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ISOZYMES AS GENETIC MARKERS IN BANANAS AND PLANTAINS[1]

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SUMMARY

Twenty-four clones of banana and plantain representing various levels of ploidy and diploid *M. balbisiana,* were analysed for enzyme variants of malate dehydrogenase, phosphoglucomutase, glutamate oxaloacetate transaminase, shikimate dehydrogenase and peroxidase. Polymorphism was detected in all 5 enzyme systems. In addition, the four principal Cavendish clones, Robusta, Giant Cavendish, Dwarf Cavendish and Pisang masak hijau were found to be monomorphic for isozymes of 10 additional enzymes. Isozymes of glutamate oxaloacetate transaminase were the most useful for discriminating among clones of a particular genomic group.

INTRODUCTION

It is estimated that in excess of 100 million people subsist on bananas and plantains as their staple carbohydrate source (ROWE, 1981). In this context, 'banana' refers to both the diploid and triploid sweet-fruited *M. acuminata* COLLA. (A genome) types whereas the plantains and other cooking-types are *M. acuminata* × *paradisiaca* L. (*M. acuminata* × *M. balbisiana* COLLA. – A × B genome) hybrids.

Bananas are generally eaten as fresh fruit whereas plantains are starchier and are typically eaten in a cooked form. Total combined world production of bananas and plantains in 1983 exceeded 5.7×10^4 metric tons (FAO, 1983). However, this estimate is considerably lower than the actual production tonnage due to the extensive 'backyard' cultivation of these crops in Latin America, Africa, Southeast Asia and the Caribbean, and due to the omission of non-plantain cooking-types (ABB and ABBB) from the production analysis. In addition to, and completely aside from its value as a food source, the economies of many countries have come to depend on the continued cultivation of these crops.

In recognition of their importance as food crops, there has been a renewed international interest in the genetic improvement of cultivated *Musa*. The application of both conventional (ROWE, 1981) and non-conventional techniques (JARRET et al., 1985) has been proposed. Pivotal to these improvement efforts is the continued collection and evaluation of *Musa* germplasm. In view of the rapid genetic erosion which is occurring

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in and around its centers of diversity and origin, (ANONYMOUS, 1984a), plant collecting activities are continuing and a series of morphological descriptors are now available (ANONYMOUS, 1984b) which should allow some estimation of the degree of genetic variation between individual clones within germplasm collections and within geographical areas targeted for future collection. However, positive identification of even the most widely cultivated clones is difficult or impossible at this time due to the multiple vernacular names for each clone and the relative lack of morphological and genetic variation between them. For example, the Cavendish cultivars which constitute the basis for today's export banana industry, arose as sports or somatic mutations from a common ancestral clone (SIMMONDS, 1962).

The use of isozymes as genetic markers for variety identification has received considerable attention (TANKSLEY & ORTON, 1983). Few studies have been conducted with *Musa*. BONNER *et al.*, (1974) examined peroxidase isozymes in various *Musa* clones and species and noted a lack of polymorphism. More recently, RIVERA (1983) examined peroxidase and polyphenoloxidase polymorphisms and was able to distinguish between the Saba (BBB) and Bluggoe (ABB) genome groups. This study was undertaken to characterize additional isozymes that could be used as genetic markers for estimating diversity within the genus, to measure somaclonal variation, to identify protoplast fusion products, for clonal identification, etc.

MATERIAL AND METHODS

Plant material for this study was collected either from the FAO-supported *Musa* germplasm collection in La Lima, Honduras or from the collection maintained at the University of Florida in Homestead, Florida as indicated in Table 1. Leaf segments from the central part of the leaf blade, or occasionally 1 cm root tips, were utilized. Extracts were prepared and were loaded onto starch or polyacrylamide gels, electrophoresed at 4 °C, and stained for shikimate dehydrogenase (SKDH), malate dehydrogenase

Table 1. *Musa* clones analyzed for enzyme polymorphism.

