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# Esterase Polymorphism Marking Cultivars of *Manihot esculenta*, Crantz

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## ABSTRACT

*Esterase isozymes were used to detected substrate-preference polymorphism in twenty cultivars of* Manihot esculenta*, and to show cultivar-specific variation of this species. A relatively complex extraction solution of proteins from leaves was needed to show a larger number of esterase isozymes. Similarity between cultivars from six groups ranged from 51 to 96%. The cultivars identified by the same name seemed to be biochemically different regarding esterase isozymes. Esterase isozyme electrophoretic patterns could, therefore, be used to discriminate the cultivars identified by the same name, and to monitor the vegetative propagation of cultivars maintained in the germplasm collection. In breeding strategies, isoesterase analysis could be used to avoid intercrossing between the similar genotypes.*

**Key words:** Cassava, isozymes, esterases, *Manihot esculenta*, cultivar-specific isoesterases

## INTRODUCTION

The ability to reliably identify different genotypes is important in breeding programs that rely on clonal propagation. An estimate of the amount of variation within a species is useful for predicting potential genetic gain in a breeding program and in testing population genetic hypothesis (Tanksley and Orton, 1983; Soltis and Soltis, 1989). Isozyme studies offer many advantages over morphological methods and have been proposed as important tools for the characterization of the genetic variation in cassava germplasm and to identify different groups of clones revealing a geographical pattern (Lefevre and Charrier, 1993; Dulloo et al., 1997). Esterase isozyme analysis is of particular interest. Esterases are proteins capable of hydrolyzing esters present in biological material of all organisms. Nonspecific esterases are usual markers in genetic studies because they are easy to detect, and appear to be highly polymorphic (Davis, 1964). For most esterases, a rather general substrate specificity is obtained, indicating that they may have a broad biological function (Aldridge, 1993). In the present study, esterase isozymes were used to detect substrate-preference polymorphism in twenty cultivars of *Manihot esculenta*, and to show the cultivar-specific variation in this species. The different *M. esculenta* cultivars have been maintained in the germplasm collection of the Agronomy Department, Maringá State University. The germplasm collection (BG) was originated from traditional cultivars collected in the southwestern and northwestern regions of the State of Paraná,

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Brazil. The cultivars have been maintained by vegetative propagation for five years and are useful in production programs (Vidigal-Filho et al., 2000). Therefore, it is important to know the genetic variation present in the collection. The hypothesis is that a single genotype might be cultivated in various places under different names, or that different genotypes might be identified by the same name in different places.

**Table 1 -** Regional origin (Southwestern: SW, and Northwestern: NW regions) of the *Manihot esculenta* cultivars maintained in the germplasm bank (BG) of the Agronomy Department, State University of Maringá, Parana, Brazil.

| Region | Cultivars | Region | Cultivars |
|---|---|---|---|
| **SW** | **Fécula Branca (BG 5)** | **NW** | **Fibra (BG 1)** |
| | Alegretti (BG 6) | | Espeto (BG 9) |
| | Amarela (BG 7) | | Vermelha (BG 10) |
| | Verdinha (BG 11) | | Branca (BG 14) |
| | Verdinha (BG 12) | | Amarela (BG 15) |
| | Amarela (BG 13) | | Araruna (BG 20) |
| | Renascença (BG 18) | | José Mendes (BG 21) |
| | Stalina (BG 19) | | Amarela (BG 23) |
| | Fécula Branca (BG 22) | | Pão-do-Chile (BG 25) |
| | Mico (BG 24) | | |
| IAC(*) | Branca de Santa Catarina (BG 2) | | |

