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CHARACTERISATION OF CACAO GERMPLASM USING ISOENZYME MARKERS. 1. A PRELIMINARY SURVEY OF DIVERSITY USING STARCH GEL ELECTROPHORESIS AND STANDARDISATION OF THE PROCEDURE

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INDEX WORDS

Theobroma cacao, cacao, germplasm conservation, genebank, diversity, isoenzymes, standardisation of procedure.

SUMMARY

A preliminary screening of isoenzymes in cacao has been carried out with a view to the use of the data in characterising cacao germplasm. Staining was carried out for 24 enzyme systems in leaf tissue. Seventeen of these showed activity, and in seven enzyme systems, reproducible variation in banding patterns was obtained. A single enzyme, peroxidase, was extracted from woody tissue and also exhibited repeatable variation. It was considered that by running extracts from leaves and wood on starch gels, and staining for these 8 enzyme systems, valuable information would be obtained. This could be used to identify clonal material for breeding. It could also be used to supplement the usual morphological descriptors for characterising material in genebanks.

INTRODUCTION

Cacao (*Theobroma cacao* L.) is a crop of great economic importance and represents a large proportion of the exports of countries in the West Indies, parts of Central and South America, West Africa and parts of southeast Asia. During the three centuries or so that it has been cultivated on a large scale, a considerable diversity of the crop has been established in these areas although the modern economic crop tends to be based on a small part of this genepool. The use of vegetatively propagated (clonal) material is a relatively recent innovation and historically, the cacao crop has been propagated by seed, thus releasing considerable variation. Disease is an important factor in cacao cultivation, and breeders have to consider the introduction of various resistance factors into cultivars. Local primitive cultivars and advanced cultivars of non local origin are an important part of the plant breeders stock. However, although valuable local collections of such germplasm exist in many cacao growing areas, catalogues of the collections are often scant or absent. Where descriptions are available,

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these are usually based on morphology, mainly relating to fruit and flower characters and often using local descriptor lists. The International Board for Plant Genetic Resources has suggested the use of a standard desciptor list (IBPGR, 1981) as part of a rationalisation programme for cacao genebanks. It would be a valuable aid to plant breeders if such lists could be supplemented by data which (1) was available from very young, sexually immature plants and (2) reflected the genotype rather more closely than morphological data. Isoenzyme banding patterns derived from vegetative tissues satisfy both of these requirements. For a useful review of the value of isoenzyme data in plant population genetic work see CRAWFORD (1983).

Another important role of cacao genebanks, which is of more urgent importance, is the collection and evaluation of wild material from the Upper Amazon region, which is usually considered to be the centre of diversity of *T. cacao*. Collection of wild cacao has had a relatively long history with early collections made by Pound between 1937 and 1943, and a very recent expedition into Ecuador between 1980 and 1983, (ALLEN & LASS, 1983). Unfortunately, cataloguing and evaluation of these accessions have lagged considerably behind collection. Incorporation, even of the Pound material, into the repertoire of the cacao breeder has been disappointingly slow. Perhaps this is in part due to the poor availability of descriptive data about these plants. Whilst collection is clearly the first priority of a germplasm conservation programme, it is essential not to lose sight of its central purpose which is to make hitherto unused sources of genetic variation available to plant breeders.

It is the aim of this paper to demonstrate that considerable isoenzyme variation occurs in cultivars and wild accessions of cacao and that this variation can be assessed rapidly. Screening has been carried out for 24 enzyme systems. Reproducible variation has been found in eight of these and is described.

MATERIALS AND METHODS

Sources of material. Sixty-five seedlings were grown in a shaded greenhouse. The provenances of the seed were as follows:

1. Amelonado from Ghana and Ivory coast farms, denoted A (24 seedlings).
2. Trinitario ICS95 from Trinidad, 2 seedlings.
3. Hybrids of unspecified origin from IRCC, Bingerville, Ivory Coast, denoted HYB, 9 seedlings.
4. Plantation grown derivatives of wild material collected in Peru by Pound, denoted MO9 (6 seedlings), MO81 (5 seedlings) and PA121 (6 seedlings).
5. Hybrids between a Criollo clone from Indonesia and selections from the progeny of a Criollo × Forastero hybrid originating from Venezuela, denoted GW1, 11 seedlings.
6. Two seedlings of unknown origin, denoted X1 and X2.

