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Multivariate analysis of polypeptide synthesis in field-grown maize inbreds and hybrids

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Summary. A leaf disc method is described to permit the localized incorporation of ^{35}S-L-methionine into polypeptides synthesized in individual leaves of maize plants grown in the field. The method of incorporation employs minimal external manipulation of the intact leaf, is simple, repeatable, and may be used at any plant age after leaf emergence. Incorporation (cpm/μg protein) in 12 leaves per plant was compared among three inbred (Oh43, W23, M14) and three F_1 hybrid (Oh43/M14, W23/M14, Oh43/W23) genotypes. The incorporation was 40% higher (hybrid versus inbred) in 9 of the 12 leaves studied. Samples from leaf 07 (7th leaf numbered from base of plant) for four inbreds (Oh43, M14, B73, Mo17) and two pairs of reciprocal F_1 hybrids (Oh43/M14, M14/Oh43; B73/Mo17, Mo17/B73) were labelled in situ using the leaf disc method. Each cultivar was sampled at three different ages in each of 1985, 1986, and 1987. High-resolution, two-dimensional isoelectric focusing sodium-dodecyl-sulfate polyacrylamide gel electrophoresis and fluorography were used to display the polypeptides synthesized in the samples. Multivariate methods – Principal Coordinate Analysis, Cluster Analysis, and Standard Deviation Distance – were used to analyze variation and to identify trends in the variation for year, genotype, and age sampled. Our analyses disclose a hierarchy to polypeptide synthesis variation in maize leaves: differences in polypeptide synthesis are greater for year-to-year comparisons than differences due to sample age, which in turn are greater than differences for inbred versus hybrid comparisons.

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Introduction

Incorporation of labelled amino acids into plant tissues has made possible the study of protein synthesis in a number of species. In most cases where label is applied to a particular plant tissue, the presentation of the label involves some type of external manipulation (e.g., wounding), which may stress the tissue and cause altered protein synthesis (Baszczynski 1984). To minimize such stress and to facilitate surface application of the label, leaf tissue was chosen for this study.

A large number of polypeptides synthesized during development may be visualized using the methodologies of two-dimensional isoelectric focusing, sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (2D-IEF-SDS-PAGE) (O'Farrell 1975) and fluorography (Bonner and Laskey 1974). Until recently, researchers have relied on visual inspection to analyse electrophoretic/fluorographic variation, particularly for single or small groups of gene products. A novel approach developed by Fewster and Walden (1987) permitted the comparison of polypeptide synthesis patterns by the partitioning of variation (eigenvector analysis) derived from Principal Coordinate Analyses (PCoA). Using a Sum of Squares Cluster Analysis (SSCA) (Orloci and Kenkal 1985) and the Standard Deviation Distance (SDD) test, statistical inference may be assigned to comparisons of patterns.

This contribution outlines a leaf disc method to label in situ samples from individual maize leaves. The incorporation of radioactive amino acid into 12 individual leaves on three inbred and three F_1 hybrid genotypes

grown in the field was assessed. A specific, representative leaf was chosen for further study in which electrophoretic, fluorographic, and the multivariate statistical methods were used to compare polypeptide synthesis patterns derived from leaves of different ages sampled from (i) three consecutive growing years, and (ii) four inbred and four F_1 hybrid genotypes. These factors are assessed to permit the rational development of a protocol for further study of field-grown genotypes.

Materials and methods

Genetic material

Inbreds Oh43, M14, W23, B73, Mo17 and F_1 hybrids Oh43/M14, M14/Oh43, B73/Mo17, Mo17/B73, Oh43/W23, W23/M14 were used in one or both studies. Each inbred has been maintained in our nursery for 25 or more generations.

