Biochemical Markers of Variety in *Cocos nucifera* L. from Yucatan[1]

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ABSTRACT

Isozyme and protein patterns were studied in local varieties of *Cocos nucifera* from Yucatan. Two acid phosphatases, two esterases, one malate dehydrogenase, and one alcohol dehydrogenase, were detected in extracts from inflorescences by isoelectrofocusing, and observed to be monomorphic for the four varieties tested: Atlantic Tall, and Malayan Green, Red, and Yellow Dwarves. Five acid phosphatases, three esterases, four malate dehydrogenases and four alcohol dehydrogenases were also found to be monomorphic for all four varieties in leaf tissue. Peroxidase isozyme patterns from both types of extracts were variable among the varieties, but this was due to unknown factors other than the genotype variety. However, analysis of the coconut endosperm of the dwarf varieties consistently revealed a peroxidase of pI ~ 4, and a silver-stained protein band of the same pI. Both of these were greatly diminished in the tall variety. Finally, sodium dodecyl sulfate-polyacrilamide gel electrophoresis revealed two polypeptides of Mrs ~ 18 000 and 38 000 that were present in the three dwarf varieties, but greatly diminished in the tall variety. These results indicate that, although the dwarf and the tall varieties of coconut are closely related, there are slight differences that permit a clear biochemical distinction between the Malayan Dwarves and the Atlantic Tall trees.

Key words: Coconut, lethal yellowing, protein markers, isozymes, endosperm.

COMPENDIO

Se analizaron patrones de proteínas o isoenzimas en variedades locales de *Cocos nucifera* de Yucatán. Se detectaron dos fosfatasas ácidas, dos esterasas, una malato y una alcohol dehidrogenasa, en extractos de inflorescencias después del isoelectroenfoque. Estas actividades fueron monomórficas en las variedades estudiadas: Alto del Atlántico y enanos malayos Verde, Rojo y Amarillo. En el tejido proveniente de hojas, cinco fosfatasas ácidas, tres esterasas, cuatro malatos y cuatro alcoholes dehidrogenasas fueron también monomórficos para todas las variedades. Los patrones de peroxidasas, en ambos tipos de extracto, fueron variables entre variedades, pero debido a factores diferentes del genótipo. Contrariamente, el análisis del endospermo de las tres variedades enanas mostró consistentemente una peroxidasa de pI ~ 4 y una proteína detectada con tinción de plata con idéntica movilidad. Ambas fueron muy débilmente detectables en la variedad Alto del Atlántico. Finalmente, una electroforesis disociante en geles de poliacrilamida mostró dos polipéptidos de Mr ~ 18000 y 38000 que estuvieron presentes en las tres variedades enanas pero disminuidas en Alto del Atlántico. Estos resultados indican que, aunque ésta como las enanas están íntimamente relacionadas, existen diferencias bioquímicas que permiten una clara distinción desde ese punto de vista entre ellas.

INTRODUCTION

Cocos nucifera is a crop with a world-wide distribution that predominates in the tropics. The modern pattern of distribution of the different varieties is due to the movement of the seeds from their center of origin to Africa and the American continent (6, 11) by the Spanish, Portuguese and Chinese (7, 14). Mexico has a rich source of germ plasm variation that still remains to be defined, and efforts have already been started (19). Classification of existing germ plasm in Mexico has been carried out through the measurement of physiological parameters (i.e., fruit form, husk/meat ratio, etc.). These methods would be greatly complemented if biochemical markers for the varieties were available to aid in the establishment of germ plasm identities and interrelationships. This characterization is even more important in view of the spread of lethal yellowing (LY) disease in Mexico (4) from the Caribbean coast. In Jamaica, LY affected coconut varieties differentially; Malayan Dwarf showed almost 100% resistance (Zizumbo Villarreal, pers. comm.), Panama Tall close to 50% (20), and Jamaica or Atlantic Tall almost no resistance (20). With

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these data, one could predic the impact of the disease on a plantation if the origin and identity of the materials were clearly established.