Genome group	Common name[1]
AA	Pisang lilin (AVP-35)[2], Pisang mas (AVP-28), Tongat (II-24)
AAA	Giant Cavendish, Pisang masak hijau, Robusta, Dwarf Cavendish, Red, Gros Michel (AVP-73), Highgate (AVP-16)
AAAA	SH 3420[2]
AB	Ney poovan (IV-13)
AAB	Mysore, Dwarf French Plantain (I-46), Horse plantain (AVP-64), Maiden plantain (AVP-67), Apple, Orinoco
ABB	Ice cream, Cardaba (II-19), Chato, Pelipita, Pisang awak (II-86)
ABBB	Klue tiparot (teparod)[1] (II-42)
BB	*M. balbisiana*

[1] SIMMONDS, N. W., 1966.

[2] Maintained in the *Musa* germplasm collection, La Lima, Honduras. Numbers in parenthesis represent the plant introduction number in La Lima. All others are from the University of Florida at Homestead collection.

Table 2. Electrophoretic systems utilized to resolve enzyme polymorphisms in leaf tissue extracts of the Cavendish banana clones.

Enzyme	Electrode buffer	Gel buffer	Matrix	Running conditions
6PGDH	0.065M L-histidine/[1] 0.007 M citrate. H_2O adjusted to pH 6.5. with citric acid	dilute electrode buffer 1:3 with H_2O	12.5% starch	350 V/12 hrs
ME PGI	0.1 M Tris/0.1 M[2] maleic acid, 0.01M Na_2EDTA, 0.01 M $MgCl_2 . 6H_2O$, adjusted to pH 7.4 with 5 N NaOH.	Dilute electrode buffer 1:9 with H_2O	12.5% starch	50 mA/4 hours
GUDH ADH TPI GDH SDH SUDH	0.19 M boric acid/[3] 0.04 M Lithium hydroxide, adjusted to pH 8.3 with LiOH.	0.05 M Tris/ 0.007 M Citric acid. H_2O, adjusted to pH 8.3 with citric acid. H_2O. Mix 9:1 with electrode buffer	7.5% T acrylamide (0.25% Bis)	15 mA/4 hours

[1] CARDY et al., 1981.
[2] SELANDER et al., 1971.
[3] SCANDALIOS, J. G., 1967.

(MDH), peroxidase (PRX), phosphoglucomutase (PGM) and glutamate oxaloacetate transminase (GOT) as described earlier (JARRET & LITZ, 1986). Extracts of the Cavendish clones (SIMMONDS, 1966) including Robusta, Giant Cavendish, Dwarf Cavendish, and Pisang masak hijau (Lacatan) were also examined for esterase (EST), tetrazolium oxidase (TO), 6-phosphogluconate dehydrogenase (6PGDH), malic enzyme (ME), phosphoglucose isomerase (PGI), alcohol dehydrogenase (ADH), triosephosphate isomerase (TPI), galactose dehydrogenase (GDH), succinate dehydrogenase (SUDH), sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GUDH). Electrophoretic conditions for isozyme separation and identification have been reported for SKDH, MDH, PRX, PGM, GOT, EST and TO (JARRET & LITZ, 1986) or are presented in Table 2. Staining procedures were those described by SHAW & PRASAD (1970) or VALLEJOS (1983) except for TO (GORMAN & KIANG, 1977). Gels were scored and photographed immediately after staining. Rf values for individual bands were calculated based on the average migration distances on 3 individual gels using extracts from 3 separate leaf samples.

RESULTS AND DISCUSSION

Examples of resolution obtained for isozymes of MDH, PGM, GOT, PRX and SKDH were reported earlier (JARRET & LITZ, 1986).

Malate dehydrogenase (MDH). Heterozygosity at Mdh-1 (Jarret and Litz, 1986) characterized the purely *acuminata*-derived cultivars (Fig. 1). This is in contrast to our earlier observations of segregation at this locus in seed-fertile diploid subspecies. MDH

