Isozyme Analyses of *Arachis* Species and Interspecific Hybrids[1]

G. D. Lacks and H. T. Stalker*[2]

ABSTRACT

To better estimate diversity within the cultivated peanut, germplasm representing 33 South American peanut accessions from six countries was evaluated for isozyme polymorphisms. Only three of 18 isozymes — glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), and phosphohexose isomerase (PHI) — were consistently variant, each displaying two banding patterns. The variant banding patterns were observed in 18, 9, and 9% of the genotypes for GOT, IDH, and PHI, respectively. Isozyme variation in *A. hypogaea* could not be associated with subspecies or botanical variety. Thirty interspecific hybrids and their parents were also evaluated for isozyme polymorphisms. Flower tissues showed variations for the following isozymes: alanine aminopeptidase (AAP), arginine aminopeptidase (AMP), glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), and phosphohexose isomerase (PHI). A specific PHI band pattern was observed in all three hybrid lines with early leafspot resistance, as well as three of the four lines associated with high yield. For seed tissue, the absence of a fast-moving leucine aminopeptidase (LAP) band was associated with three of the four high-yielding lines. A comparison of flower and seed isozyme banding patterns revealed that the banding pattern was different for GOT, IDH, LAP, MDH, and PHI. IDH and MDH were variant in seeds but not flowers, and GOT was more polymorphic in flowers than seeds. The investigation indicates that isozymes may serve as molecular markers for interspecific hybrid identification and gene introgression to the *A. hypogaea* genome, and possibly for identifying lines with useful resistances.

Key Words: Isozymes, *Arachis* species, interspecific hybrids, disease resistance, insect resistance.

Because most economically important traits of agricultural crops are inherited quantitatively, a better understanding of the genes affecting these traits should provide the plant breeder with useful information for plant improvement. Isozymes, restriction fragment length polymorphisms (RFLP's), and other molecular markers are being utilized to locate quantitative trait loci (QTLs). Through the use of marker loci, genes affecting quantitative traits can be mapped and their effects on quantitative traits determined (Stuber and Edwards, 1986). Isozyme techniques have been utilized because of their ease of preparation and low cost. Because the electrophoretic gel is stained for a specific enzyme activity, it is possible to distinguish allelic from nonallelic proteins after genetic analyses. Isozyme techniques have been used to evaluate more than 30 crop species (Tanksley, 1983).

Grieshammer and Wynne (1990) evaluated starch gel electrophoresis and 25 isozyme staining systems using seed

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[2]Former Graduate Research Assistant and Professor of Crop Science, North Carolina State University, Raleigh, NC 27695-7629.

*Corresponding author.

tissues and screened 61 U.S. peanut cultivars, one breeding line, and six exotic peanut introductions representing three botanical varieties — *hypogaea*, *fastigiata*, and *vulgaris* — that are of the virginia, valencia, and spanish market types, respectively. Of the 25 isozyme systems evaluated, only three were found to be consistently polymorphic: glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), and phosphohexose isomerase (PHI). The banding patterns for GOT and PHI distinguished spanish and valencia market types (which are members of subspecies *fastigiata*) from virginia genotypes, which belong to subspecies *hypogaea*. IDH was not polymorphic for the spanish and valencia market types, whereas many virginia-type cultivars showed an extra band. Because Grieshammer and Wynne (1990) used many cultivars with complex pedigrees, they concluded that variation reflected the narrow genetic base of most spanish cultivars and the broader genetic base that was utilized for the pedigrees of virginia cultivars.

Stalker *et al.* (1990) used starch gel electrophoresis and 17 enzyme-staining systems to evaluate seed of 113 wild *Arachis* accessions and the cultivar NC 4. Polymorphisms were found for most isozyme systems among species belonging to both different and the same sections, between accessions of the same species, and even between individual seeds of single accessions. This suggests that original seed collections may be highly variable even though the species analyzed are diploid and self-pollinating. They concluded that germplasm should be maintained as bulk seed lots of many individuals rather than as progenies from single plants.

The areas where greatest isozyme diversity in the genus are found appear to be the Mato Grosso region of Brazil and eastern Bolivia (Lacks *et al.*, 1991). Due to the large amount of polymorphism exhibited between *Arachis* species as compared to within *A. hypogaea*, isozymes have potential use as markers to follow introgression patterns in interspecific hybrids. The objectives of this study were to a) develop a nondestructive isozyme extraction technique for peanut that is neither cost- nor time-limiting, b) determine if isozyme polymorphisms exist in an array of South American introductions of *A. hypogaea*, c) evaluate whether isozymes have potential for use in genetic and germplasm introgression studies, and d) compare isozyme banding patterns between seeds and flowers.