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## MATERIAL AND METHODS

Table 1 show the regional origin of the different *M. esculenta* cultivars used as samples.The cultivars were planted yearly at the Iguatemi Experimental Farm of the Maringá State University. The electrophoretic evaluations were carried out in December/1998 and January/1999. Samples of young unexpanded leaves (10-20 mm length) of each *M. esculenta* cultivar were individually homogenized with a glass rod in an Eppendorf tube using 80 μL of a 1.0 M phosphate buffer, pH 7.0, and three different proportions of phenol-complexing agent and antioxidant agents: a) 5% PVP-40 (polyvinylpyrrolydone), 0.5% β-mercaptoethanol solution, and 0.1% Triton X-100; b) 10% PVP, 1.0 mM EDTA, 1.0% β-mercaptoethanol solution, and 0.1% Triton X-100; c) 5% PVP-40, 0.01 M DTT (dithiothreitol), 10 mM sodium metabisulfite, 50 mM ascorbic acid, 1.0 mM EDTA, and 0.5% β-mercaptoethanol solution. After homogenization, the samples were centrifuged at 14,000 rpm for 30 minutes at 4 °C in a Beckman GS-15R centrifuge. The supernatants were absorbed with Watman n$^{o.}$ 3 paper strips (5 x 6 mm), which were vertically inserted into a 14% starch gel (penetrose-30), prepared in 0.01 M Tris and 0.0028 M citric acid buffer, pH 7.5. In the electrode chambers 0.1 M Tris and 0.028 M citric acid, pH 7.5, was used. Electrophoresis was carried out at low temperature (10 °C), for approximately 5-6 hours, at 35 mA (8.5 V/cm of gel). The esterase isozymes were visualized by procedures originally described by Hopkinson et al. (1973) and Coates et al. (1975), and modified in the present study. 4-Methylumbelliferyl esters (acetate, propionate, butyrate) and fluorescein diacetate, and α– and β–naphthyl acetate, α–naphthyl propionate, and α– and β-naphthyl butyrate were utilized as substrates (Tashian, 1969). 4-Methylumbelliferyl ester substrates (4 mg) were dissolved separately in 500 μL acetone and the volume completed to 10 mL using twice-distilled water. After staining with 4-methylumbelliferyl acetate, propionate and butyrate, the gels were washed with tap water and incubated for 30-60 min in a solution containing 50 mL 0.05 M sodium acetate, pH 6.5, 40 mg fast blue RR salt and 4 mL 1% α-naphthyl acetate, β- naphthyl acetate, α-naphthyl butyrate, β-naphthyl butyrate, and α-naphthyl propionate. The α- and β-naphthyl esters were prepared in 50 mL acetone and the volume completed to 100 mL with twice-distilled water. Stained gels were fixed in methanol, twice-distilled water and acetic acid (5:5:1) for 3 min, and washed with tap water. Data were analyzed by comparing the esterase patterns on the basis of presence or absence of each esterase isozyme. The similarity between strains was calculated using Jaccard's coefficient;

UPGMA cluster analysis was performed using the NTSYS-pc software (Rohlf, 1989).

## RESULTS

At least five plants of each *M. esculenta* cultivar were used with the eight esterase substrate to check the consistency of isozyme banding patterns. A higher number of esterase isozymes were identified as more strongly stained bands in C extraction solution. In A and B extraction solutions, a lower number of isoesterases were observed as diffused and faintly stained bands, using 4-methylumbelliferyl esters. In A and B extraction solutions, only three and five esterase-activity regions, respectively, were detected using 4- methylumbelliferyl acetate, while fourteen isoesterases were observed in C extraction solution. The esterase isozyme patterns observed with 4-methylumbelliferyl acetate, propionate, or butyrate, and fluorescein diacetate, and α-naphthyl acetate, β-naphthyl acetate, α-naphthyl propionate, α-naphthyl butyrate, and β-naphthyl butyrate produced a total of 82 esterase isozymes, 32 of which were consistent in all cultivars, while 50 were polymorphic (Table 2).

**Table 2 -** Esterase polymorphism in the *Manihot esculenta* cultivars detected with the use of 4-methylumbelliferyl esters (acetate, propionate, butyrate), fluorescein diacetate, and α- and β-naphthyl esters (acetate, propionate, butyrate).

| Substrates | Polymorphic Isoesterases | Total Isoesterases |
|---|---|---|
| 4-Methylumbelliferyl acetate | 8 | 14 |
| 4-Methylumbelliferyl propionate | 8 | 14 |
| 4-Methylumbelliferyl butyrate | 4 | 5 |
| Fluorescein diacetate | 6 | 10 |
| α-Naphthyl acetate | 7 | 11 |
| α-Naphthyl propionate | 5 | 10 |
| α-Naphthyl butyrate | 5 | 6 |
| β-Naphthyl acetate | 5 | 9 |
| β-Naphthyl butyrate | 2 | 3 |
| **Total** | **50** | **82** |

