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EVALUATION OF MAIZE GERMPLASM BASED ON ZEIN POLYMORPHISM FROM THE ARCHIPELAGO OF MADEIRA

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ABSTRACT - Zein polypeptides are a group of proteins that accumulate in maize endosperm during seed development, representing more than 60% of the total endosperm proteins in the mature seeds. To evaluate genotype variability of Madeiran maize germplasm, a biochemical study was conducted based on the prolamins of maize, zeins, extracted from endosperm meal of 43 populations of *Zea mays* L. maintained in the Germplasm bank of Madeira University along with the inbreed W64A which was used as a polymorphic zein polypeptide standard profile. The zein polymorphism of these 44 maize populations were compared using two different electrophoresis techniques, SDS-PAGE in 15% discontinuous polyacrylamide gel and Acid-PAGE in 10% continuous polyacrylamide gel. SDS-PAGE allowed up to 16 polypeptides to be identified with apparent molecular mass ranging from 28-kDa to 10-kDa. Acid-PAGE allowed up to 20 zein fractions to be identified. The data was submitted to principal component analysis (PCA) and canonical discriminate and similarity analysis. The SDS-PAGE zein polymorphism allowed us to detect 6 groups, assembling all maize populations and explaining 55.32% of all variability. The similarity analysis of zein patterns obtained by Acid-PAGE showed that among regional maize germplasm, 22.5 % of all population seems to be related and have a common ancestor. The ISOP71 seems to be the population more closed to the common ancestor and appears related with the remaining maize populations, excluding the ISOP125. The obtained results and the importance of zein polymorphism in the evaluation of maize germplasm from Madeiran Archipelago are discussed.

KEY WORDS: Seed storage proteins; Zein; SDS-PAGE; Acid-PAGE, Maize germplasm; Archipelago of Madeira.

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INTRODUCTION

The major storage proteins of maize, the highest-yielding cereal in the world, are contained in the prolamin fraction known as zeins (for a recent review, see MOTTO *et al.*, 1996). These proteins are synthesized in the developing endosperm between 15 and 50 days post pollination and account for 50% or more of the total protein in the mature seed (for a review see MOTTO *et al.*, 1997). All polypeptides of this fraction are synthesized by membrane-bound polysomes and stored in protein bodies, where these proteins are exclusively found (cfr. COLEMAN *et al.*, 1997).

The zein family of proteins consists of a mixture of alcohol-soluble polypeptides that can be resolved by SDS-polyacrylamide gel electrophoresis into up to ten components with apparent molecular masses of about 28, 23, 22, 21 (22-kDa class), 20, 19, 18 (20 kDa class), 16, 14 (10 kDa class) and 10 kDa class (GIANAZZA *et al.*, 1976). SHEWRY and MIFLIN (1985) and SHEWRY and HALFORD (2002) proposed to group these polypeptides in A, B, C and D classes instead of being referred according to their apparent molecular weight classes while ESEN (1987) divide the proteins into classes designated α-, β-, and γ-zeins that correspond to the 22000 and 20000, the 16000 and 14000, and the 28000 Da components, respectively. However, the 16-kDa proteins are structurally more similar to the 28-kDa proteins than the 14-kDa proteins (PRAT *et al.*, 1985). The 10-kDa proteins are structurally different from the others (KIRIHARA *et al.,* 1988) and constitute a fourth class, δ-zein (WALLACE *et al.*, 1988). Zein proteins with small mass differences are difficult to separate by sodium dodecyl sulfate polyacrylamide gel electrophoresis and can be resolved through mass spectrometry. Mass signals corresponding to 10-kDa δ-, 15-kDa β-, 16-kDa γ-, 27-kDa γ-, and several 19 and 22-kDa α-zeins

were detected (ADAMS *et al.*, 2004). The α-zeins are the products of a large multigene family clustered in at last three genomic regions, whereas the other zein proteins are encoded by gene present in a few copies (reviewed by MOTTO *et al.*, 1997). These two classes of genes arise from an ancestral gene followed by further divergence through point and chromosomal (mainly duplication) mutations (RUBENSTEIN and GERAGHTY, 1986).