Materials and Methods

Extraction Technique

Various extraction techniques were evaluated for leaf and flower tissues including enzyme digestion, centrifugation, maceration, and the use of an automatic shaker (Lacks, 1993). Initial experiments were conducted to obtain scorable zymograms from leaf tissue using systems reported by Grieshammer and Wynne (1990) for the following: aconitase (ACO), alcohol dehydrogenase (ADH), GOT, IDH, and phosphoglucomutase (PGM). They also reported scorable zymograms for ACO, esterase (EST), IDH, and PGM. Scorable zymograms using leaf tissue were not obtained by Grieshammer and Wynne. However, by adjusting the extraction buffer recipes of Arulsekar and Parfitt (1986) and Wendel and Weeden (1989) as presented in Table 1, 18 isozyme systems from peanut flower tissue were developed (Table 2). The procedures utilized for starch gel preparation and electrophoresis were those described by Stuber *et al.* (1988). The staining solutions used to detect zones of enzymatic activity were described by Grieshammer and Wynne (1990). After the appropriate staining incubation time, the gels were rinsed with water and were either scored immediately or preserved for several days by fixing them in a 1:5:5 mixture of glacil acetic acid, absolute methanol, and distilled water.

A. hypogaea and Interspecific Hybrid Germplasm

Germplasm representing 33 South American *A. hypogaea* lines collected in six countries were obtained from the Southern Regional Plant Introduction Station in Griffin, GA during March 1990 (Table 3). These genotypes had previously been scored for plant and seed types, and the germplasm was selected based on the wide divergence in morphological traits and geographical origin. The mixture of plant/pod types (Table 3) is presumably from ancient hybridization and subsequent preservation of phenotypes by South American peoples. Five seedlings per genotype were potted in a 1:1:1 ratio of soil:sand:Metro-Mix and maintained in the greenhouse. Approximately 4 weeks after planting, corolla tissue of each accession was collected and used to extract isozymes. Tissues were macerated by mortar and pestle in one of two extraction buffers at 4C, depending on the isozyme evaluated (Tables 1 and 2).

Thirty lines developed by interspecific hybridization and their parents (Table 4) were screened for isozyme variation. The female parent was *A. hypogaea* subspecies *fastigiata* var. *fastigiata* (PI 261942 or PI 261943) ($2n = 4x = 40$) and the male parent was the diploid ($2n = 2x = 20$) species *A. cardenasii* Krap. et Greg. *nom. nud.* (GKP 10017/PI 262141). Hybrids ($2n = 3x = 30$) were initially produced during the mid-1960s and then hexaploids ($2n = 6x - 60$) produced through colchicine treatments were

Table 1. Extraction buffer recipes used for *Arachis* flower tissues.

Modified Arulsekar/Parfitt extraction buffer		Modified Wendel/Weeden extraction buffer	
H_2O	100 ml	H_2O	100 ml
Tris	0.61 g	Tris	0.91 g
Citric acid	0.15 g	Sucrose	5.0 g
Cysteine HCl	0.10 g	PVP-40	5.0 g
Ascorbic acid	0.10 g	Ascorbic acid	0.80 g
Polyethylene glycol	1.00 g	Diethyldithiocarbamate	0.17 g
Polyvinyl-polypyrrolidone	1.0 g	Bovine serum albumin	0.10 g
		Tween 80	3 drops
- Adjust to pH 7.5 - Keep refrigerated		- Adjust to pH 7.5 - Keep refrigerated	

Note: Arulsekar/Parfitt extraction buffer according to reference except for the pH, no β-merceptoethanol, and reduced polyvinyl polypyrrolidone.

Table 2. Isozyme and appropriate extraction buffer for *Arachis* flower tissues.