Sample preparation, staining and electrophoresis. Within 30 minutes of collection, leaves were washed in tap and distilled water and a piece of tissue approximately 1 cm square was excised from the lamina. This was ground (on ice) with approximately 0.4 ml of buffer (0.1 M tris-HCl, pH 7.8 containing 2% calcium chloride, 4% Triton X100, 5% soluble polyvinylpyrrolidone (PVP-40), 1% 2-mercaptoethanol). This was

ground by hand with a pestle and mortar for 1 minute,after which the extract was absorbed onto filter paper (Whatman 3M). Wicks were placed in a slit in a 1 cm thick 12% starch gel and subjected to electrophoresis. After electrophoresis for the required time (see below), gels were cut into five slices, each being stained separately.

Bark and outer wood were removed with a razor blade. Strips approximately 20 × 5 mm were ground in about 0.4 ml of the same extraction solution for 1 minute and treated in the same way as the leaf extracts.

Five buffer systems were used in screening enzymes:

1. Gel buffer 0.046 M tris, 0.14 M citric acid, 0.02 M boric acid, 0.003 M lithium hydroxide, pH 8.3. Tank buffer 0.2 M boric acid, 0.03 M lithium hydroxide, pH 8.3.
2. Gel buffer 0.07 M histidine-HCl, pH 7.0. Tank buffer 0.04 M citric acid, 0.13 M tris, pH 7.0.
3. Gel buffer 0.12 M tris, pH 8.8. Tank buffer 0.025 M tris, 0.192 M glycine, pH 8.8.
4. Gel buffer 0.005 M histidine, 0.0025 M sodium chloride, pH 6.0. Tank buffer 0.41 M sodium citrate/citric acid, pH 6.0.
5. Gel buffer 0.009 M tris, 0.0036 M citric acid, pH 6.2. Tank buffer 0.11 M tris, 0.04 M citric acid, pH 6.2.

Systems 1, 2 and 3 were run using bromophenol blue to mark the front. Systems 4 and 5 were run for fixed times of 6 hours and 18 hours respectively. System 1, 2, 3 and 4 gels were run at 28 mA and system 5 gels at 8 mA.

Localisation was attempted for 24 enzyme systems in leaf extracts. Staining methods are only given for those 17 systems which showed activity (see Table 1).

– Acid phosphatase (VALLEJOS, 1983). 0.2 M sodium acetate/acetic acid buffer, pH 5.5, containing 0.03% B-naphthyl acid phosphate, 0.2% magnesium chloride ($6H_2O$) and 0.1% Fast Black K salt.

– Alcohol dehydrogenase (VALLLEJOS, 1983). 0.1 M tris-HCl buffer, pH 7.2 containing 0.03% NAD, 0.02% MTT and 0.004% phenazine methosulphate. Immediately before use, 6 ml of 95% ethanol was added per 100 ml of staining solution.

– α and β naphthyl esterase (SHAW & PRASAD, 1970). 0.05 M tris-HCl, pH 7.1 containing 0.03% α-naphthyl acetate, 0.03% β-naphthyl acetate (both dissolved initially in acetone) and 0.1% Fast Blue RR salt.

– α-D-galactosidase (VALLEJOS, 1983). 0.1 M sodium acetate/acetic acid buffer, pH 5.0 containing 0.03% α-naphthyl-α-D-galactopyranoside dissolved in acetone, and 0.1% Fast Garnet GBC salt.

– Glucose-6-phospate dehydrogenase (SOLTIS et al., 1983). 0.1 M Tris-HCl buffer, pH 8.3 containing,0.1% glucose-6-phosphate, disodium salt, 0.02% NADP, 0.01% MTT and 0.002% phenazine methosulphate.

– Glutamic oxaloacetate transaminase (SHAW & PRASAD, 1970). 0.1 M phosphate buffer, pH 7.0 containing 0.5% L-aspartic acid and 0.07% α-ketoglutaric acid, 0.05% pyridoxal-5-phosphate and 0.2% Fast Violet B.