Sampling

Samples were collected from 12 individual leaves on each of three inbred (Oh43, M14, W23) and three F_1 hybrid (Oh43/M14, Oh43/W23, W23/M14) genotypes in 1985 or from leaf 07 on four inbred (Oh43, M14, B73, Mo17) and four F_1 hybrid (Oh43/M14, M14/Oh43, B73/Mo17, Mo17/B73) genotypes in 1985, 1986, and 1987. Three different sampling times in each of an early and late planting were utilized. Mean values ± standard error (SE) were calculated to compare ^{35}S-L-methionine uptake between the inbred and hybrid genotypes collected in 1985. Leaf 07 was the 7th leaf from the base of the plant. For Oh43, M14, Oh43/M14, M14/Oh43, samples were collected in 1985 at 60, 90, and 119 days after planting (dap); in 1986 at 52, 76, and 110 dap; and in 1987 at 61, 77, and 92 dap. For B73, Mo17, B73/Mo17, Mo17/B73, samples were collected in 1985 at 66, 85, and 103 dap; in 1986 at 54, 82, and 110 dap; and in 1987 at 66, 78, and 97 dap. Days after planting were further defined for the eight genotypes by the following: 52–66 dap = early; 76–90 dap = intermediate; and 92–119 dap = late sampling age.

The details of sampling, meteorological, and agronomic data pertaining to the three growing seasons were collected and recorded (Crowe 1988).

Leaf disc method

(i) Application of amino acid. The introduction of the radioactive 25% (v/v) solution of ^{35}S-L-methionine (630 μCi/ml; New England Nuclear, Dupont 1097 Ci/mmol) in sterile, distilled water containing 2% (v/v) Tween-20 surfactant (BioRad) into a maize leaf in situ was accomplished through direct application (Crowe and Walden 1986, 1987). An area encompassing the midpoint of the maize leaf was wiped gently with a 0.5% Javex solution prior to a thorough rinse with distilled water. A 2.5-cm diameter rubber ring was affixed to the dry leaf surface on one side of the midrib with a small amount of Thomas Lubriseal (Canlab) (Fig. 1). A 20 μl aliquot of the ^{35}S-L-methionine solution was applied at 1,000 h for 1.5 h. At the end of the 1.5-h incubation, the stalk was cut at ground level and transported to the laboratory. A 1.5-cm diameter leaf disc, within the labelled area, was excised using a sterile cork borer. The leaf disc was rinsed with distilled water and placed in a cold 45-ml ceramic mortar.

(ii) Extraction. A 250-μl aliquot of plant extraction buffer (Hughes et al. 1981) modified by the addition of 0.1 mg/ml in-

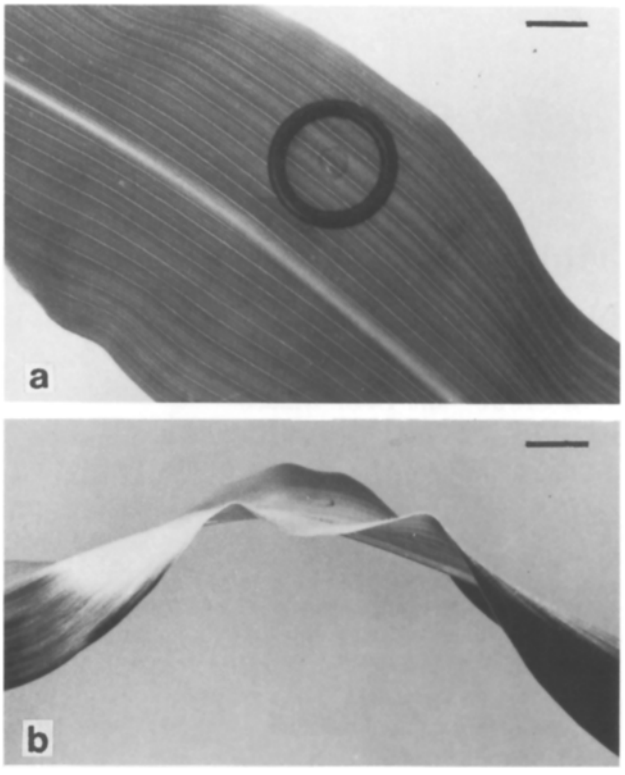

Fig. 1 a and b. Photograph of the upper surface of the maize leaf showing the position of the rubber confinement ring (diameter 2.5 cm). A 20-μl aliquot of ^{35}S-L-methionine solution was applied within the ring. **a** Top view of leaf (bar = 17 mm). **b** Side view of leaf, confinement ring removed (bar = 40 mm)