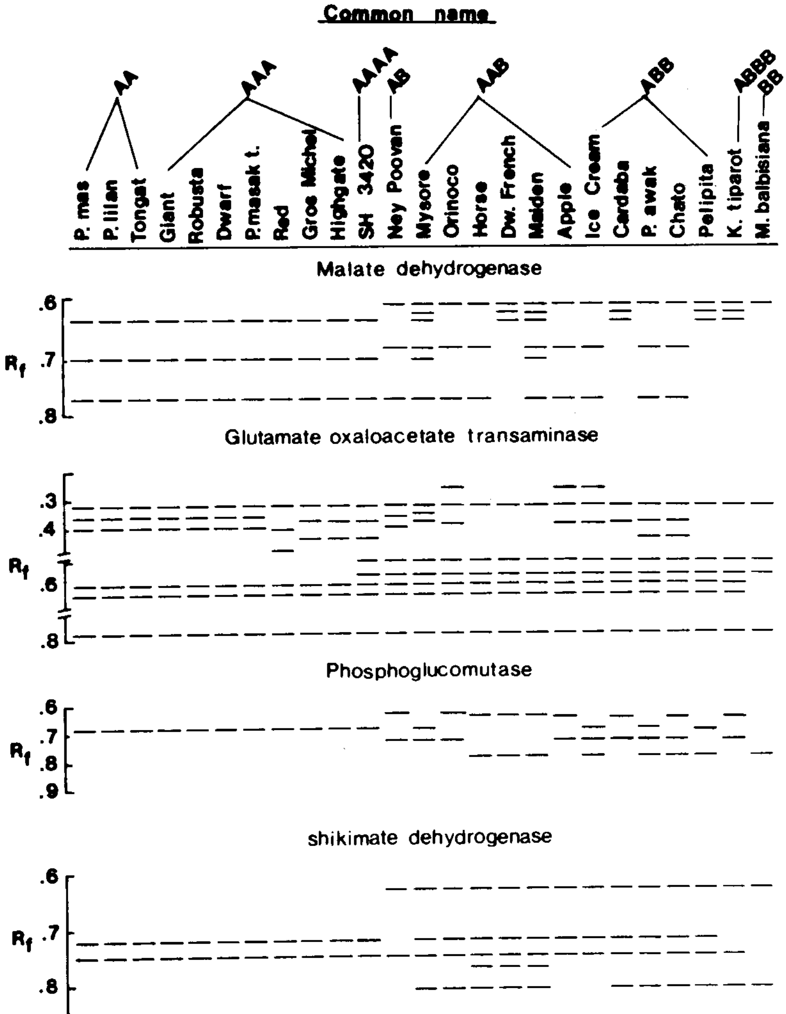

Fig. 1. Schematic representation of enzyme polymorphisms for MDH, PGM, SKDH and GOT.

is a dimeric protein in *M. acuminata* and the banding patterns of the purely *acuminata* derived clones reflect this. For example, the bands of MDH activity of Rf 0.63 and 0.77 represent the homodimeric allozymes at the Mdh-1 locus and the band of intermediate mobility (Rf 0.70) represents the intralocus heterodimer. The *acuminata*-derived clones are all believed to be of Malayan origin (SIMMONDS, 1966). The isozyme unique to *M. balbisiana* (Rf 0.6) was identified in extracts of all clones purported to be of bispecific origin. Banding patterns of the AB, AAB, ABB, and ABBB genome groups also reflected the dimeric nature of MDH. For example, consider 'Ney Poovan'an AB diploid (Fig. 1). The band of activity at Rf 0.6 represents the homodimeric form of MDH characteristic of *M. balbisiana*. The band of activity at Rf 0.77 reflects the more rapidly migrating homodimeric form of MDH characteristic of *M. acuminata*. The band of intermediate mobility at Rf 0.68, in this case, reflects the formation of

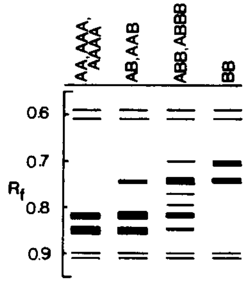

Fig. 2. Schematic representation of peroxidase zymotypes of various genome groups in the cultivated *Musa*.

an interlocus heterodimeric form of this enzyme. The banding pattern of 'Ney Poovan' is identical to the progeny of *M. acuminata* diploid II-357 × *M. balbisiana* (R. JARRET, unpublished data). Resolution of MDH isozymes in extracts of 'Mysore' and 'Maiden' plantain (Fig. 1) was poor and usually streaked or with low activity which precluded accurate scoring on occasion. The polymorphism at this locus (Mdh-1) will be useful for clonal identification.