It is shown here that, although the Dwarf and Tall varieties are closely interrelated in Yucatan, one can distinguish the Malayan Dwarf varieties from the Atlantic Tall variety through the biochemical analysis of protein and isozyme patterns.

MATERIALS AND METHODS

Plant material

All dwarf varieties were collected from commercial plantations located in San Crisanto, Yucatan. The tall variety was collected from different plantations along the coast of Yucatan where LY symptoms were absent (4). The internal rachillae from the oldest unopened inflorescences and basal pinnae from basal leaves were used for the analysis. In addition, mature fruits were collected from all varieties following the criteria described by Harries (6), and from which the solid endosperm was used for the analyses.

Tissue extractions

Two tissue extraction were employed. Nondenaturing extractions methods were carried out by grinding 1 g of tissue in 1 ml of 10% (v/v) glycerol, 1 mM sodium ascorbate, 3 mM l-cysteine, 10 mM Tris-HCl, pH 6.0, in a mortar and pestle at 4°C. The homogenates were centrifuged at 14 000 x g, for 5 min at 4°C and the supernatants stored at -70°C until analyzed. Denaturing extractions were performed by a modification of the procedure of Wu and Wang (18). One gram of tissue was frozen with liquid nitrogen and ground in a mortar and pestle to a fine powder. The powder was added to 10 vol of 10% (v/v) trichloroacetic acid (TCA) and incubated 30 min in darkness. After incubation, the slurry was filtered through two layers of cheese-cloth and the flowthrough was centrifuged at 27 000 x g, for 15 min al 4°C. The pellet was washed three times by resuspension in 1:1 ethanol-ether and recentrifuged at 27 000 x g. Proteins in the pellet were resolubilized in 1% (w/v) sodium dodecyl sulfate (SDS), 1.5 mM B–mercaptoethanol, 10% (v/v) glycerol, 10 mM Tris-HCl, pH 6.8, and the 27 000 x g supernatant collected for further analysis.

Protein determination

The protein content in the extracts was determined by the method of Markwell *et al.* (9), which is designed to detect the presence of SDS in the samples.

Isoelectrofocusing-polyacrylamide gel electrophoresis

Isoelectrofocusing-polyacrylamide gel electrophoresis (IEF– PAGE) was carried out by blotting the nondenatured extracts on to 5 x 10 mm squares of Whatman paper and placing them on LKB isoelectrofocusing polyacrylamide gels (LKB Bromma) with ampholytes with a pH range of 3.5 to 9.5. The gels were run at 400 v, 25 mA, for 2 h 30 minutes. Enzyme activities were detected on the gels as follows: esterases, acording to Shaw and Prasad (12); acid phosphatases, Scandalios (13); peroxidases, Villanueva and Malek–Hedayat (15); malate (MDH) and alcohol (ADH) dehydrogenases, Brown, Zohary, and Nevo (3); and phosphoglucoisomerases (PGI), Vallejos (16).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were adjusted to 2.3% (w/v) SDS, 5% (v/v) B-mercaptoethanol, 10% (v/v) glycerol, 10 mM Tris-HCl, pH 6.8, and boiled for 5 minutes. The samples were run on 15% polyacrilamide gels according to Laemmli (8). The proteins were stained with 0.1% (w/v) Brilliant Coomassie Blue in 5:5:2 water– methanol-acetic acid (10), or with ammoniacal silver (17).

RESULTS

Although minor bands sometimes appeared in the gel patterns, our assessment of differences among the varieties were focused on major, reproducible bands.