soluble polyvinylpyrolidine (Polyclar AT, BDH) was added to the mortar, and the sample was ground to yield a uniform extract. Insoluble polyvinyl-pyrolidine was included in the extraction buffer to precipitate phenolic compounds and prevent matting of the sample during isoelectric focusing. The extraction was transferred to a 400-μl Eppendorf microtube; the sample was placed in a boiling water bath for 3 min and centrifuged at 16,000 × *g* for 5 min. The supernatant was drawn off and placed in a 400-μl Eppendorf microtube. Prior to storage at –20 °C, a 10-μl aliquot was spotted onto a Whatmann glass filter disc and the incorporation of radioactive amino acid into trichloroacetic acid (TCA) precipitated material was determined (Mans and Novelli 1960).

(iii) Protein determination. The protein was determined by the TCA turbidimetric assay (Comings and Tack 1972) using bovine serum albumin (BSA) (Sigma) as a standard for protein concentration.

Electrophoresis and fluorography

High-resolution 2D-IEF-SDS-PAGE (O'Farrell 1975) and fluorography (Bonner and Laskey 1974) were performed (Boothe and Walden 1989). Duplicate polyacrylamide gels were run for each individual sample. Both a light and a dark fluorographic X-ray film exposure were produced from each gel to ensure complete visualization of all labelled polypeptides within a single sample.

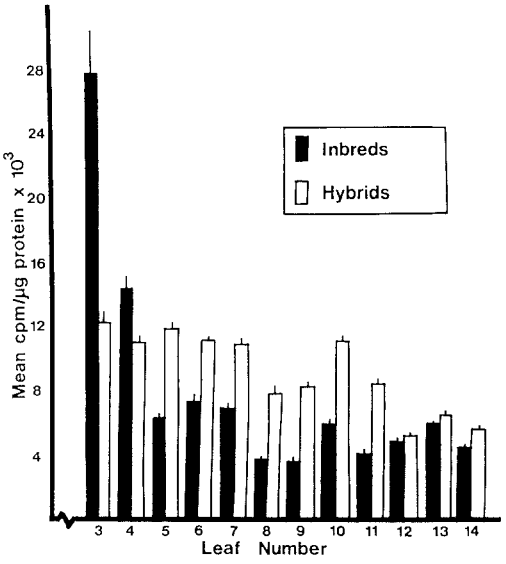

Fig. 2. Incorporation of ^{35}S-L-methionine into individual maize leaves for grouped inbred and hybrid genotypes. (Mean ± SE)

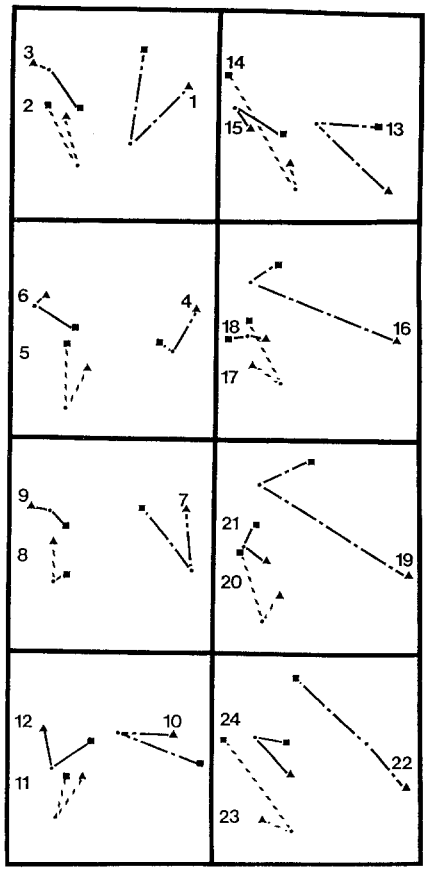

Fig. 3. Individual trajectory diagrams for PCoA-I and PCoA-II. The abscissa is the first principal coordinate axis and the ordinate is the second. Trajectories and symbols are identified in Table 2