Glutamate oxaloacetate transmaninase (GOT). Three loci coding for GOT activity were evident on polyacrylamide gels (Fig. 1). Got-1 was monomorphic under our running conditions. Got-2 was also monomorphic within the *acuminata*-derived clones. An additional doublet of bands, characteristic of the *M. balbisiana* genome was present in extracts of the remaining cultivars. Got-3 was polymorphic. Polymorphism at this locus allowed differentiation within the *acuminata* types. 'Red' was distinguishable from 'Gros Michel', 'Highgate' and 'SH3420', and the latter 3 from the Cavendish group and the *acuminata* diploids. 'Highgate' is a dwarf mutant of 'Gros Michel' and 'SH3420' is a sib selection from a 3X × 2X hybridization utilizing 'Gros Michel' as the seed parent. We observed several banding patterns at Got-3 not previously recorded in the seed-fertile subspecies. This emphasizes the need for a more in-depth investigation of isozyme polymorphism in the diploids and for further collection and characterization of these materials. No consistent patterns were evident by which the Bluggoe (ABB) types could be differentiated from the plantains (AAB). Nevertheless, heterogeneity for GOT banding patterns suggests that polymorphism at Got-3 will facilitate efforts to estimate taxonomic relationships and will be useful for clonal identification within the genus. Isozyme banding patterns for GOT have been used with considerable success in evolutionary studies in *Capsicum* (MCLEOD et al., 1983).

Phosphoglucomutase (PGM). This typically monomeric enzyme (TANKSLEY & ORTON, 1983) was also monomorphic within and among *acuminata*-derived clones of various ploidy (Fig. 1). This is in contrast to the polymorphism characteristic of seed-fertile *acuminata* subspecies and suggests an extremely narrow genetic base for the dessert-type clones. Within the A × B genome groups, PGM banding allowed for differentiation of 'Horse', 'Dwarf', 'French' and 'Maiden' plantains from other AAB cultivars.

Members of the plantain subgroup have been divided into French and Horn types

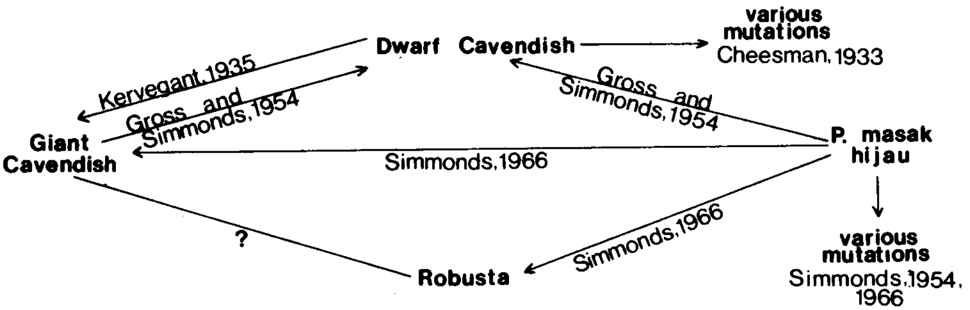

Fig. 3. Schematic representation of the genetic base and origins of the 4 principle Cavendish banana cultivars.

based on the persistence of the male axis (SIMMONDS, 1966), although this arbitrary division has been questioned by DELANGHE (1961). Horn plantain types have been noted to arise spontaneously as somatic mutations from French plantain types, and have subsequently been propagated as sectoral or mericlinal chimeras. The incidence of this type of interconversion has been accelerated by in vitro culture (H. IRIZARRY, 1985-personal communication). Our results also suggest a close relationship between members of the French and Horn subgroups. We were unable, based on PGM banding patterns, to differentiate between these subgroups, however, our sample size was small.

Shikimate dehydrogenase (SKDH). Six banding patterns were observed for SKDH activity. Five of these were present in the cultivated clones and the sixth was unique to *M. balbisiana*. The *acuminata*-derived clones were monomorphic for isozymes of SKDH. In contrast, the plantain and other cooking-types showed a diversity in origin not evident in the dessert banana clones. The plantains 'Maiden', 'Dwarf' and 'Horse' possessed an additional band of SKDH activity not present in the AAB or ABB groups suggesting a unique evolutionary/geographic origin for these types. The band of SKDH activity at Rf 0.63 is characteristic of *M. balbisiana* and should be useful as a species-specific marker.