Substrate preference for both 4-methylumbelliferyl esters and α- or β-naphthyl esters was observed; Fig. 1 shows the substrate-preference polymorphism obtained with 4-methylumbelliferyl esters and α- or β-naphthyl esters. A clear cultivar-specific pattern was maintained despite the wide divergence of the cultivars, with 39% of isoesterases shared by all cultivars. The number of isoesterases for each substrate varied from 3 (β-naphthyl butyrate) to 14 (4-methylumbelliferyl acetate or propionate, Table 2). α- and β-Naphthyl butyrate produced the least stained isoesterase patterns. The esterase isozyme phenotypes of the 20 *M. esculenta* cultivars were compared, and variation in the isoesterase patterns was observed, indicating genomic variability among the cassava cultivars. The relationship between cultivars was estimated using Jaccard's coefficient of similarity. Similarity between cultivars from the six groups ranged from 51% (BG 13 and BG 25 cultivars) to 96% (BG 21 and BG 25 cultivars). A dendrogram produced by cluster analysis shows the differences among the *M. esculenta* cultivars (Fig. 2). The dendrogram shows cultivar BG 11 (Verdinha), as the most divergent. The most similar (96%) were cultivars BG 21 (José Mendes) and BG 25 (Pão-do-Chile). The majority of the cultivars clustered together at 88% similarity.

## DISCUSSION

The use of the different extraction solutions to homogenize young leaves of *Manihot esculenta* cultivars, allowed the identification of a higher number of esterase isozymes than described in other studies with *M. esculenta* species or other species of the *Manihot* genus (Lefevre and Charrier, 1993; Borsoi-Filho, 1995).