Table 1. Eigenvector analysis values

Analysis	Genotype	Axis[a]	RGU[b]	Information %	Cumulative %
PCoA-I	Oh43, W23,	1	1, 6, 12, 16	35	–
	Oh43/W23,	2	2, 6, 10, 12, 13	17	52
	W23/Oh43	3	1, 2, 4	12	64
PCoA-II	B73, Mo17,	1	1, 6, 12, 16	35	–
	B73/Mo17,	2	2, 6, 12	21	56
	Mo17/B73	3	1, 2, 9, 12, 16	8	64

[a] Axis – Principal coordinate axis
[b] RGU – Relevant grid units (Fig. 1, Boothe and Walden 1989)

Multivariate statistical analyses

A 4 × 4 grid matrix was placed over each fluorogram; a score that derived from the number of proteins (polypeptides) and their intensity on the fluorogram was recorded from each grid (Fewster and Walden 1987; Boothe and Walden 1989). The multivariate statistical analyses performed included Principal Coordinate Analysis (PCoA)/eigenvector analysis (Fewster and Walden 1987), Sum of Squares Cluster Analysis (SSCA) (Orloci and Kenkal 1985), and Standard Deviation Distance (SDD) (Jancey 1966a; Crowe 1988). Results are presented graphically as trajectory diagrams. A trajectory is a straight line determined by joining two or more eigenanalysis-derived points (e.g., from a dap sequence). The relative position of the points in the trajectory is the result of the multidimensional relationship calculated by the PCoA. SSCA results are presented as cluster group diagrams. SDD calculations are statistics used to determine cluster group relatedness.

Results

To analyze the isotope uptake data and test for homogeneity, the samples collected from 12 individual leaves on six genotypes were grouped into one data set. The incorporation of ^{35}S-L-methionine into protein in individual leaves of the inbreds (Oh43, M14, W23) and hybrids (Oh43/M13, Oh43/W23, W23/M14) is presented in Fig. 2. Uptake in leaves 05-14 is significantly ($P = 0.01$) greater (approximately 40%) for hybrids ($8.80 \times 10^3 \pm 0.25 \times 10^3$ cpm/µg protein) than for inbreds ($5.28 \times 10^3 \pm 0.25 \times 10^3$ cpm/µg protein). In the lower leaves (03, 04), mean incorporation is 1.8 times greater in the inbred than in the hybrid leaves.

The results from eigenvector analyses from two sets of two inbreds and their F_1 reciprocal hybrids, analyzed separately, are presented in Table 1. In each analysis, 64% of the information is contained within the first three principal coordinate axes.

The individual year-to-year trajectories for the first analysis, PCoA-I (Oh43, M14, Oh43/M14, M14/Oh43) and the second analysis, PCoA-II (B73, Mo17, B73/Mo17, Mo17/B73) are presented in Fig. 3. The year-to-year trajectories for the combined PCoA-I genotypes and

Table 2. Trajectory characteristics

Trajectory number	Genotype	Sample			
		Year	Days after planting		
			Early ■	Intermediate ●	Late ▲
1	Oh43	1985	60	90	119
2	Oh43	1986	52	76	110
3	Oh43	1987	61	77	92
4	M14	1985	60	90	119
5	M14	1986	52	76	110
6	M14	1987	61	77	92
7	Oh43/M14	1985	60	90	119
8	Oh43/M14	1986	52	76	110
9	Oh43/M14	1987	61	77	92
10	M14/Oh43	1985	60	90	119
11	M14/Oh43	1986	52	76	110
12	M14/Oh43	1987	61	77	92
13	B73	1985	66	85	103
14	B73	1986	54	82	110
15	B73	1987	66	77	97
16	Mo17	1985	66	85	103
17	Mo17	1986	54	82	110
18	Mo17	1987	66	78	97
19	B73/Mo17	1985	66	85	103
20	B73/Mo17	1986	54	82	110
21	B73/Mo17	1987	66	78	97
22	Mo17/B73	1985	66	85	103
23	Mo17/B73	1986	54	82	110
24	Mo17/B73	1987	66	78	97