– Hexokinase (SOLTIS et al., 1983). 0.1 M tris-HCl buffer, pH 8.3 containing 0.045% glucose, 0.2% magnesium chloride ($6H_2O$), 0.02% EDTA disodium salt, 0.005% NAD, 0.008% MTT, 20 units of Glucose-6-phosphate dehydrogenase (Sigma Chemical Co., Type XXIII), 0.013% ATP and 0.001% phenazine methosulphate.

– Isocitrate dehydrogenase (SOLTIS et al., 1983). 0.1. M Tris-HCl, pH 7.2 containing

Table 1. Leaf enzyme systems which were screened showing the buffer systems used, whether activity was detected and whether variability was observed. NA: no activity; V: variable; NV: not variable.

Enzyme	Buffer systems[a]	Activity
Aconitase	1	NA
Acid phosphatase	1 (4)	V[b]
Alcohol dehydrogenase	1 (3, 4, 5)	V[b]
α-amylase	1	NA
Catalase	1	NA
α and β naphthyl esterase	1 (3)	V[b]
Fluorescent esterase	1	NA
α-D-galactosidase	1	NV
Glucose-6-phosphate dehydrogenase	(1, 4)	NV
Glutamic oxaloacetate transaminase	1	NV
Hexokinase	1	NV
Isocitrate dehydrogenase	1, 4 (5)	V
Leucine aminopeptidase	1	NV
Malate dehydrogenase	4 (1, 2, 5)	V[b]
Malic enzyme	4	V
Menadione reductase	4 (1,5)	V[b]
Nitrate reductase	1	NA
Peroxidase	1	NV
Phosphoglucoisomerase	5 (1, 2, 3, 4)	V[b]
Phosphoglucomutase	1, 4 (3)	V[b]
Polyphenol oxidase	1	NV
Shikimate dehydrogenase	4 (1)	NV
Succinate dehydrogenase	4	NA
Xanthine dehydrogenase	1	NA

[a] The buffer system is given which yielded optimal resolution and separation. Other buffer systems which were tried are given in parentheses.
[b] Enzymes showing repeatable variability and described in the text.

0.1% isocitric acid, trisodium salt, 1% magnesium chloride ($6H_2O$), 0.01% NADP, 0.015% MTT and 0.002% phenazine methosulphate.
– Leucine aminopeptidase (SHAW & PRASAD, 1970) 0.025 M tris-maleate buffer, pH 6.0 containing 0.05% Fast Black K and 0.02% L-leucyl-B-naphthylamide.
– Malate dehydrogenase (VALLEJOS, 1983 with modifications after SOLTIS et al., 1983). 0.1 M tris-HCl, pH 8.3 containing 0.4% DL-malic acid, 0.03% NAD, 0.02% MTT and 0.004% phenazine methosulphate.
– Malic enzyme (SOLTIS et al., 1983). 0.1 M Tris-HCl buffer, pH 8.3 containing 0.4% DL-malic acid, 0.4% magnesium chloride ($6H_2O$), 0.02% NADP, 0.02% MTT and 0.002% phenazine methosulphate.
– Menadione reductase (PRENTICE, 1984). 0.1 M sodium phosphate buffer, pH 7.0 containing 0.06% menadione dissolved in acetone, 0.05% NADH and 0.04% MTT.
– Peroxidase (SHAW & PRASAD, 1970). 0.2 M acetate buffer, pH 5.0 containing 0.025% 3-amino-9-ethylcarbazole dissolved in dimethyl formamide. Hydrogen peroxide (0.25 ml of a 3% aqueous solution) was added immediately prior to use.
– Phosphoglucoisomerase (SOLTIS et al., 1983). 0.1 M Tris-HCl buffer, pH 8.3 containing 0.2% magnesium chloride ($6H_2O$), 0.03% fructose-6-phosphate, disodium salt, 40

units of glucose-6-phosphate dehydrogenase (Sigma Type XXIII), 0.01% NAD, 0.2% MTT and 0.002% phenazine methosulphate.
- Phosphoglucomutase (SOLTIS et al., 1983). 0.1 M tris-HCl, pH 8.3 containing 0.4% magnesium chloride ($6H_2O$), 0.1% Glucose-1-phosphate disodium salt, 40 units of Glucose-6-phosphate dehydrogenase (Sigma Type XXIII), 0.01% NAD, 0.02% MTT and 0.002% phenazine methosulphate.
- Polyphenol oxidase (VALLEJOS, 1983). 0.1 M phosphate buffer, pH 6.8 containing 0.015% catechol and 0.05% sulphanilic acid.
- Shikimate dehydrogenase (VALLEJOS, 1983 with modifications after SOLTIS et al, 1983). 0.1 M tris-HCl, pH 8.3 containing 0.1% shikimic acid, 0.015% NADP, 0.02% MTT and 0.004% phenazine methosulphate.