The 22- and 20-kDa α-zeins constitute about 70% of the α fraction and comprise a complex group of polypeptides that vary in size (VIOTTI *et al.*, 1985) and charge (SOAVE *et al.*, 1979). At least 15-20 components are usually resolved by isoelectric focusing (IEF), differing both qualitatively and quantitatively among maize lines (GIANAZZA *et al.*, 1976). The IEF heterogeneity is genotype-specific and is inherited codominantly in a simple Mendelian fashion (RIGHETTI *et al.*, 1977). Heterogeneity has been useful in distinguishing among various genotypes (NUCCA *et al.*, 1978; WILSON, 1992).

In crop plants intraspecific taxonomy has been successfully and widely utilized by employing proteins for varietial classifications especially in wheat (MENKE *et al.*, 1973) and sorghum (SHECHTER and DE WET, 1975). Among the plant proteins, seed storage proteins have been preferred because they are readily obtainable and extractable. In cereals, the seed prolamins have been adopted as taxonomic traits in barley (SHEWRY *et al.*, 1978) and wheat (AUTRAN and BOURDET, 1975). Our group is mainly involved in the evaluation of germplasm diversity of maize and wheat using morphological, biochemical and molecular approaches with special reference to stress tolerance (PINHEIRO DE CARVALHO *et al.*, 2003, 2004). This work presents data on the evaluation of Madeiran maize germplasm based on zein protein polymorphism according to standard procedures.

MATERIALS AND METHODS

A sample consisting of 43 populations of maize from the Germplasm Bank of University of Madeira (Portugal) and an inbreed W64A obtained from the Istituto Sperimentale per la Cerealicoltura, Bergamo (Italy) as standard were used in the present study (Table 1). The storage protein, zein was extracted from the endosperm meal following the protocol described by NUCCA *et al.* (1978). The zein polymorphism analysis was performed using SDS and Acid polyacrylamide gel electrophoresis according to the modified protocols of LAEMMLI (1970) and MOREL (1994) respectively.

Ten kernels from each population were separately crushed and extracted with 70% ethanol (v/v) and 2% DTT for 2 hr 20 min. at 60ºC, centrifuged at 12,000 rpm for 10 min. and precipitated with acetone (1:4, v/v). The pellet was allowed to dry under a hood for 30 min. and solubilized with the respective sample buffer (0.1% SDS, 1M Tris pH 6.8, 2-mercato-ethanol, 60% glycerol) at a ratio of 1:4 and heated at 100ºC for three min. prior to loading. Twenty µg of zein extracts from each sample were loaded in the presence of W64A and molecular size marker (Amersham Biosciences) as standards. The LAEMMLI buffer system (1970) was used with 15% polyacrylamide gels (160 x 180 x

TABLE 1 - *List of 43 maize populations used in the evaluation of zein electrophoretic patterns corresponding to a collection maintained at the ISOPlexis Germplasm Bank of Madeira University.*

Sample number	Accession Number, ISOP	Collection Site
1	61	Santa Cruz
2	62	Faial
3	63	Canhas
4	64	Estreito da Calheta
5	65	São Roque do Faial
6	66	Quinta Grande
7	68	Santo António
8	69	Santana
9	70	Faial
10	71	Ilha
11	75	Santo da Serra
12	125	Ponta do Pargo
13	128	Ribeira Brava
14	129	S. Vicente
15	130	Ribeira Brava
16	131	Ribeira Brava
17	132	Ribeira Brava
18	133	S. Vicente
19	134	P. Santo
20	135	S. Vicente
21	136	S. Vicente
22	137	Santa Cruz
23	138	Machico
24	139	Machico
25	140	Porto Moniz
26	141	Calheta
27	142	Calheta
28	143	Porto Moniz
29	146	São Roque do Faial
30	147	Canhas
31	148	Calheta
32	149	Ponta do Sol
33	150	Ribeira Brava
34	151	Câmara de Lobos
35	152	Câmara de Lobos
36	153	Ponta do Sol
37	154	Câmara de Lobos
38	155	Santana
39	156	Santana
40	157	Santana
41	158	Machico
42	159	Ponta do Sol
43	160	Porto Moniz

0.75mm) and was run at 20 mA per gel for 4 hr 30 min at constant temperature. After electrophoresis, the gels were silver stained according to BLUNT *et al.* (1987) and dried on a Dry gel system for 24 hr.