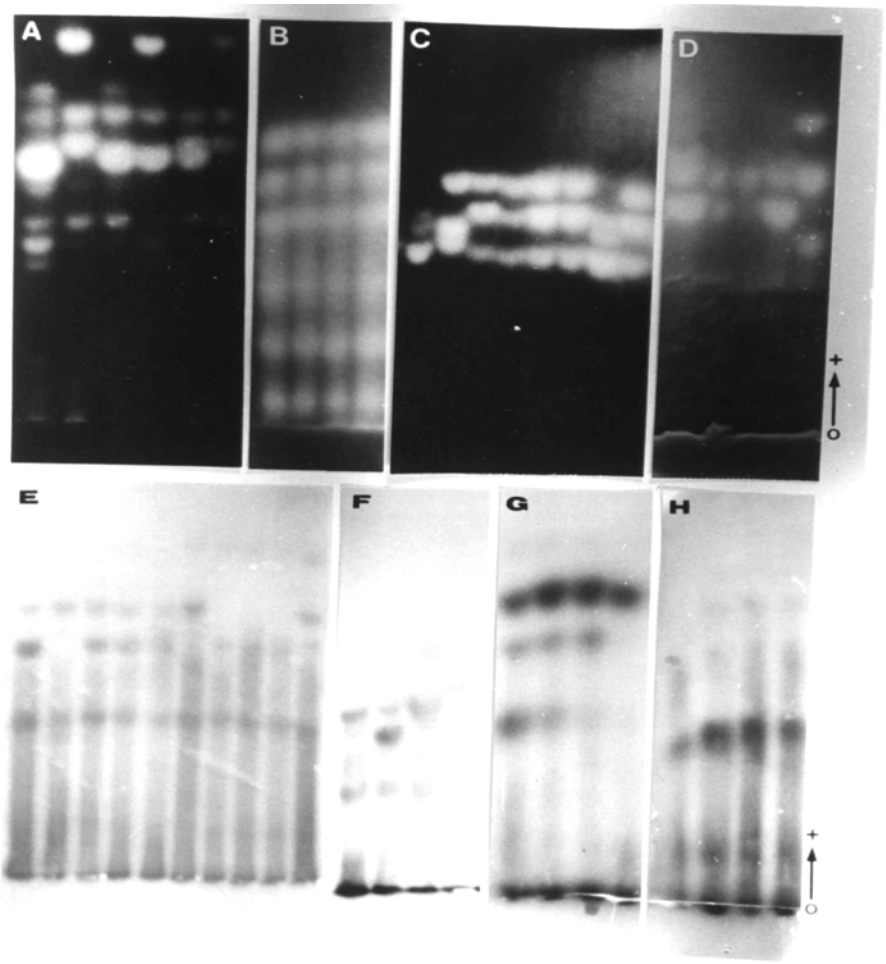


**Figure 1 -** Esterase polymorphism of the *Manihot esculenta* cultivars detected with 4-methylumbelliferyl acetate (A: samples 1-5 correspond to cultivars BG20-BG25), 4-methylumbelliferyl propionate (B: samples 1-4 correspond to cultivar BG 19), 4-methylumbelliferyl butyrate (C: samples 1-8 correspond to cultivars BG 11-BG 15, BG 5-BG 7; D: samples 1-5 correspond to cultivars BG 1, BG 2, BG 19-BG 21), α- and β-naphthyl acetate (E: samples 1-10 correspond to cultivars BG 20-BG 25, BG 1, BG 2, BG 9, BG 10), α-naphthyl acetate (F: samples 1-4 correspond to cultivars BG 10-BG 13), α-naphthyl propionate (G: samples 1-4 correspond to BG 20-BG 23), and α-naphthyl butyrate (H: samples 1-4 correspond to BG 20-BG 23).

Our results showed that additives such as sodium metabisulfite and ascorbic acid, which are important to reverse or prevent the oxidative formation of quinones (Loomis, 1974), were needed to solubilize and maintain a higher number of esterase isozymes. Metabisulfite can form a stable covalent complex with phenols that does not react with PVP-40 (Arulsebar et al., 1986; Buckley et al., 1988); ascorbic acid will reduce quinones (Loomis, 1969), not reducible by β-mercaptoethanol (Shaw et al., 1987; Buckley et al., 1988; Hickey et al., 1989; Krebs and Hancock, 1989; Laguidah and Hanna, 1989). Thus, the extraction solution for esterase analysis in young leaves of *M. esculenta* required two phenol-complexing agent and four antioxidant agents. The development of protocols for identifying the esterase isozymes using different esterase substrates, provided an enzymatic system of relatively reduced cost that cold be used to monitor the various *M. esculenta* cultivars maintained by vegetative propagation in

germplasm banks. The observed esterase substrate-specific polymorphisms showed that the species *M. esculenta* is heterogeneous, with wide cultivar-specific variation. The clear difference between the 20 *M. esculenta* cultivars studied permited their identification even in mixed cultivars. Use of 4-methylumbelliferyl acetate or propionate revealed the highest number of isoesterases among the substrates used, and therefore seem to be the substrates indicated to discriminate among *M. esculenta* cultivars.

The two Verdinha cultivars (BG 11 and BG 12) and the two Fécula Branca cultivars (BG5 and BG 22) did not cluster together. The four Amarela cultivars (BG 7, BG 13, BG 15 and BG 23) also did not cluster together. This is a clear indication that marker isoesterases for *M. esculenta*, are able to reflect the assignment of genomes may exist. In contrast, genotype duplicates of cassava in accessions from Central and South America were identified through isozyme and AFLP profiles (Chavarriaga-Aguirre et al., 1999). There was no obvious geographic differentiation among the *M. esculenta* cultivars. The isoesterase electrophoretic patterns detected within the subset of traditional cultivars from the southwestern region of the State of Paraná did not differ from those observed in the northwestern region of Paraná. Branca de Santa Catarina cultivar (BG 2), which is considered a well adapted and highly productive cultivar produced in the cassava breeding program of the Agronomic Institute of Campinas (IAC), and the Branca variety obtained from the northwestern region of Paraná, clustered together, showing 82.8% of similarity.