Isozyme	Extraction buffer[a]
Aconitase (ACO)	1
Alanine aminopeptidase (AAP)	2
Alcohol dehydrogenase (ADH)	1
Aldolase (ALD)	1
Arginine aminopeptidase (AMP)	1
Catalase (CAT)	1
Esterase (EST)	1
Glutamate hydrogenase (GDH)	1
Glutamate oxaloacetate transaminase (GOT)	1
Isocitrate dehydrogenase (IDH)	1
Leucine aminopeptidase (LAP)	2
Malate dehydrogenase (MDH)	2
Menadione reductase (MNR)	2
Phosphoglucomutase (PGM)	2
Phosphohexose isomerase (PHI)	2
Shikimate dehydrogenase (SAD)	2
Superoxide dismutase (SOD)	1
Triose phosphate isomerase (TPI)	1

[a]1 = Arulsekar and Parfitt (1986), 2 = Modified Wendel and Weeden (1989; see Table 2).

Table 3. South American *A. hypogaea* germplasm used for isozyme studies.

PI no.	Plant type	Pod type	Country of origin	Area/province
339971	Valencia	Valencia	Bolivia	Yacuiba, Tarija
261910	Virginia	Virginia	"	Samaipata
262111	"	Spanish	"	Santa Cruz
262093	Spanish	Valencia	"	"
117846	Valencia	Valencia	Brazil	Sao Paulo
118991	"	Virginia	"	Porto Alegre
119060	"	Mixed	"	"
338559	"	Valencia-Spanish	"	Ceara
118472	Chinese	Valencia	"	Sao Paulo
152109	"	Chinese	"	"
152127	"	Spanish	"	Campinas, Sao Paulo
152129	"	Virginia	"	"
119063	Spanish	Mixed	"	Porto Alegre
119073	"	Valencia	"	"
149639	"	Spanish	"	Sao Paulo
149643	"	Virginia	"	"
152105	"	Chinese	"	Campinas, Sao Paulo
119876	Valencia	Mixed	Paraguay	Colorado
119880	"	Spanish	"	Villa Rica
121493	"	Valencia	"	Tobaty
155244	Virginia	"	"	Asuncion
155247	Spanish	"	"	"
230329	Valencia	Valencia	Peru	Lima
215724	"	Mixed	"	"
331336	"	Virginia	"	--
262126	Spanish	Valencia	"	Lima
155108	Valencia	Valencia	Uruguay	La Estanzuela
155107	Virginia	Spanish	"	"
155112	"	Valencia	"	Montevideo
119238	Spanish	Spanish	"	Rivera
119239	"	Valencia	"	"
119240	Valencia	Spanish	"	"
338336	Valencia	"	Venezuela	Maracay

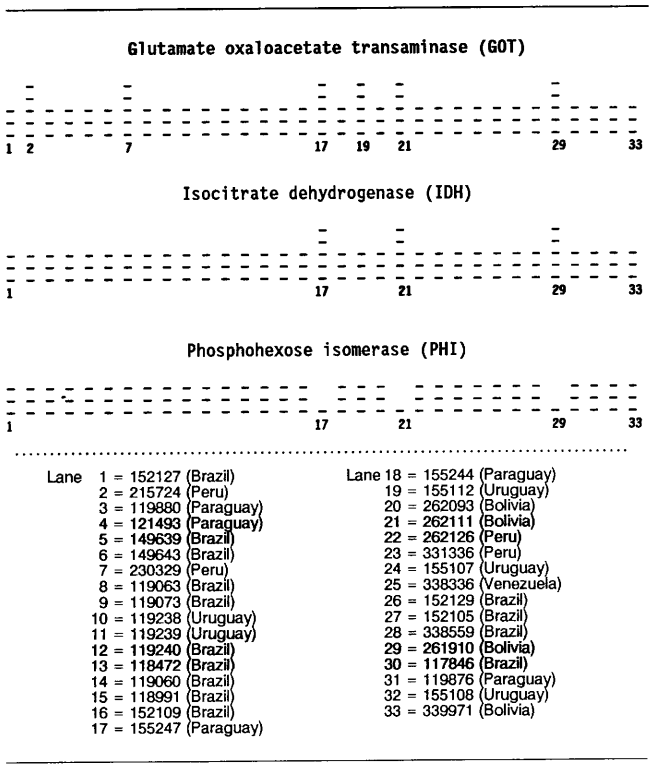

Fig. 1. Variation observed for isozyme patterns in flowers of South American cultivars.

fertile. The hexaploids were self-pollinated and presumably underwent spontaneous chromosome loss after several selfing generations until plants reached the tetraploid level. F_5-derived lines were produced from selfed seed of 40-chromosome plants identified by Stalker *et al.* (1979). Selections were made among these highly polymorphic tetraploid lines for leafspot (Stalker, 1984; Moss, 1985) and insect (Stalker and Campbell, 1983; H. T. Stalker, unpubl. data) resistances, and yield (Guok *et al.*, 1986). The lines have since been self-pollinated for an additional 10 generations, and they are believed to be homozygous. At least three seedlings per genotype were potted and maintained in the greenhouses located at NCSU, Raleigh, NC and evaluated for isozyme variation.