An aliquot of 150 µl of reduced zein was precipitated with cold acetone (1ml), centrifuged at 12000 rpm for 15 min at 0ºC and dried under a hood for 1h. The dried pellet was resuspended in sample buffer consisting of 6 M urea, 30% (w/v) glycerol and 0.025 M acetic acid. Fifteen µl aliquots of the extracts were loaded in the presence of W64A on the gel and fractionated according to the procedure of MOREL (1994). Acid-PAGE of 10% polyacrylamide gels was achieved as described by REDAELLI *et al.* (1994). The gels were stained in 12.5% (w/v) trichloroacetic acid, 0.01% (w/v) Coomassie Brilliant Blue R250 and distained with distilled water (REDAELLI *et al.*, 1994).

The relative electrophoretic mobility of zein bands in protein patterns of 10 individuals per population has been determined based on the analysis of SDS-PAGE and Acid-PAGE. The individual zein patterns were compared and results of the same have been used in the elaboration of consensus zein pattern according to KONAREV *et al.* (1979). The results were statistically analysed through multivariate analysis. A canonical variable analysis was employed to ordinate population means considering variance and covariance among characters. Hierarchical cluster analysis using the UPGMA method and coefficient of Squared Euclidean distances were performed considering variability within populations. Principal Components Analysis (PCA) – principal factor analysis – was used as an objective method to summarise variation when a prior knowledge of population to which certain individuals belonged was disregarded. Factor analysis of mean values and standard deviations were performed based on EIGEN coefficient and Varimax with Kaiser Normalization, using SPPS for Windows version 9.0 (KINNEAR and GRAY, 1999). Similarity matrix was determined according to AUTRAN and BOURDET (1975).

RESULTS AND DISCUSSION

A large variability in SDS polyacrylamide gel banding pattern of zein were observed after an extensive analysis of the 43 maize populations (Fig. 1). Four major groups of zeins consisting of about 14 polypeptides for all populations and 9 to 12 polypeptides per population are found in SDS gels with molecular masses of about 22-, 20-, 16-14 and 10-kDa and along with a non-zein rsp proteins soluble in alcoholic solutions (WILSON, 1984). Based on ESEN (1987) classification, the zein patterns obtained for Madeiran maize on a SDS-PAGE allowed us to identify at least four additional γ-zeins, with molecular masses of 26.76-kDa; 26.34-kDa; 25.56-kDa and 24.6-kDa.

The data was submitted to principal component analysis, which revealed that the polymorphism could be explained by 14 variables. However, only two are necessary to summarise 55.32% of the total variation. These results suggest a strong correlation between the zein polymorphism and the uniform distribution of variance among the different variables (Table 2). This analysis allowed us to conclude that 14-kDa and 15-kDa β-zeins, and 25.56-

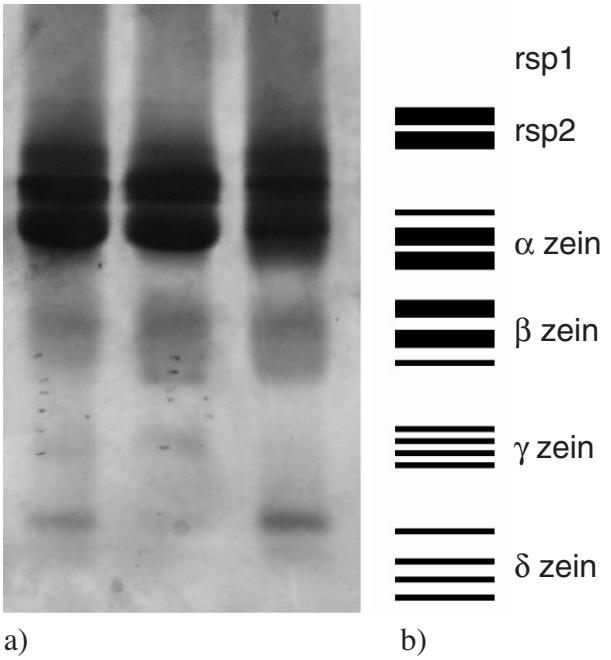

FIGURE 1 - Example of Zein pattern fractions for two Madeiran populations obtained by SDS-PAGE. a) Lane 1: Isop146 and Lane 2: Isop148 in regard to the W64A standard (Lane 3). b) Schematic diagram of the pattern of zeins on SDS-PAGE.