RESULTS

1. Leaf enzymes

The variation of 7 enzyme systems for which reproducibility has been observed is described below.

(i) Acid phosphatase. Only on buffer system 1 was clear banding demonstrated. Both α and β naphthyl acid phosphate were used as substrates. A single extra band was present when the β form was used. The region between Rf 0.3 and 0.7 showed variation but the genetic basis of this was not clear (Fig. 1).

(ii) Alcohol dehydrogenase. This enzyme was only resolved into bands on system 1 gels. Three patterns were observed (Fig. 2): A single band at Rf 0.36 (aa), a single band at Rf 0.38 (bb), (a and b denoting the two allelic forms). The third pattern was shown either as 3 bands (the intermediate one being a 'hybrid' band) between Rf 0.36 and 0.38 or as apparently continuous staining in the same region. These patterns are not easily distinguished and are presumed to reflect the same situation, (ab). It is suggested that the banding of this enzyme represents a single locus (Adh-1) coding for a dimeric enzyme.

(iii) α and β naphthyl esterase. Two buffer systems were tried for this enzyme. Band

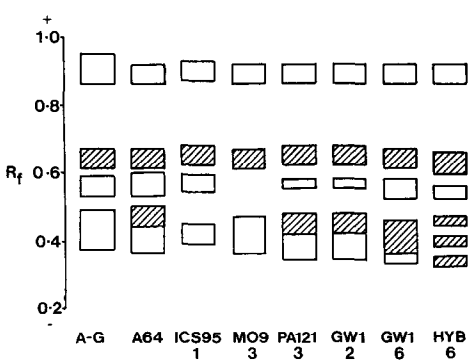

Fig. 1. β-acid phosphatase extracted from leaf tissue. R_f values for a 6 hour run on system 1 gel.

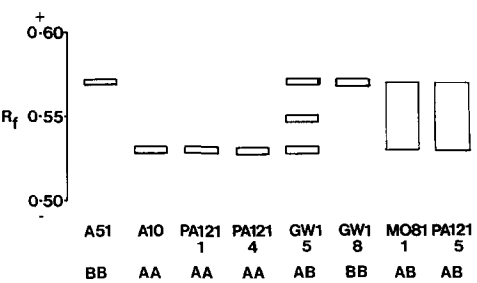

Fig. 2. Alcohol dehydrogenase extracted from leaf tissue. R_f values for a 6 hour run on system 1 gel.

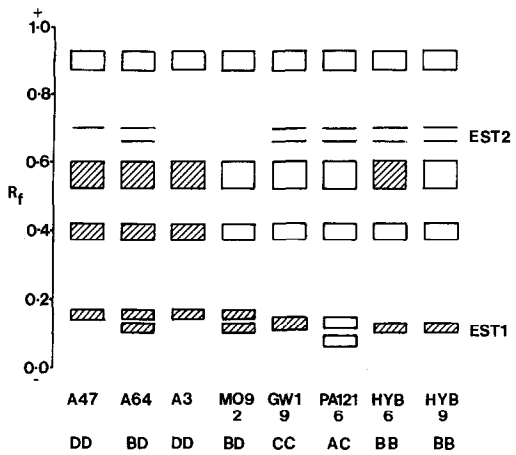

Fig. 3. α and β-naphthyl esterase extracted from leaf tissue. R_f values for a 6 hour run on system 1 gel.

resolution was very poor with system 3. On system 1 a large number of bands was observed, which could be grouped into three invariant and two varying regions, Fig. 3). The most cathodal of the variable regions, Est-1 showed either one or two prominent black bands. The implication of this is that Est-1 codes for a monomeric enzyme. Four bands, denoted a, b, c and d have been observed here. The other variable region, Est-2 consisted of one or two thin faint bands. The number of alleles represented by these latter bands and their mode of inheritance has not yet been ascertained.