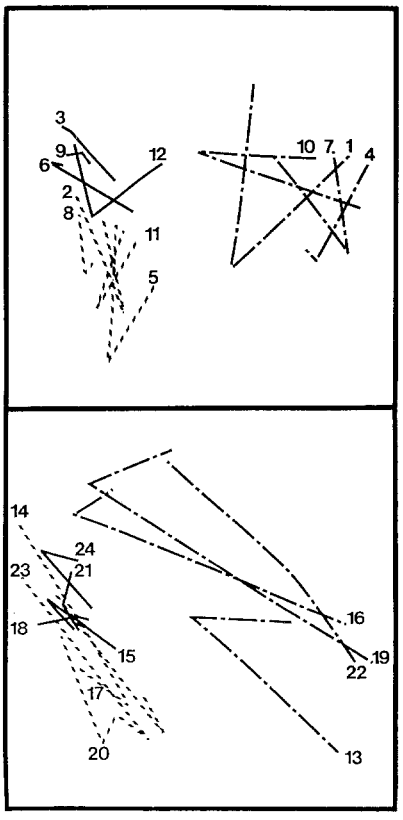

Fig. 4. Combined genotype trajectory diagrams for PCoA-I and PCoA-II. The abscissa is the first principal coordinate axis and the ordinate is the second. Trajectories and symbols are identified in Table 2

combined PCoA-II genotypes are presented in Fig. 4. Each trajectory is numbered and characterized (Table 2).

In Fig. 3, the individual PCoA-I trajectories (1–12) are separate for the 3 years. Some individual PCoA-II trajectories intersect for the years 1986 and 1987 (trajectories 14–15, 17–18) but are separate for 1985; however, the individual F_1 reciprocal hybrid trajectories are separate for each of the 3 years.

The combined genotype trajectories from PCoA-I and PCoA-II (Fig. 4) reveal that the four 1985 trajectories are more dispersed, while those for 1986 and 1987 are grouped more tightly. In the combined PCoA-I results there is little overall similarity in the individual trajectory patterns for any single genotype in the 3-year comparisons. However, in 1985, all four genotype trajectories (1, 4, 7, 10) are grouped at the early age. In 1986, the late age sampled is grouped for M14 and M14/Oh43 genotypes (trajectories 5, 11). Similar groupings are shown for the inbred genotypes in 1987. In the combined PCoA-II results, the trajectories (16, 19) for Mo17 and B73/Mo17 genotypes are similar in overall pattern in 1985 (Fig. 4, symbols in Fig. 3, Table 2). In 1986, the F_1 reciprocal

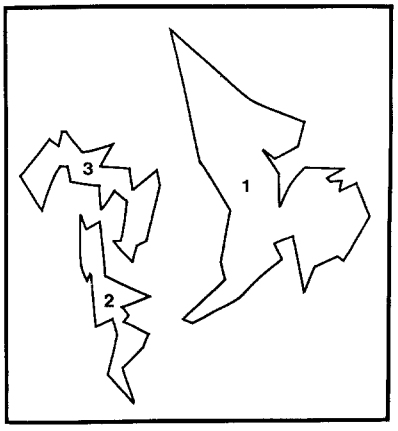

Fig. 5. Cluster diagram for the genotypes in PCoA-I. The abscissa is the first principal coordinate axis and the ordinate is the second. Individual groups are determined by statistical cluster analysis methods. Groups are spatially organized from three axes flattened into a two-dimensional diagram. The outhermost points were joined to give a perimeter outline. Group 1 – 1985 samples, all four genotypes, all ages; group 2 – 1986 samples, all four genotypes, all ages; group 3 – 1987 samples, all four genotypes, all ages

Table 3. Standard deviation distance statistics for two cluster analyses

Analysis	Group	Centroid[a]			SD_i[b]	Δ_{ij}[c]	SDD[d]
		$\bar{X}$	$\bar{Y}$	$\bar{Z}$			
I (Fig. 5)	1[e]	0.195	0.021	−0.023	0.092	1, 1 = 0.000[e]	1, 1 = 0.0
	2	−0.117	−0.122	0.039	0.055	1, 2 = 0.348	1, 2 = 3.8
	3	−0.141	0.065	−0.002	0.036	1, 3 = 0.339	1, 3 = 3.7
II (Fig. 6)	1	0.305	−0.001	0.000	0.055	2, 1 = 0.428	2, 1 = 6.2
	2[e]	−0.119	0.046	0.036	0.069	2, 2 = 0.000[e]	2, 2 = 0.0
	3	0.006	0.260	−0.102	0.045	2, 3 = 0.283	2, 3 = 4.1
	4	−0.063	−0.121	−0.010	0.055	2, 4 = 0.182	2, 4 = 2.6