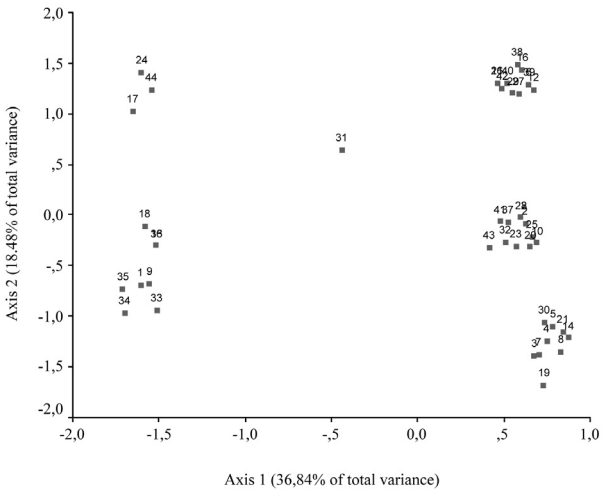

FIGURE 2 - Principal component analysis on the 43 madeiran maize populations and inbreed line W64 based on zein polymorphism observed on 15% SDS-PAGE. PCA variation explains along the first axis is 36.84% and along the second axis is 18.48%.

TABLE 2 – *Distribution of variance among 14 variables according to principal component analysis of zein polymorphisms on SDS-PAGE and among 7 variables on Acid-PAGE.*.

		Component													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
SDS-PAGE	% Total Variance	36.83	18.47	12.18	9.11	7.07	5.30	3.35	2.83	2.29	1.19	0.56	0.39	0.26	0.11
	Cumulative % of Total Variance	36.83	55.31	67.49	76.61	83.68	88.90	92.34	95.17	97.46	98.66	99.23	99.62	99.88	100
Acid-PAGE	% Total Variance	23.22	20.42	14.50	8.34	6.38	6.29	5.27							
	Cumulative % of Total Variance	23.22	43.63	58.14	66.48	72.86	79.15	84.42							

kDa γ-zein were weakly correlated with the observed variation because all of them are present in the populations studied, while rsp_1, rsp_2, 16-kDa β-zein and 26.34-kDa γ-zein were strongly correlated with the observed variability. PCA analysis showed an agglomeration of the 43 maize populations into six groups (Fig. 2), explaining first component (axis) 36.84% of cumulative variance and the second component (axis) 18.48% of cumulative variance.

Discriminate analysis data had 93.2% of cross-validated group cases correctly classified and three major variables (genotype formula, 26.34-kDa γ-zein and 24.6-kDa γ-zein contributing to the total variability were identified. The dendogram with 43 maize populations display based on between-cluster variance determined a distance coefficient lower 25%, which allow us to conclude that all maize populations are closely related (Fig. 3).

A considerable polymorphism resulting from Acid polyacrylamide gel banding pattern of zein were observed after an extensive analysis of 43 maize populations (Fig. 4). For all populations up to 20 polypeptides were identified, with a variation of 5 to 10 polypeptides per population. Acid-PAGE data was submitted to similarity matrix analysis. Similarity values between maize populations vary from 0.12 to 1.0. In 22.5% of the cases, the similarity values vary between 0.8 and 1.0, suggesting that they are closely related and has a common ancestor. The ISOP71 population shows similarity values of 0.85 and 1.0 with all Madeiran populations excluding the ISOP125.