(iv) Malate dehydrogenase. Attemps were made to localise this enzyme system on buffer system 1, 2, 4 and 5 gels. Bands were discernible but unclear on system 1 and 2 gels and better resolved on system 5 gels. System 4 gels gave superior resolution.

Of the 16 plants which were tested for this enzyme system, 14 yielded the same banding pattern. This is shown in Fig. 4 as a pair of heavily stained bands in region 1, a single faint band in region 2 and a pair of bands in region 3. The remaining two plants (X1 and X2) showed differences in regions 1 and 2.

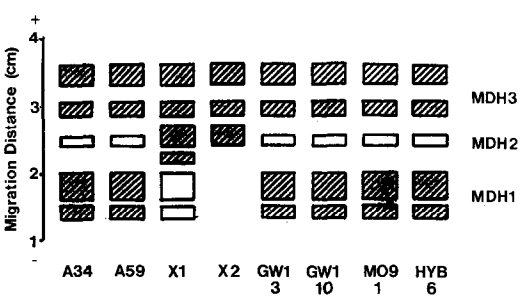

Fig. 4. Malate dehydrogenase extracted from leaf tissue. Absolute migration distances, in centimetres, for a 4 hour run on system 4 gel.

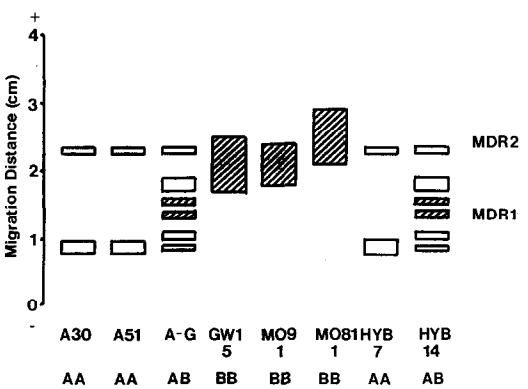

Fig. 5. Menadione reductase extracted from leaf tissue. Absolute migration distances, in centimetres, for a 4 hour run on system 4 gel.

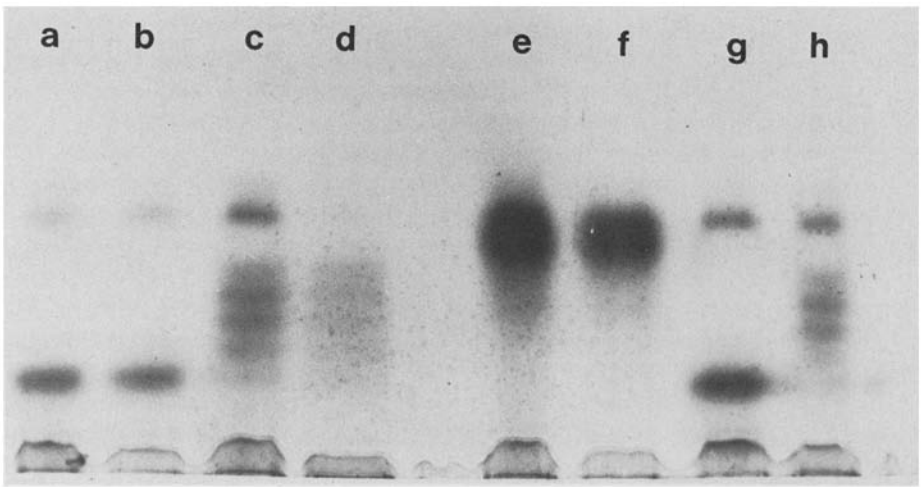

Fig. 6. Menadione reductase. 6 hour run on a system 5 gel. a) A30 b) A51 c) A–G d) X1 e) GW1-5 f) MO9-1 g) HYB-7 h) HYB-14.

(v) Menadione reductase. Three buffer systems were tried for this enzyme. System 4 gave the sharpest bands and greatest separation. Band sharpness on system 1 was slightly less and on system 5, considerably less.

The following tentative hypothesis is suggested for the interpretation of this enzyme system (Fig. 5 and 6). There are 2 loci, Mdr-1 and Mdr-2. Mdr-1 has (on the material tested) 2 alleles, a and b. The aa homozygote appears as a single band. The bb homozygote fuses with the Mdr-2 band to give a heavily staining broad band. The ab heterozygote appears as a series of five bands, which suggests that the molecule coded by this locus is tetrameric. Mdr-2 has one invariant allele, appearing as a single band.