[a] Centroid – The center of a particular cluster group with regard to the first three principal coordinate axes, X, Y, and Z
[b] SD_i – The standard deviation value for group i
[c] Δ_{ij} – The euclidian distance between the centroid of group i and the centroid of group j
[d] SDD – The standard deviation distance ($SDD = \Delta_{ij}/SD_i$) [adapted from Jancey (1966b)]
[e] Reference group – The group with the largest SD_i value used to calculate SDD

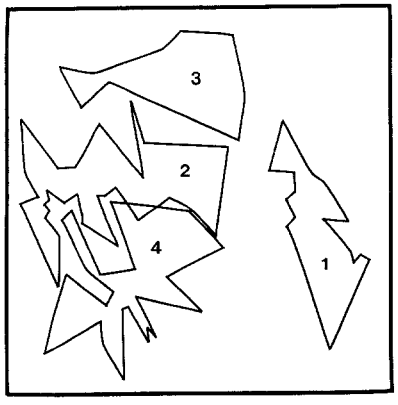

Fig. 6. Cluster diagram for the genotypes in PCoA-II. The abscissa is the first principal coordinate axis and the ordinate is the second. Individual groups are determined by statistical cluster analysis methods. Groups are spatially organized from three axes flattened into a two-dimensional diagram. The outermost points were joined to give a perimeter outline. Group 1 – 1985 samples, all four genotypes, 66 dap age; group 2 – 1987 samples, all four genotypes, all ages; group 3 – 1985 samples, all four genotypes, 85 dap and 103 dap ages; group 4 – 1986 samples, all four genotypes, all ages

hybrids (trajectories 20, 23) are identical at the early sample age. In 1987, there is very little overall trajectory pattern similarity for the four genotypes.

Utilizing the Sum of Squares Clustering method, all four genotypes (Oh43, M14, Oh43/M14, and M14/Oh43) cluster according to year (Fig. 5). Four major groups were determined by the SSCA (Fig. 6) for the genotypes B73, Mo17, B73/Mo17, and Mo17/B73. The 1985 samples were divided between groups 1 and 3; groups 2 and 4 contain the four genotypes sampled in 1986 and 1987.

The Standard Deviation Distance calculations for the two cluster analyses (Figs. 5 and 6) are listed in Table 3. The SDD calculations provide an estimate of the degree of similarity between two cluster groups (Jancey 1966b). If two groups differ by at least two standard deviation distances, the two groups are ($P = 0.05$) significantly distinct (Crowe 1988). In all the clustered groups compared (Figs. 5 and 6) the centroids are significantly distinct.

Discussion

The purpose of this report is (1) to describe a method for in situ application of labelled amino acids, permitting 2D-IEF-SDS-PAGE separation of proteins and fluorography, and (2) to identify the sources of variation inherent in the application of the method.

The in situ application of ^{35}S-L-methionine has been described.

Based upon observations of the incorporation data obtained from samples drawn from three replicates and two plantings, and the analysis of several plant parameters (Crowe 1988), these data were combined in Fig. 2. Figure 2 reveals that leaf 07 exhibits consistent uptake in hybrids and inbreds and is near the midpoint of the plant, often the leaf attached to the top ear-bearing node. Hybrids took up significantly more label than inbreds in leaves 05–14 (Fig. 2). This increased uptake of amino acid by hybrids did not occur in leaves 03 and 04. Age, physiological, and/or metabolic differences between inbreds and hybrids appear to influence ^{35}S-L-methionine uptake. Shading due to leaf position and leaf surface wax differences may also contribute to genotypic differences in amino acid uptake.