Acid-PAGE data was also submitted to principal component analysis, being all the polymorphism explained by 6 variables. However, only three variables are needed to summarise 58.14% of the total

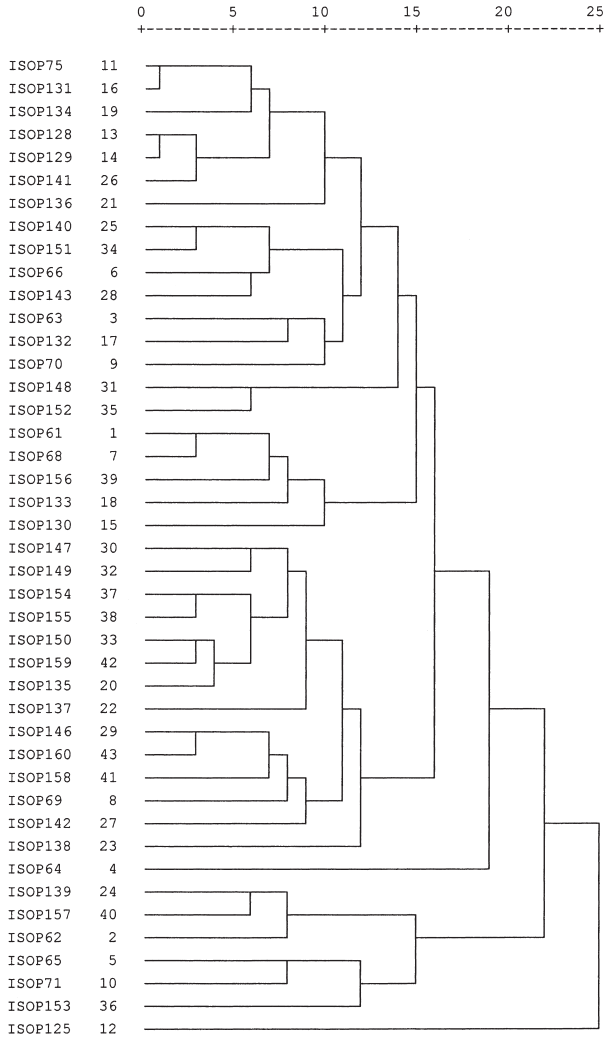

FIGURE 3 - Tree diagram of the 43 populations of maize according to zein pattern observed on 15% SDS electrophoresis separation. The UPGMA method and the Squared Euclidean distance Coefficient were employed in this analysis.

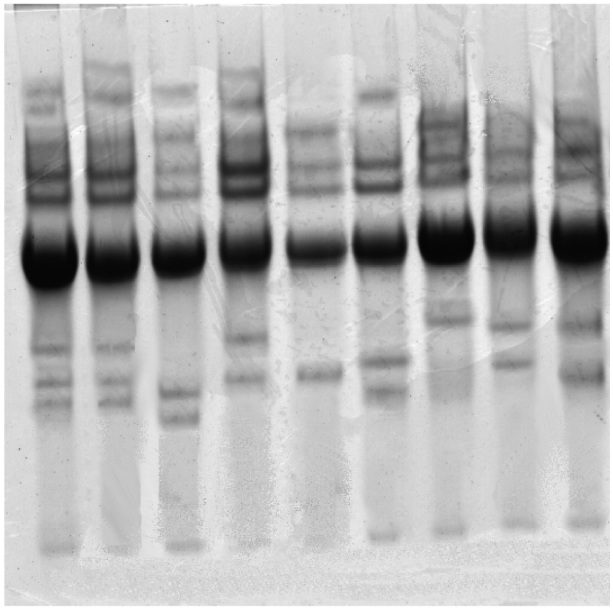

FIGURE 4 - An example of maize zein pattern obtained by Acid-PAGE separation of extracts of 9 Madeiran populations. From left to the right: Lane 1, ISOP61; Lane 2, ISOP68; Lane 3, ISOP70; Lane 4, ISOP129; Lane 5, ISOP140; Lane 6, ISOP150; Lane 7, ISOP155; Lane 8, ISOP159 and Lane 9, ISOP160.