Results and Discussion

South American Cultivar Study

Fifteen of 18 isozyme systems evaluated in flower tissue from 33 South American peanut genotypes gave monomorphic zymograms. Only GOT, IDH, and PHI were found to be variant. Each of these displayed two banding patterns, the difference being the presence or the absence of two slow bands (Fig. 1).

GOT was variant in six of the 33 genotypes and was the most variable isozyme observed. The geographical areas where the GOT variant was observed included the central area of Bolivia (PIs 262111 and 261910), the southern area of Paraguay (PI 155247), the central coastal area of Peru (PIs 215724 and 230329), and the southern coastal area of Uruguay (PI 155112). These six genotypes include several botanical types (Table 3), so variation in GOT was not associated with a specific subspecies or group.

Unique banding patterns were observed for IDH and PHI in three of six genotypes identified as variant for GOT (Paraguay, PI 155247; Bolivia, PI 262111 and PI 261910). The presence (IDH) and absence (PHI) of two slow bands in these three genotypes separated them from other cultivars (Fig. 1). The plant types were two virginia (Bolivia) and one spanish (Paraguay). The pod types were virginia (Bolivia PI 261910), spanish (Bolivia PI 262111), and valencia (Paraguay, PI 155247) (Table 3). Thus, IDH and PHI variation in *A. hypogaea* at least can be found in central Bolivia and southern Paraguay but does not appear to be associated with subspecies or botanical variety.

Analyses of seed tissues of U.S. cultivars showed variation for the same three isozymes (Grieshammer and Wynne, 1990) as flower tissues in this study. All genotypes in both studies exhibited either the presence or absence of two slow bands. However, the IDH variant for seed tissue was the presence vs. the absence of one band (Grieshammer and Wynne, 1990), while the IDH variant for flower tissue was the presence vs. the absence of two slow bands. Whether the differences of the extra slow band in IDH is due to flower vs. seed tissue or to the genotypes used to derive U.S. cultivars vs. the South American genotypes analyzed here cannot be answered based on available data. In summary, small amounts of isozyme variation were observed using flower tissue to screen South American peanut genotypes, which supports the work of Grieshammer and Wynne (1990) who observed little isozyme variation in seeds among *A. hypogaea* cultivars developed in the U.S.

Interspecific Hybrid Study

Flower Tissue. The *A. hypogaea* (PI 261942 or PI 261943) and *A. cardenasii* (PI 262141) parents expressed variation in flower tissue for alanine aminopeptidase (AAP), arginine aminopeptidase (AMP), GOT, malate dehydrogenase (MDH), and PHI (Fig. 2). *Arachis cardenasii* did not express the slow moving band for AAP, AMP, and MDH as was found in *A. hypogaea*. All hybrids showed the same banding pattern for these three isozymes as *A. hypogaea*, and no