The pattern of the four enzyme activities in inflorescence extracts analyzed by IEF-PAGE was identical for all four varieties studied. The acid phosphatase isozymes consistently showed two bands with enzymatic activity in all four varieties studied (Table 1), although one band of pI~4 showed inconsistently in either the Malayan Green or the Malayan Red Dwarf

varieties (data not shown). The esterase isozymes also showed an identical pattern of two bands in all four varieties analyzed (Table 1), but only one ADH and one MDH with identical pIs were detected (Table 1). The activity of peroxidase isozymes was also studied in the same extracts, but the patterns were very different in each of five experiments (data not shown), suggesting that, rather than polymorphism due to variety, external factors influenced the expression of these enzyme activities.

Table 1. Isoelectric points of acid phosphatases, esterases, MDH and ADH from inflorescence extracts of different varieties of *Cocos nucifera* from Yucatan.

	acid phosphatase	esterases	MDH	ADH
Malayan Yellow Dwarf	6.6, 6.9	6, 6.9	6.9	6.9
Malayan Green Dwarf	6.6, 6.9	6, 6.9	6.9	6.9
Malayan Red Dwarf	6.6, 6.9	6, 6.9	6.9	6.9
Atlantic Tall	6.6, 6.9	6, 6.9	6.9	6.9

The same enzyme activities were analyzed in extracts of pinnae from leaves. Although the number of major bands varied in some cases compared to the ones detected in inflorescence extracts, the patterns of MDH, ADH, esterases and acid phosphatases were identical in all four varieties analyzed (Table 2). Five acid phosphatase bands were observed in all four varieties (Table 2). Contrary to the inflorescence extract patterns, three esterase bands consistently observed in the leaf extracts of the four varieties analyzed (Table 2). Finally, four bands with ADH and MDH activities with identical pIs were detected in all four varieties (Table 2). The proteins with peroxidase activity in these extracts were also irreproducible in all four varieties tested.

Table 2. Isoelectric points of acid phosphatases, esterases, MDH and ADH from leaf extracts of different varieties of *Cocos nucifera* from Yucatan.

	acid phosphatase	esterases	MDH	ADH
Malayan Dwarves All varieties (Yellow, Green, Red)	2.6, 6.6, 7.0, 7.3, 7.5	6.8, 7.1, 7.4	2.9, 6.9, 7.3, 7.5	3.0, 6.7 7.2, 7.5
Atlantic Tall	2.6, 6.6 7.0, 7.3 7.5	6.8, 7.1 7.4	2.9, 6.9 7.3, 7.5	3.0, 6.7 7.2, 7.5

Due to the high degree of monomorphism found with the enzyme activities tested in inflorescence and leaf extracts, and the high variation that the peroxidase activities displayed, analysis was focused on fruit endosperm, since it is the organ with the most constant physiological stage at maturity. Mature fruits of all four varieties were analyzed and selected according to the criteria described by Harries (6). The extracts from the endosperm were extracted under nondenaturing conditions and analyzed by IEF-PAGE. The resulting gels were incubated in substrates to detect PGI and peroxidase activities. The pattern obtained for the PGI activities was identical for all four varieties analyzed (Fig. 1).