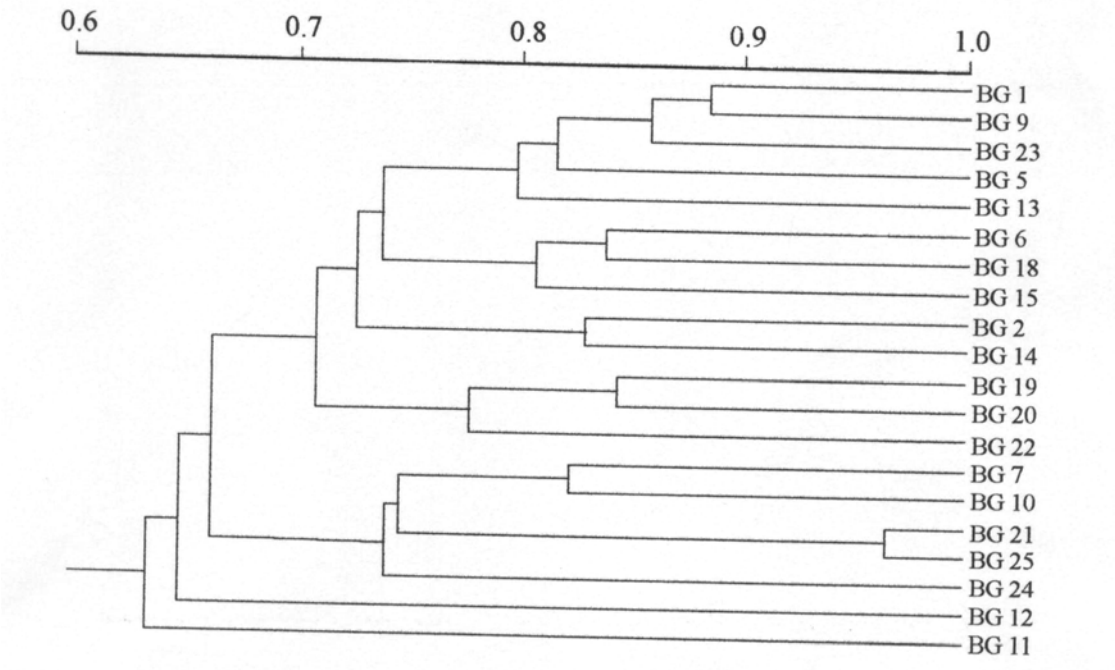


**Figure 2 -** Dendrogram representating the relationship between the twenty *Manihot esculenta* cultivars (BG 1: Fibra, BG 2: Branca de Santa Catarina, BG 5: Fécula Branca, BG 6: Alegretti, BG 7: Amarela, BG 9: Espeto, BG 10: Vermelha, BG 11: Verdinha, BG 12: Verdinha, BG 13: Amarela do Mato Grosso, BG 14: Branca, BG 15: Amarela, BG 18: Renascença, BG 19: Stalina, BG 20: Araruna, BG 21: José Mendes, BG 22: Fécula Branca, BG 23: Amarela de Mandaguaçu, BG 24: Mico, BG 25: Pão do Chile) based on UPMGA cluster analysis of the esterase isozymes detected with substrates using Jaccard´s similarity coefficient.

Despite the vegetative propagation of genotypes in the southwestern and northwestern regions as well as in the experimental germplasm bank, we identified different groups of genotypes among the *M. esculenta* cultivars. Thus, the present report indicates that a relatively more complex extraction solution for proteins of *M. esculenta* leaves is needed to show a higher number of esterase isozymes. Esterase analysis showed a wide biochemical variation between *M. esculenta* cutivars collected in the southwestern and northwestern regions of the State of Paraná. The cultivars identified by the same name seem to be biochemically different regarding esterase isozymes. Therefore, esterase isozyme electrophoretic patterns can be used to discriminate the cultivars identified by the same name and to monitor the vegetative propagation of these cultivars maintained in the BG collection. In breeding strategies, isoesterase analysis could be used to avoid intercrossing between similar genotypes.

## RESUMO

Isoenzimas esterases foram usadas no presente estudo, para detectar polimorfismos específicos para diferentes substratos em vinte cultivares de *Manihot esculenta*, e para mostrar variações específicas de cultivares nesta espécie. Os diferentes cultivares de *M. esculenta* tem sido mantidos na coleção de germoplasma do Departamento de Agronomia da Universidade Estadual de Maringá (Maringá, PR), e foram provenientes de cultivares tradicionais coletados nas regiões sudoeste e noroeste do Estado. Foi necessário a utilização de uma solução de extração de proteínas relativamente mais complexa, para evidenciar um maior número de isoenzimas esterases. A similaridade entre os cultivares variou de 51 a 96%. Cultivares identificados pelo mesmo nome parecem ser bioquimicamente diferentes para as isoenzimas esterases. Os padrões eletroforéticos das isoesterases podem, portanto, serem usados para discriminar os cultivares que são identificados pelo mesmo nome, e para monitorar a propagação vegetativa dos cultivares mantidos na coleção de germoplasma. A análise das isoesterases pode também ser usada para evitar cruzamentos entre genótipos mais similares em programas de melhoramento.

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