Shikimate dehydrogenase is typically functional in plant tissues as a monomeric enzyme (TANKSLEY & ORTON, 1983). Complete interpretation of SKDH banding patterns awaits additional genetic data.

SIMMONDS (1966) has hypothesized that the movement of diploid *acuminata* (AA) into areas occupied by *balbisiana* resulted in a profusion of AB hybrid clones. While of limited value economically (i.e. 'Ney Poovan') these hybrids, upon backcrossing to either *acuminata* or *balbisiana* in conjunction with female restitution, result in AAB and ABB types respectively. A thorough knowledge of the genetic mechanisms governing the expression of SKDH and other monomeric enzymes may yield significant insight into the evolution of both plantains and other cooking-type bananas.

Peroxidase (PRX). Considerable activity for this enzyme remained unresolved under our electrophoretic conditions. Nevertheless, peroxidase polymorphisms were found to be useful in our efforts to group cultivars by genomic constitution (AA, AAB, etc.).

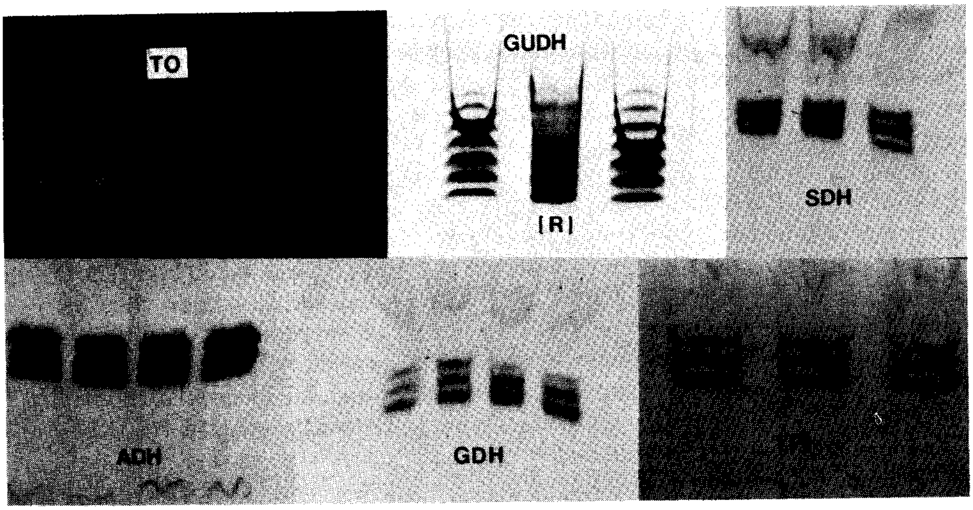

Fig. 4. Examples of the resolution achieved for enzyme variants in leaf extracts (or root – R) from the Cavendish banana cultivars (except TO – a variety of triploid clones).

Four distinct banding patterns were evident and these were found to correspond, without exception, to the genomic groups as indicated in Fig. 2. A doublet of Rf 0.82 and 0.85 typified *acuminata* clones whereas the doublet at Rf 0.71 and 0.75 was characteristic of *M. balbisiana*. Bispecific clones (A × B) were distinguished both by their banding pattern and the intensity of individual bands which presumably reflects genome dosage. The genetic/biochemical basis for the formation of bands of intermediate mobility has yet to be elucidated. We are investigating the feasibility of differentiating between the AA, AAA and AAAA groups based on band intensity (i.e. gene dosage) with the aid of a densitometer. This capability would allow for a rapid method to categorize individual clones targeted for collection and would complement the existing method which is based upon a series of morphological characteristics (SIMMONDS & SHEPHERD, 1955), and which occasionally produces ambiguous results. Such a technique may help clarify the genetic relationships among clones of the Saba group. The term Saba used in a generic sense, refers to a complex of closely related Philippine cultivars which were originally classified as ABB (SIMMONDS, 1966). However, several investigators including ALLEN (1965), BREWBAKER & UMALI (1956), and RIVERA (1983) have suggested the probability that individual clones within this complex are triploid *balbisiana* (BBB). When several of these clones are scored on the basis of morphological descriptors according to the method of SIMMONDS & SHEPHERD (1955), values are obtained that are nearly identical to those of *M. balbisiana* (BB). RIVERA (1983) studied polyphenol oxidase, peroxidase and protein banding patterns and found 'Cardaba', a member of the Saba complex, to be BBB. Our results suggest that 'Cardaba', as we know it, is indeed an ABB as originally classified, based on species-specific alleles at Pgm-1, Got-2, Mdh-1, and for PRX polymorphisms. A closer examination of this group of clones, utilizing species-specific isozyme markers would reveal true BBB types should they exist.