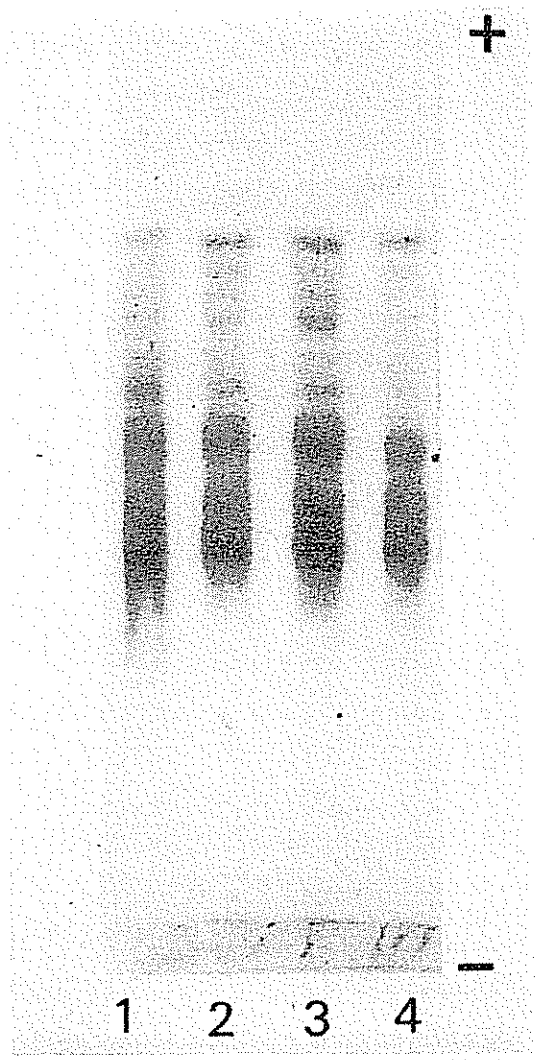

Fig. 1. Isozyme pattern of phosphogluco-isomerase activities from mature solid endosperm from *Cocos nucifera* after isoelectric focusing on polyacrylamide gels. The lanes correspond to extracts from the varieties: 1, Atlantic Tall; 2, Malayan Red Dwarf; 3, Malayan Green Dwarf; and 4, Malayan Yellow Dwarf. The symbols represent the acidic (+), and basic (-) ends of the gel.

However, the peroxidase activity pattern showed a band with a pI~4 that was present only in the three Malayan Dwarf extracts (Fig. 2, lanes 1-3), and completely absent in the Tall variety (Fig. 2, lane 4). This pattern was consistent in five independent experiments. In a parallel analysis, IEF-PAGE gels were stained with ammoniacal silver to detect total protein. A protein band of pI~4 that was present in all three Malayan Dwarf varieties was again observed (Fig. 3, lanes 1-3), but absent in the Tall variety (Fig. 3, lane 4). It is important to note that all three Malayan Dwarf varieties had identical patterns in all the analyses.

In addition to the analysis carried out by IEF-PAGE, differences at the level of total protein on SDS-PAGE gels were studied. The proteins were run and stained with Coomassie Blue. The analysis showed consistency in the protein patterns of the Malayan Dwarf varieties; however, all three varieties consistently showed two polypeptides of Mrs ~18 000 and 38 000 (Fig. 4, lanes 2-4) that were almost not able to be detected in the Tall variety (Fig. 4, lane 1).

