de los demás, por la carencia de alelo *pgi-2* y *pgi-3*. Los alelos g_6*pdh-l*, *me-1* y *skdh-3*, podían servir de marcadores que diferenciaron los genotipos investigados. Esto significa que los resultados obtenidos pueden utilizarse para el análisis genético de genotipos, finger printing.

ISOENZYMES EN TANT QUE MARQUEURS DE DIFFÉRENCIATION DE GÉNOTYPES DE TOURNESOL

RÉSUMÉ

Comme le tournesol (*Helianthus annuus* L.) est une plante fécondable par croisement, la contamination génétique est un problème constant et la description variétale est complexe. Une recherche a été faite sur la variabilité des isoenzymes dans les hybrides, les lignes parentales et les sortes de tournesol dans le but d'identifier les génotypes et d'établir une relation phylogénétique entre eux. Des données sur six isoenzymes ont été analysées à l'aide du logiciel Popgene (Yeh, 1997). Il a été établi que les génotypes qui étaient étroitement liés au niveau morphologique ne l'étaient pas au niveau biochimique. L'hybride KBSH-42 se différenciait des autres par l'absence d'allèles *pgi-2* et *pgi-3*. Les allèles g_6*pdh-1*, *me-1* et *skdh-3* ont pu servir de marqueurs qui différenciaient les génotypes examinés. Cela signifie que les résultats obtenus peuvent être utilisés dans l'analyse des génotypes par "finger printing".