The eigenvector values recorded in Table 1 identify the grid units from the fluorograms which contribute the major variation for each of the first three principal coordinate axes. PCoA partitions the variation such that the

greatest percentage of information is contained in the first axis, the next largest in the second, and so on, decreasing until all the information is partitioned (16 grid units providing 16 principal coordinate axes).

The trajectory diagrams demonstrate that the genotypes Oh43, M14, Oh43/M14, M14/Oh43, B73/Mo17, and Mo17/B73 (Fig. 3) are separated during the 3 years. For the inbreds B73 and Mo17, the trajectories for 1986 and 1987 samples overlap. When any two points in a trajectory are separate, there is a greater amount of variation present than if two points are in close proximity to or overlap one another. For the purpose of this study, tightly grouped or overlapping trajectories show greater similarity in polypeptide synthesis as determined by the PCoA.

The PCoA trajectory results identify variation in polypeptide synthesis that appears to be year specific. Additional individual inbred and hybrid comparisons reveal some evidence that variation was age specific per genotype.

Since trajectory values derive from a qualitative assessment, the data sets were analyzed further using the Sum of Squares clustering procedure and Standard Deviation Distance test calculations. The SSCA (Figs. 5 and 6) revealed that the majority of the clustering was due to year-to-year variation. As revealed earlier, the only exception found (Fig. 5) was for M14 and M14/Oh43 genotypes sampled in 1986, where the 110-dap (late) sample clusters with the 1987 samples in group 3. Individuals group according to sample age as well as year in Fig. 6.

Standard Deviation Distances calculated for the centroids of the cluster groups (Table 3) reveal that the centroid of each cluster group is significantly distinct, thereby providing an independent statistical confirmation of the interpretations offered from the examination of the trajectories (PCoA).

The sampling and methodologies used in this study are sufficient to yield fluorographic displays of polypeptide synthesis in maize leaves and to permit the visualization of variation in polypeptide synthesis among genotypes. Multivariate analyses allow this variation to be classified, leading to the following hierarchy for polypeptide synthesis variation in maize leaves: differences in polypeptide synthesis are greater for year-to-year comparisons than differences due to sample age, which in turn are greater than differences for inbred versus hybrid comparisons.

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References

Baszczynski CL (1984) Thermal shift induction and regulation of gene expression in maize. PhD thesis, University of Western Ontario, London, Canada

Bonner WM, Laskey RA (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46:83–88

Boothe JG, Walden DB (1989) Multivariate analyses of polypeptide synthesis in developing maize embryos. Theor Appl Genet 77:495–500

Comings DE, Tack LC (1972) Similarities in the cytoplasmic proteins of different organs and species examined by SDS gel electrophoresis. Exp Cell Res 75:73–78

Crowe TG (1988) Variation in polypeptide synthesis for select maize cultivars. MS thesis, University of Western Ontario, London, Canada

Crowe TG, Walden DB (1986) Use of leaf discs to monitor protein synthesis under field conditions. Maize Genet Coop Newsl 60:92–93

Crowe TG, Walden DB (1987) Use of leaf discs to monitor protein synthesis under field conditions. Maize Genet Coop Newsl 61:72–73

Fewster PH, Walden DB (1987) Multivariate analysis of data from two-dimensional electrophoretic separation of macromolecules. Comput Biol Med 17[1]:29–35

Hughes WG, Baszczynski CL, Ketola-Pirie C (1981) Improved conditions for the extraction of maize polypeptides. Maize Genet Coop Newsl 55:116–117

Jancey RC (1966a) Multidimensional group analysis. Aust J Bot 14:127–130

Jancey RC (1966b) The application of numerical methods of data analysis to the genus *Phylotta benth.* In New South Wales. Aust J Bot 14:131–149

Mans RJ, Novelli DG (1960) A convenient, rapid, and sensitive method for measuring the incorporation of radioactive amino acids into protein. Biochem Biophys Res Commun 3:540–548

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250 [10]:4007–4021

Orloci L, Kenkal NC (1985) In: Statistical ecology monographs, vol 1. Introduction to data analysis. International Cooperative Publishing House, Fairland MD, pp 340