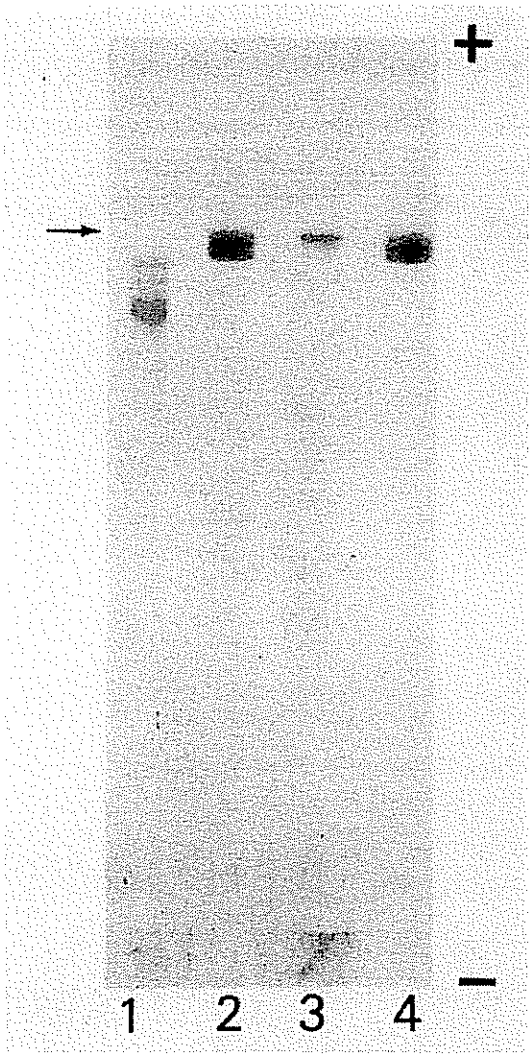

Fig. 2. Isozyme pattern of peroxidase activities from mature solid endosperm from *Cocos nucifera* after isoelectric focusing on polyacrylamide gels. The lanes correspond to extracts from the varieties: 1, Atlantic Tall; 2, Malayan Red Dwarf; 3, Malayan Green Dwarf; and 4, Malayan Yellow Dwarf. The arrow indicates the position of electrofocusing of a protein of pI ~ 4.0. The symbols represent the acidic (+), and basic (-) ends of the gel

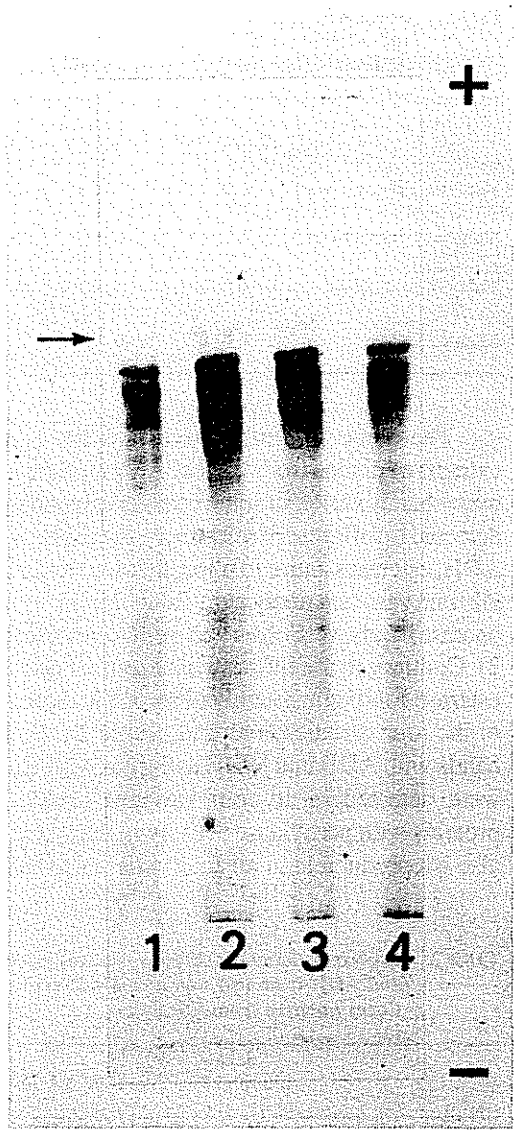

Fig. 3. Total protein pattern from mature solid endosperm from *Cocos nucifera* after isoelectric focusing on polyacrylamide gels, and subsequent silver staining. The lanes correspond to extracts from the varieties: 1, Malayan Yellow Dwarf; 2, Malayan Green Dwarf; 3, Malayan Red Dwarf; and 4, Atlantic Tall. The arrow indicates the position of electrofocusing of a protein of pI~4.0. The symbols represent the acidic (+), and basic (-) ends of the gel.