(vi) Phosphoglucoisomerase. This enzyme was localised on gels run under all five buffer systems. One of these, (system 4), was considered by LANAUD & BERTHAUD (1984) to be the most suitable for this enzyme. However, in the present work, system 4 gave erratic results. Sometimes bands were well separated and at other times were very close. Bands on this buffer system were always poorly defined. On buffer system 1, although the bands were very sharp they were not very well separated. The best and most consistent separation of bands, with adequate definition, was given by system 5. On all the buffer systems tested, the same basic banding pattern was observed: One variable locus Pgi-1 and one invariant locus represented by a single band (Pgi-2).

Pgi-1 is represented either by a single band (in one of three positions denoted a, b, c) or by three bands (Fig. 7). This implies the production of a dimeric molecule. LANAUD & BERTHAUD (1984) observed 4 alleles for this enzyme but only 3 have been observed in this study.

(vii) Phosphoglucomutase. Staining of this enzyme on buffer system 4 is very diffuse. Systems 1 and 5 give good separation, system 5 being the superior (Fig. 8).

One variable locus, Pgm-1 and one invariable locus, Pgm-2 are seen. Two alleles have been observed for Pgm-1 and are denoted a and b. Either a single band or a pair of bands are present, which suggests that the enzyme is a monomer.

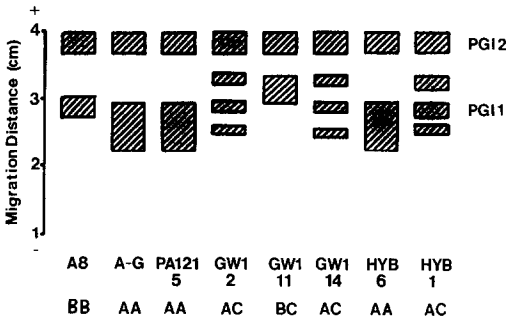

Fig. 7. Phosphoglucoisomerease extracted from leaf tissue. Absolute migration distances, in centimetres, for an 18 hour run on system 5 gel.

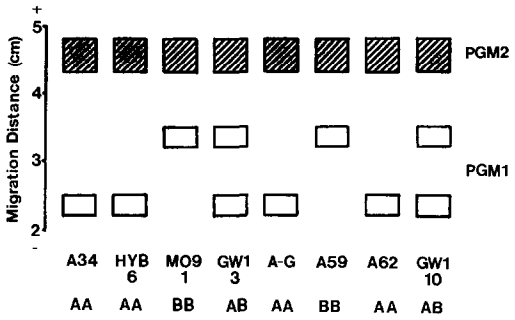

Fig. 8. Phosphoglucomutase extracted from leaf tissue. Absolute migration distances, in centimetres, for an 18 hour run on system 5 gel.

Effect of leaf age on enzyme activity. Several developmental stages of leaves were tested for their enzyme activity on starch gels. As far as phosphoglucoisomerase, phosphoglucomutase and leucine aminopeptidase were concerned, there was no difference between very young, unhardened leaves and matured hardened leaves. However, alcohol dehydrogenase and isocitrate deydrogenase activity were drastically reduced in hardened leaves.

Effect of freezing leaf material. A preliminary study was made of three enzymes: Esterase, β-acid phosphatase and alcohol dehydrogenase. Leaves were subjected to two treatments with liquid nitrogen (−196 °C); (1) Immersing whole leaves for a few minutes before grinding a part of the leaf. (2) Grinding fresh leaf tissue and freezing the extract in niquid nitrogen for a few minutes. Neither of these treatments had any discernible effect on the staining activity or banding patterns of the three enzymes compared with fresh leaf tissue. In contrast, banding patterns were modified by freezing and storage for several days at −20 °.

2. Wood enzymes

Staining for five enzyme systems was attempted for this tissue; peroxidase, esterase, leucine aminopeptidase, glutamic oxaloacetate transaminase and malate dehydrogenase. Only peroxidase showed any activity.