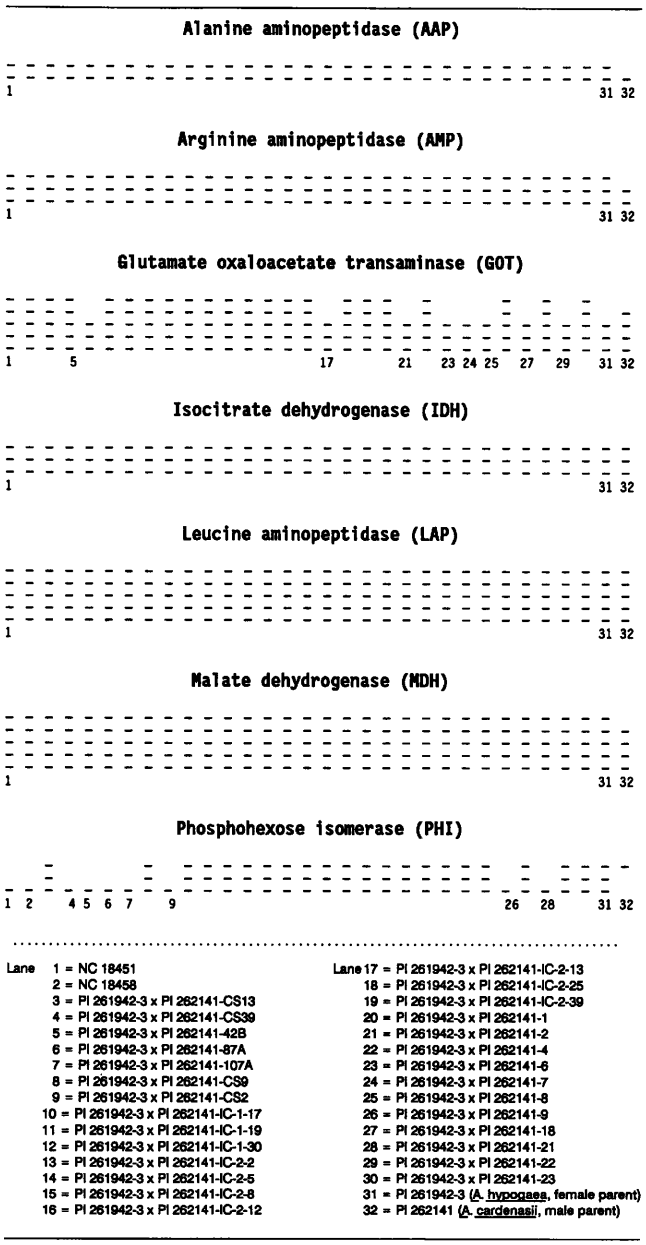

Fig. 2. Variation observed for isozyme patterns in flower tissue of interspecific hybrids (IDH and LAP were included in the figure in order to make easier comparisons with polymorphic patterns from seed tissue).

conclusions can be made regarding introgression of the *A. cardenasii* genes for these enzymes.

Eight interspecific lines had the same GOT banding pattern as the cultivated parent with three bands, whereas 22 other interspecific lines had the banding pattern of *A. cardenasii* plus an extra slow band (Fig. 2). The presence of this second slowest band suggests that a genetic transfer occurred from *A. cardenasii* into the *A. hypogaea* genome. The slowest GOT band (Fig. 2) was not found in either *A. cardenasii* or *A. hypogaea*. Because these isozyme analyses were conducted with seeds different from the one plant used to create the interspecific hybrid, the slowest moving GOT band may have originated from a variant *A. cardenasii* genotype or perhaps from epistatic effects between the *A. hypogaea* and *A. cardenasii* genomes. Regardless, the slowest GOT band is not found in *A. hypogaea*, and its presence in the interspecific hybrids represents interaction and/or gene transfer from an *Arachis* species to the cultivated peanut.

Nine interspecific hybrids had a banding pattern unlike either of the two parents for PHI. The cultivated parent and 21 hybrids contained three bands, *A. cardenasii* only contained the slowest band, and the nine hybrids lacked the two slowest bands (Fig. 2). All bands observed in interspecific hybrids were found in the *A. hypogaea* parent and, therefore, no conclusions regarding gene transfer can be made regarding this isozyme.

The flower banding pattern for GOT did not specifically identify any lines with distinguishing characteristics of agronomic value because the variation was scattered throughout the lines which previously had been selected for different disease and insect resistance traits. However, one PHI variant (a pattern with one fast band only) was observed in all three hybrid lines with early leafspot resistance as well in three of the four lines associated with high yield. Additional studies will be needed to confirm possible relationships between banding patterns and resistances, and segregation patterns of crosses will need to be evaluated before conclusions can be made.

Seed Tissue. Variation was observed in seed tissue

Table 4. Interspecific hybrid germplasm sources used in isozyme studies.