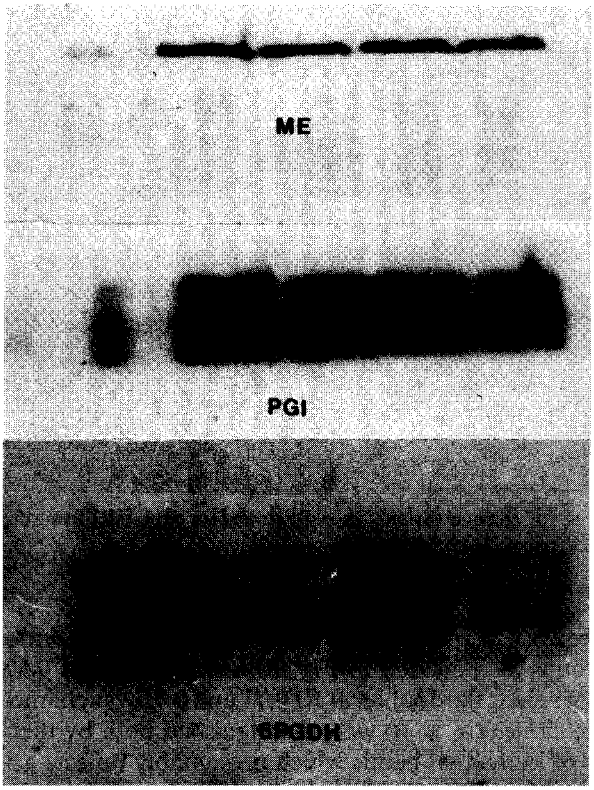

Fig. 5. Examples of resolution achieved for enzyme variants of 6PGDH and PGI using the electrophoresis conditions described in Table 2. Extracts are from L to R 'Robusta', 'Giant Cavendish', 'Dwarf Cavendish', and 'Pisang masak hijau'.

In general the zymograms of the clones of bispecific origin additively express enzymes present individually in the ancestral diploids. This behavior has been observed previously (CHERRY et al., 1972; GOTTLIEB, 1973, 1977).

It is theorized that members of the Cavendish group of banana cultivars originated as bud mutations and that 'Pisang masak hijau' or a similar clone represents the parental type from which the others arose (SIMMONDS, 1966) (Fig. 3). The 4 principal Cavendish clones, Robusta, Giant Cavendish, Dwarf Cavendish and Pisang masak hijau account for virtually all the commercial dessert banana production worldwide, having replaced the cultivar Gros Michel as a result of its susceptibility to Panama disease (caused by *Fusarium oxysporum* f. *cubense*). Although the Cavendish clones are relatively immune to Panama disease, they are highly susceptible to leaf spot, nematodes and a variety of diseases and pests which continually threaten their monoculture. Somatic mutations within this group are common and include, for example, the mutation of 'Giant Cavendish' to 'Dwarf Cavendish' and its reversion to the tall form (Fig. 3). All members of the Cavendish group are generally distinguishable from one another based on differences in plant stature; however, misidentifications do occur as plant height and other vegetative characteristics may vary considerably due to environmental effects. Therefore, a means to unambiguously identify individual members of this group is desirable. To this end we examined the 4 principal Cavendish clones for polymorphism in an additional 10 enzymes. Results of typical resolutions achieved for these enzymes are presented in Figs. 4 and 5.