This enzyme showed cathodal as well as anodal staining activity. All the plants tested from amongst the progeny of MO9, MO81, Amelonado and PA121 showed the same banding pattern, with 2 anodal and 2 cathodal bands (Fig. 9). All the plants tested from the progenies of GW1 and HYB had these bands together with a third, intermediate cathodal band (Arrowed in Fig. 9). No other repeatable differences have been observed. Most of the screening was carried out on system 1 gels. System 3 gels performed similarly.

This enzyme showed the unusual property, compared with most of the variable leaf enzymes studied, that all the plants within a progeny that were tested showed the same banding pattern. All the leaf enzymes, except MDH showed variability within progenies.

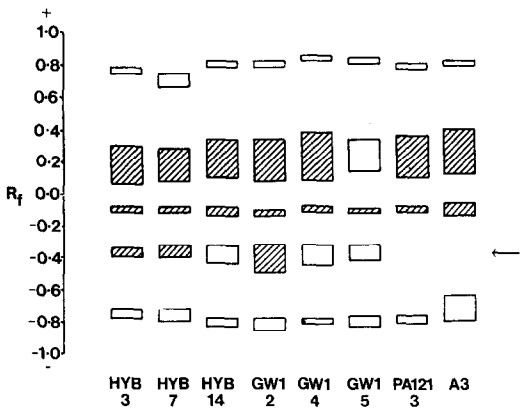

Fig. 9. Peroxidase extracted from woody tissue. R_f values for a 3 hour run on system 1 gel.

DISCUSSION

Of the 17 leaf enzyme systems which showed activity, 9 exhibited variation and in 7 of the enzymes, the variation was reproducible (Table 1). It is entirely possible that by screening a wider range of genotypes, variation might be apparent in other loci. It is also very likely that some of the loci shown here to be variable will yield more alleles when sampling is extended. However, 'fingerprinting' of genotypes is feasible even with this relatively small number of variable loci. Addition of new alleles and new variable loci will tend to increase the number of genotypes which can be identified.

In order to confirm the genetic hypotheses advanced here, it is necessary at least to compare the electrophoretic patterns of progeny with those of their parents, but this was outside the scope of the present investigation. Such work was carried out by AMEFIA et al. (1984) in a large scale study of the inheritance of cacao esterases visualised on polyacrylamide gels. An investigation into the genetic basis of polymorphism in three cacao enzymes was made by LANAUD & BERTHAUD (1984).

One immediate practical use of this type of information afforded by isoenzyme analysis would be to aid the identification of clones in commercial use, particularly those used as breeding stock. Reliable identification of material is of great importance to cacao breeders and is at present carried out by measurement and scoring of morphological descriptors of which the fruit and flower are the principal sources. (For descriptor lists see IBPGR (1981) and SORIA & ENRIQUEZ (1981) and for discussion of morphological descriptors, see ENGELS et al. (1980). It is worth stressing here that, since the usual descriptors tend to rely heavily on fruit and flower characters which are not apparent until the plants are several years old, isoenzyme characters have the advantage that they can be scored when the plants have produced their first leaves. Given that the usual descriptors have an unknown relation to the genotype, isoenzyme markers allow one to measure, more directly, genotypic variation.

Now that the importance of characterisation of germplasm is being more widely appreciated, it may be possible to introduce a standard protocol for isoenzyme screening into several genebanks. Standardisation of the procedure would increase the value of cultivar catalogues thus produced. The starch gel electrophoresis method presented

here has the advantage of being relatively inexpensive (as compared to more sensitive procedures such as isoelectric focusing) and requires correspondingly less specialised equipment.

Apart from identification of clones for breeding purposes, one could suggest a number of further uses to which isoenzyme data derived from gene bank material could be put. Firstly, there is the use of the data for characterising plants other than the usual commercial and breeding clones, i.e.: wild and primitive cultivar material in genebanks. Since space is at a premium in many field genebanks, particularly if new collections are being incorporated, discarding of parts of collections with low genotypic variation may be a necessary part of curation. One is concerned, after all, about conserving genotypic and not phenotypic variation.

Secondly, collection of cacao from wild and old cultivar populations is a subjective activity as far as the capturing of variation is concerned. Any information about genetic variation of such populations which could be made available by an electrophoretic survey, and which would improve the efficiency of their collecting strategy, would increase the cost effectiveness of such expeditions.

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