Lines	Characteristics of lines
NC 18451	High yield, large seed
NC 18458	"
PI 261942-3	*A. hypogaea*, female parent
PI 262141	*A. cardenasii*, male parent
PI 261942-3 x PI 262141-CS13	High yield, large seed
PI 261942-3 x PI 262141-CS39	High yield, large seed, late leafspot resistance[a]
PI 261942-3 x PI 262141-42B	Early leafspot resistance[b]
PI 261942-3 x PI 262141-87A	"
PI 261942-3 x PI 262141-107A	"
PI 261942-3 x PI 262141-CS9	Rust, late leafspot resistance
PI 261942-3 x PI 262141-CS2	Rust resistance
PI 261942-3 x PI 262141-IC-1-17	Insect resistance[c], nematode resistance[d]
PI 261942-3 x PI 262441-IC-1-30	"
PI 261942-3 x PI 262141-IC-1-19	"
PI 261942-3 x PI 262141-IC-2-2	Insect resistant[c]
PI 261942-3 x PI 262141-IC-2-5	"
PI 261942-3 x PI 262141-IC-2-8	"
PI 261942-3 x PI 262141-IC-2-12	"
PI 261942-3 x PI 262141-IC-2-13	"
PI 261942-3 x PI 262141-IC-2-25	"
PI 261942-3 x PI 262141-IC-2-39	"
PI 261942-3 x PI 262141-1	Randomly chosen line
PI 261942-3 x PI 262141-2	"
PI 261942-3 x PI 262141-4	"
PI 261942-3 x PI 262141-6	"
PI 261942-3 x PI 262141-7	"
PI 261942-3 x PI 262141-8	"
PI 261942-3 x PI 262141-9	"
PI 261942-3 x PI 262141-18	"
PI 261942-3 x PI 262141-21	"
PI 261942-3 x PI 262141-22	"
PI 261942-3 x PI 262141-23	"

NC 18451 = *A. hypogaea* PI 261942-3/*A. cardenasii* 10017, ACRS C1P04 (4x selection)//ACRS C1P06 (same source as ACRS C1P04); pedigree SO-B-B-03-01.

NC 18458 = *A. hypogaea* PI 261942-3/*A. cardenasii* 10017, ACRS C1P05 (4x selection)//ACRS C1P04 (same source as ACRS C1P05); pedigree SO-B-B-01-02.

[a]Late leafspot caused by *Cercosporidium personatum* (Berk. et Curt.) Deighton.

[b]Early leafspot caused by *Cercospora arachidicola* Hori.

[c]Resistant to leafhopper (*Empoasca fabae* Harris), corn earworms (*Heliothis zea* Bodie), and southern corn rootworm (*Diabrotica undecimpunctata howardii* Barber).

[d]Resistant to *Meloidogyne arenaria* (Neal) Chitwood.

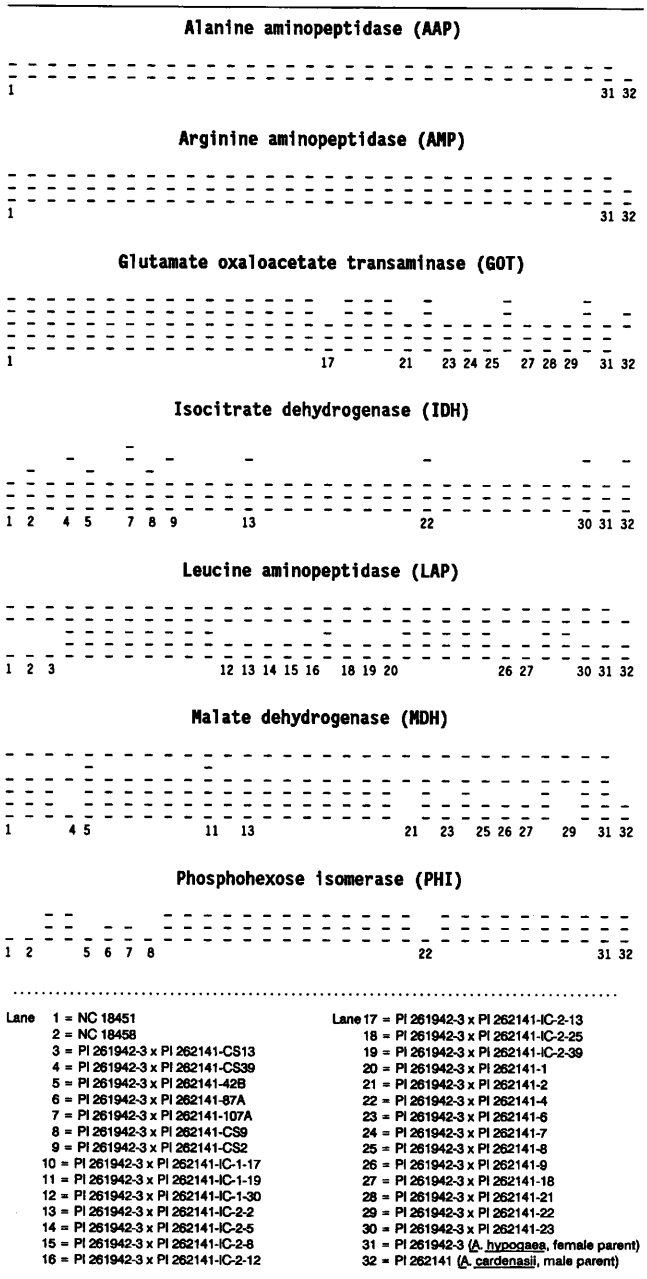

Fig. 3. Variation observed for isozyme patterns in seed tissue of interspecific hybrids.