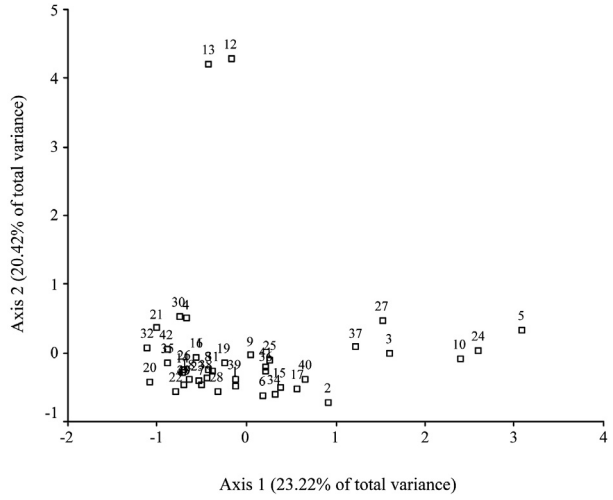

FIGURE 5 - Principal component analysis on the 43 madeiran maize populations based on zein polymorphism observed on 10% Acid-PAGE. PCA variation explains along the first axis is 23.22% and along the second axis is 20.42%.

variability (Table 2). PCA analysis showed an agglomeration of the 43 maize populations into four groups (Fig. 5), explaining first component axis 23.22% of cumulative variance and the second component axis 20.42% of cumulative variance.

Discriminate analysis of the data on Acid–PAGE had 76.7% of cross-validated group cases correctly classified and two major variables (B4 and B7) contributing to the total variability were identified. The hierarchical cluster analysis with the 43 maize populations display based on between-cluster variance determined a distance coefficient lower than 25%, again suggesting a strong correlation between the zein polymorphism and the uniform distribution of the variance among the different variables, allowing us to conclude that all maize populations are closely related (Fig. 6).

The seed storage polymorphism is currently used in crop variety identification (MENKE *et al*., 1973; KONAREV *et al*., 1979) or to evaluate its germplasm (SHEWRY *et al*., 1978). Zein peptides represent the product of several structural genes located in different places of the genome (cfr. MOTTO *et al*., 1989) accounting this has an explanation of their reliability in measuring the genetic similarity or variability among maize populations (NUCCA *et al*., 1978). Inheritance of zein components in maize has been studied by acidic continuous polyacrylamide gel electrophoresis revealing extraordinarily high polymorphisms of zeins. (ZAYAKINA and SOZINOV, 2000).

In this report, the zein patterns obtained through electrophoresis separation in SDS-PAGE and Acid-PAGE has been used to evaluate the germplasm of Madeiran maize. The maize in the Archipelago of Madeira being one of the oldest crops has accumulated a substantial genetic diversity. Fourteen zein fractions have been detected in the analysed maize populations. This is in agreement with a similar zein polymorphism and fraction composition reported by ADAMS *et al.* (2004) and SALAMINI *et al.* (1985).

The evaluation of maize germplasm using these zein fractions as markers allow us to detect six distinct groups among Madeiran maize, explained by 55.32 and 58.14% of accumulated variability using SDS and Acid-PAGE analyses, respectively. This variability is related with the observed variations in zein through SDS-PAGE and rsp pattern composition. However, special maize population separations based on Acid-PAGE zein patterns are less evident than in the case of SDS-PAGE.

The rsps are zein associate proteins and have been located in the periphery of protein bodies by immunocytochemical labelling. The zeins represent a multigenic family, with being silent genes, pseudogenes or individual genes. The amount of zein fractions in the seeds depends upon the num-