6 Phosphogluconate dehydrogenase (6PGDH). Activity of 6PGDH in leaf extracts was generally sufficient immediately after extraction but diminished during storage at -30 °C (Fig. 5). A 4-banded zymotype (monomorphic within the Cavendish group) was typically observed. Resolution was adequate using the His/Cit (pH 6.5) buffer system (Table 2). However, resolution varied considerably as a result of starch concentration, type and batch as observed previously in maize (GOODMAN & STUBER, 1983). The cathodal cluster of 3 bands is suggestive of heterozygosity at a single 6PGDH locus. 6PGDH is typically functional as a dimer (GOODMAN & STUBER, 1983).

Malic enzyme (ME). Malic enzyme was resolved as a single band of intense activity under a wide array (pH, gel matrice) of electrophoretic conditions. This enzyme was monomorphic within the Cavendish clones examined (Fig. 5). Additional mobility alleles have been observed in other *Musa* species which we have examined.

Phosphoglucose isomerase (PGI). This enzyme was resolved as a triplet of bands using the pH 7.4 buffer system (Table 2) (Fig. 5). Additional areas of activity were evident, but poorly resolved using the His/Cit pH 6.5 buffer system. Phosphoglucose isomerase was monomorphic within the Cavendish group and its banding pattern suggests heterozygosity for this typically dimeric (TANKSLEY, 1980) protein.

Alcohol dehydrogenase (ADH). Activity of this enzyme was resolved as a 3-banded zymotype in leaf tissue extracts from all 4 of the Cavendish clones using either the pH 7.4 or the pH 8.3 buffer systems (Table 2). Expression of ADH activity was noticeably enhanced when leaf tissue was maintained under anaerobic conditions (submerged in H_2O) for 8–12 hours prior to extraction. An additional locus was expressed in anaerobically treated root extracts; however, resolution of additional ADH isozymes was inadequate for accurate scoring using the buffer systems described in this report.

Triosephosphate isomerase (TPI), galactose dehydrogenase (GDH), glutamate dehydrogenase (GUDH), sorbitol dehydrogenase (SDH), and succinate dehydrogenase (SUDH). The zymotypes of these (except GUDH) were resolved as triplets of bands in all cases on the pH 8.3 buffer system (Table 2). Glutamate dehydrogenase activity was more clearly resolved in root extracts when compared to leaf extracts (Fig. 2). The banding pattern for these 4 enzymes was monomorphic within the Cavendish clones.

Tetrazolium oxidase (TO). Banding patterns resulting from this non-specific staining procedure, were complex as reported earlier (JARRET & LITZ, 1986). Three major areas of activity were evident on polyacrylamide gels as well as multiple minor bands. TO activity was monomorphic within the Cavendish clones but considerable heterogeneity is evident within and between other genome groups suggesting a role for TO in clonal identification.

The Cavendish group is apparently monomorphic for variants in all the enzyme systems that we have examined. However, genetic regulation of many individual isozymes awaits demonstration and is essential in order that artifacts or incompletely resolved loci can be identified. Such a study is being conducted utilizing seed-fertile diploid *acuminata* subspecies. The use of fertile diploid clones also allows for rapid differentiation of isozymes from allozymes using gametic and somatic tissue extracts (Weeden & Gottlieb, 1979). Furthermore, we are continuing to develop techniques for identification of additional electrophoretic variants.

Simmonds (1966) has estimated that perhaps 50% of today's cultivated banana cultivars originated as somatic mutations. Therefore, the limited amount of genetic diversity within a genome group which we observed is to be expected. The genetic base of this crop, especially within the *acuminata* types, is much narrower than morphology suggests. Weeden & Lamb (1985) were unable to distinguish sports of apple (*Malus domestica*) by isozyme analysis. We also were unable to differentiate within the Cavendish group or between the cvs. Gros Michel and Highgate. However, the sensitivity of this technique is a function of the number of polymorphic loci which can be resolved. Therefore, it is essential that a greater number of polymorphic loci should be identified and characterisized. Vallejos (1983) and Shaw & Prasad (1970) list staining procedures for 57 and 50 enzyme systems respectively. Nevertheless, the use of isozymes as genetic markers will provide estimates of genetic diversity within the cultivated and wild members of this genus, facilitate clonal identification and should provide valuable evidence on the origins of the triploid cultivars. Differentiation of somatic mutations from their parental clone may occasionally be detected by isozymes analysis (Brettell et al., 1986) or may require more sophisticated techniques (Myers et al., 1985; Orton, 1983).

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