between *A. cardenasii* and *A. hypogaea* for isozyme staining patterns of AAP, AMP, GOT, IDH, leucine aminopeptidase (LAP), and MDH (Fig. 3). As compared to *A. hypogaea*, *A. cardenasii* lacked the slow-moving band for AAP, AMP and LAP; lacked three slow-moving bands for MDH; and had an extra band in GOT and IDH. All hybrid lines showed the same banding pattern as *A. hypogaea* for AAP and AMP, so conclusions cannot be made regarding gene transfer from *A. cardenasii* for these isozymes.

Arachis cardenasii had two GOT bands, whereas *A. hypogaea* and eight interspecific hybrid lines each had the same three bands. Twenty-two other hybrid lines contained five bands, one of which was likely derived from *A. cardenasii*, whereas the slowest moving band was unique (Fig. 3). The variation was scattered among both resistant genotypes for diseases and insects plus randomly selected lines, so useful associations were not apparent which would allow GOT to be used as a marker in a breeding program.

Four unique banding patterns were observed for IDH, with a maximum of five bands in single plants. The cultivated parent had the three fastest moving bands; *A. cardenasii* had four bands, of which one was slower moving. Five interspecific hybrids had the same banding pattern as *A. cardenasii*, 21 had the banding pattern of *A. hypogaea*, and four hybrids had unique patterns (Fig. 3). The presence of a band at the same location as found in *A. cardenasii* in interspecific hybrids is evidence for germplasm introgression; but, because the extra band was randomly distributed across previously selected lines with variation for yield and resistance, IDH was not a useful selectable marker for the traits. The additional bands not found in either parent are believed to have come from variants of *A. cardenasii* used for the original cross vs. the seeds analyzed in this study.

The exact banding pattern observed in *A. cardenasii* for MDH was not found in hybrids, whereas 21 hybrids were identical to *A. hypogaea*. Two hybrids expressed an extra slow band which was not observed in either parent, and six others were missing either 1, 2, or 3 bands present in the cultivar (Fig. 3). Although *A. hypogaea* and *A. cardenasii* had identical banding patterns for PHI, bands were missing in seven hybrids as compared to either parent. These two isozyme systems will not be useful for identifying resistant genotypes.

None of the hybrids had the banding pattern for LAP of *A. cardenasii* (three bands), whereas 11 hybrids had the banding pattern of *A. hypogaea* (four bands). Three of four large-seeded, high yielding hybrids were missing the second fastest moving band. The remaining 16 hybrids had five bands, including an extra band not observed in either parent (Fig. 3). Again, the extra band likely was derived from a variant *A. cardenasii* used in the original hybrids.

In a comparison of flower to seed isozyme zymograms, seeds provided variation for the isozymes IDH and MDH that flower tissue did not show; however, GOT was more variant in flowers than seeds. The differences observed between variant patterns of seed and flower in interspecific hybrids for GOT, IDH, LAP, MDH, and PHI are most likely due to developmental differences for expression. Unfortunately, little isozyme variation was observed in *A. hypogaea* in this or other studies (Grieshammer and Wynne, 1990). A similar trend has been reported for RFLPs (Kochert *et al.*, 1991) and randomly amplified DNA (RAPD) markers (Halward *et al.*, 1991, 1992). Thus, making a genetic map for cultivated peanut likely will be difficult using isozymes or other molecular markers.

The use of the flower isozyme extraction techniques described in this paper provide relatively fast, inexpensive, and nondestructive methods to screen a large number of genotypes for isozyme polymorphisms. The technique has potential for identifying lines with interspecific gene transfers. This will become more important in peanut as the number and array of wide hybrids are being increased and evaluated for disease and insect resistances and when (if) patterns of variability can be associated with desired traits. This study indicated that specific PHI bands may be associated with high yields, seed size, or leafspot resistance.

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