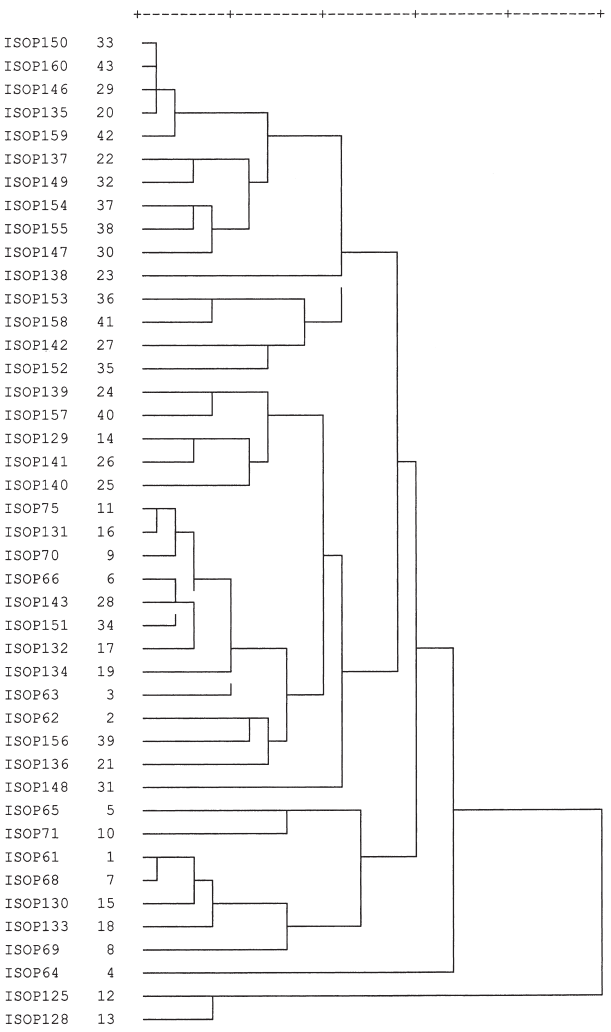

FIGURE 6 - Tree diagram of 43 populations of maize according to zein pattern observed on 10% Acid-PAGE separation. The UPGMA method and the Squared Euclidean distance Coefficient were employed in this analysis.

ber of genes for the zein present in that genome (WILSON, 1984; SOAVE and SALAMINI, 1984). Several studies employing allele mutants of zein genes have revealed that in protein synthesis multiple regulatory pathways are active, acting either through the reduction of the synthesis or of the storage deposition (SONG and MESSING, 2003). These pathways act on all zein fractions or on some zein groups preferentially to a different extent (WOO *et al.*, 2001; SALAMINI *et al.*, 1985). These facts led us to conclude that observed zein polymorphism among Madeiran maize could be the result of the action of unknown regulatory mechanisms controlling the rate of zein synthesis and deposition or the result of variability created by open-pollination of maize populations. The maize is one of the oldest crops cultivated in the Archipelago of Madeira, with the first introductions occurring during the eighteen century (SILVA and MENESES, 1984). Studied maize collection was composed of seed populations maintained by farmers for several generations. The PCA analysis shows a strong correlation between observed maize variability and zein polymorphism. The discriminate analysis determined that in 93.2% of the cases the maize populations are correctly correlated. At the same time, obtained values for distance coefficient, lower than 25% shows that all maize populations are closely related. This observation is consistent with a model of Madeiran maize germplasm evolved from a small number of introductions on the Island. ACID-PAGE zein polymorphism was performed based on the similarity analysis also confirms this model. According to parental analysis carried out 22.5% of all populations are related and share a common ancestor. A maize population, the ISOP71, appears related with all crop populations, excluding the ISOP125. The ISOP71 seem to be the closest maize populations to this common ancestor. If consider that the ISOP71 is originated from Ilha, Santana and that the maize was for the first time introduced in the county of Santana (SILVA and MENESES, 1984) and we can have a confirmation of the model of Madeiran maize origin. Despite of the fact that discriminate analysis shows more than 77% of maize populations are not related with the remaining populations and their zein polymorphism can be the result of open-pollinated variability.

Our data on zein polymorphism show that in spite the distance proximity among Madeiran maize populations determined by SDS-PAGE analysis, according to Acid-PAGE data a high diversity also exists. The detected Madeiran maize diversity through zein polymorphisms can be compared with the diversity observed in relation to aluminium tolerance, where a high variability in the crop responses to aluminium tolerance was identified (PINHEIRO DE CARVALHO *et al.*, 2004).

Further perspectives would be the comparison of this seed protein profiles with a morphologic characterization of the same populations in order to identify a possible relationship between genotype and phenotype. The zein polymorphism seems to be a powerful tool in the evaluation of maize germplasm and in the establishment of core collections. The analysis of zein patterns according to

KONAREV *et al.* (1979) allows us to obtain the genotype formula used in the elaboration of germplasm passports and population identification. The identification of maize varieties and landraces allow us to determine cultivated forms of maize existing within them.

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