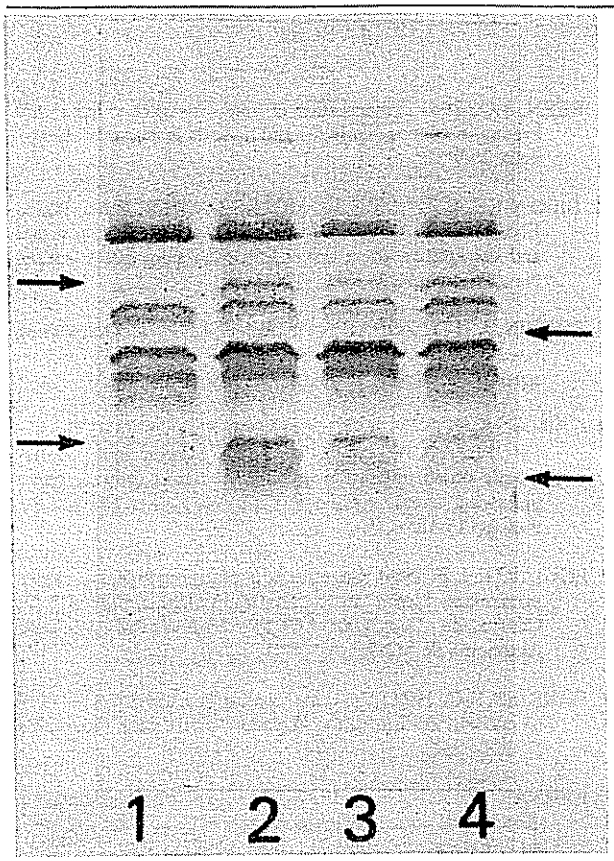

Fig. 4. Total protein pattern from mature solid endosperm from *Cocos nucifera* after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequent coomassie blue staining. The lanes correspond to extracts from the varieties: 1, Atlantic Tall; 2, Malayan Red Dwarf; 3, Malayan Yellow Dwarf; and 4, Malayan Green Dwarf. The arrows on the right indicate, from top to bottom, the molecular weight markers: carbonic anhydrase, 29 000; cytochrome c, 13 000. The arrows on the left indicate, from top to bottom, proteins with a relative mobility of 38 000, and 18 000.

Results demonstrate a close interrelationship among all four varieties of coconut, especially among the Malayan Dwarf varieties. However, slight differences in the protein expression allow the researcher to distinguish between a Malayan Dwarf and an Atlantic Tall tree.

DISCUSSION

Among the varieties of *Cocos nucifera* in Yucatan, the Malayan Dwarf and the Atlantic Tall varieties predominate. In addition to the phenotype, biochemical criteria for the assessment of the particular variety are necessary. Further, a catalog of biochemical markers can be most helpful in the establishment of a taxonomical system that will allow the classification of all existing germ plasm in Mexico.

In this study, the existence of such biochemical markers of variety in the coconut trees that predominate in the coast of Yucatan were sought. Two types of analyses were carried out: one to determine isozyme patterns in the four varieties mentioned, in order to observe differences in the expression of these proteins; the other, to look for differences at the level of total proteins, both under nondenaturing and denaturing conditions.

Analysis of isoenzyme patterns in both inflorescence and leaf extracts showed that in all four varieties of coconut the expression of these proteins is well conserved, indicating a close evolutionary interrelationship. The acid phosphatases and the esterases showed a similar pattern in the extracts from inflorescences, suggesting that they may be equivalent enzyme activities, as esterases have a broad substrate specificity (13). On the other hand, the ADH and MDH identical isozyme patterns may be distinct proteins, unless they are the result of general dehydrogenase activities with a broad substrate specificity. The peroxidase activities seemed to show a high degree of polymorphism at first, but subsequent experiments showed that the patterns were not consistent. This suggests that the expression of these enzymes is highly influenced by external factors and/or the physiological stage of the plant. Due to this, and based on a previous report of taxonomical studies carried out on the Palmaceae plant, *Astrocaryum mexicanum* (5), analyses were focused on fruit which was less susceptible to variation in protein expression due to external factors.

Since all four enzyme activities except peroxidases, presented monomorphism, the analysis of the activities of PGIs and peroxidases by IEF-PAGE in fruit endosperm was chosen. In five independent experiments, it was found that the PGI activity bands were identical in the four varieties analyzed. However, the peroxidase activities, although identical in all three Malayan Dwarf varieties, showed an activity band of pI~4 that was present in the dwarves but absent in the tall variety. A similar result was obtained when total protein was analyzed in parallel gels; a protein band of pI~4 was also detected in all three Malayan Dwarf varieties, but was absent in the tall variety. Although they have similar pIs, we do not know whether the protein detected by the silver stain is the same one that displays peroxidase activity.

Alternatively, the analysis on SDS-PAGE gels of the total proteins of all four varieties under denaturing

conditions showed an identical pattern in the three Malayan Dwarf varieties; however, two polypeptides of Mrs 18 000 and 38 000 were virtually absent in the tall variety.

Our results indicate that the four varieties of *C. nucifera* are closely interrelated, with the tall variety diverging slightly in the expression of some proteins. We do not know that the significance of extra proteins in dwarf varieties is, although we might speculate that, for example, the extra peroxidase activity provides protection against the attack of pathogens (2). This initial finding of biochemical differences between dwarf and tall varieties of *C. nucifera* indicates that differences among varieties and ecotypes can be established further by two-dimensional gel electrophoresis or by molecular analysis at the gene level. In addition, these data could be used to certify either Malayan Dwarf or Atlantic Tall trees, as well as establish a taxonomical catalog for coconut varieties in Mexico.

CONCLUSIONS

1. IEF-PAGE analysis of leaf and inflorescence extracts yielded acid phosphatase, esterase, malate dehydrogenase, and alcohol dehydrogenase isozymes which were monomorphic in Atlantic Tall, and Malayan Red, Green and Yellow Dwarf varieties of coconut from Yucatan.

2. IEF' and SDS-PAGE analyses from fruit endosperm extracts showed that the Atlantic Tall can be distinguished from the three Malayan Dwarf varieties by the following: a) a peroxidase of pI~4 predominantly present in the Malayan dwarves; b) a silver-stained polypeptide of the same pI also present in the dwarf varieties; and c) two polypeptides of Mrs~18 000 and 28 000 predominantly present in the dwarf varieties.

3. Malayan Red, Green and Yellow Dwarf varieties were indistinguishable among themselves by any of the analytical procedures.

4. Although all four varieties are closely related, slight differences in their isozyme and protein patterns could be detected (i.e., one protein band by IEF page and two by SDS-PAGE), which suggests that further analysis could be used for classification of other varieties.

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Este volumen presenta el progreso de los estudios sobre materia orgánica en suelos y su aplicabilidad agrícola. Los aspectos básicos y el impacto ambiental, especialmente en condiciones de las zonas templadas, son también considerados.

El material está organizado en cinco secciones, cada una con un comentario introductorio a cargo de un conocido especialista en el campo.

En la primera sección, casi una cuarta parte del volumen, se examina la información reciente sobre la composición química y estructura de la materia orgánica. Se dedica gran atención al estudio de los métodos instrumentales recientes como el de la resonancia magnética nuclear.

En una segunda sección más breve se trata de la relación entre materia orgánica y calidad de agua. Se examinan los problemas ocasionados por los plaguicidas en el agua y, especialmente, el transporte de nitratos.

En la tercera sección se analiza la relación entre materia orgánica en suelos y su estructura, con énfasis especial en la estabilidad de agregados secundarios, tanto en suelos de regiones templadas como en los trópicos. Este, como los otros capítulos, se distinguen por contener un gran volumen de resultados experimentales de campo, citados en apoyo a las presentaciones principales.

En la cuarta sección se informa sobre las transformaciones de la materia orgánica en suelos. Se examinan aspectos novedosos como el efecto de niveles aumentados del CO_2 atsmosférico y el uso del C^{14} producidos por explosiones nucleares, como indicador de la descomposición de la materia orgánica.

La quinta sección examina el efecto de la materia orgánica sobre la fertilidad de suelos; el efecto de residuos agrícolas como de los urbanos e industriales. Se examina también el papel central desempeñado por la materia orgánica en la agricultura orgánica.

El volumen concluye con un amplio índice de materiales; bien documentado y editado sobre el estado actual de la investigación sobre materia orgánica.

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