ISOENZYMATIC ANALYSIS FOR DETECTING *In Vitro* VARIABILITY AND/OR STABILITY OF ECONOMICALLY IMPORTANT CROPS

Regla M. Lara, Marilyn Florido✉, Dagmara Plana, Olivia Moré, María E. González, Marta Álvarez and María M. Hernández

ABSTRACT. One of the most frequent problems for developing tissue culture techniques is the loss of genetic stability, because micropropagated materials under these conditions usually produce non-normal regenerating plants or somaclonal variants of the original varieties. However, sometimes, much advantage from these genetic disorders is taken in different plant breeding programs. It is, for this reason, important to monitor genetic stability of the material obtained, isoenzymatic techniques being very useful for this purpose. The objective of this work was to study the stability and/or genetic variability generated under these conditions. For doing so, electrophoretic analyses were performed to different enzymatic systems coming from potato embryogenic calluses, as well as to leaf tissue of tomato, soybean and coffee vitroseedlings, regenerated under *in vitro* conditions. The analysis was carried out using polyacrilamide gels at different concentrations, depending on the revealed enzymatic system, using a Mighty Small II vertical electrophoresis unit from "Pharmacia Biotech". With the use of isoenzymes, monomorphism was detected in tomato, coffee and soybean electrophoretic patterns, which allowed corroborating the genetic stability in these crops. On the other hand, potato calluses showed certain variations in the electrophoretic patterns of peroxidases and esterases; by means of such systems, it is feasible to detect any possible somaclonal variant in this crop. The results also allowed corroborating the importance of using such techniques as an auxiliary tool for carrying out *in vitro* regeneration processes, as well as their application for monitoring genetic stability in other micropropagated vegetable species under these conditions.

Key words: isoenzymes, electrophoresis, micropropagation, genetic stability

RESUMEN. Uno de los problemas más frecuentes cuando se utilizan las técnicas de cultivo de tejidos es la pérdida de estabilidad genética, pues muchas veces los materiales micropropagados en estas condiciones dan lugar a regenerantes no normales o a variantes somaclonales de las variedades de origen, aunque en ocasiones estas alteraciones genéticas son aprovechadas en diversos programas de mejoramiento. Es por ello que resulta de suma importancia cuando se utilizan estas técnicas de cultivo, monitorear la estabilidad genética del material que se obtenga, siendo las isoenzimas muy útiles para estos propósitos, por lo que el objetivo de este trabajo fue el estudio de la estabilidad y/o variabilidad genética generada por estas condiciones. Para ello se realizaron análisis electroforéticos en diferentes sistemas enzimáticos provenientes de callos embriogénicos de papa, así como tejido foliar de vitroplántulas de tomate, soya y café regeneradas y desarrolladas en condiciones *in vitro*. El análisis se efectuó utilizando geles de poliacrilamida a diferentes concentraciones, en dependencia del sistema enzimático revelado, en cámara de electroforesis vertical *Migthy Small II* de *Pharmacia Biotech*. Con el uso de las isoenzimas se detectó monomorfismo en los patrones electroforéticos de tomate, café y soya, lo cual permitió corroborar la estabilidad genética en estos cultivos; los callos de papa, por su parte, mostraron ciertas variaciones en los patrones electroforéticos de peroxidasas y esterasas, siendo factible, mediante estos sistemas, detectar posibles variantes somaclonales en este cultivo. Los resultados obtenidos permitieron corroborar la importancia del uso de esta técnica, como herramienta auxiliar en los procesos de regeneración *in vitro*, y su aplicación para monitorear la estabilidad genética en otras especies vegetales micropropagadas en estas condiciones.

Palabras clave: isoenzimas, electroforesis, micropropagación, estabilidad genética

INTRODUCTION

Tissue culture can produce genetic stability changes in different crops, and this is an essential element to take into account when developing breeding programs, with the objective of improving features of some plant crops (1).

Nowadays, tissue culture efficiency has increased a great deal through the use of new growth bioregulators of national production, as well as through the tune-up of new regeneration protocols, making these processes cheaper to a great extent (2, 3, 4).

In this sense, it is essential to make use of fast and accurate techniques for detecting crop genetic stability, which can serve as a base for certifying seeds, varieties

Ms.C. Regla M. Lara, Especialista; Ms.C. Marilyn Florido, Investigador Agregado; Ms.C. Dagmara Plana y Ms.C. Olivia Moré, Investigadoras y Dra.C. María E. González, Investigador Auxiliar; Dra.C. Marta Álvarez y Dra.C. María M. Hernández, Investigadoras Titulares del Departamento de Genética y Mejoramiento Vegetal, Instituto Nacional de Ciencias Agrícolas, Gaveta Postal 1, San José de las Lajas, La Habana, Cuba, CP 32 700

✉ mflorido@inca.edu.cu

and hybrids, as well as for an efficient development of genetic breeding programs, applied to economically important crops (5, 6, 7).

Several techniques, such as enzymatic and molecular markers, the study of karyotype and others, have been applied for measuring the genetic variability produced in regeneration processes (2, 5, 6).

During growth and development processes, many plants present dramatic changes in their enzymatic expression. The use of isoenzymes as markers is well documented and their genetically defined variants have evidenced their importance for evaluating genetic variability in some crops, such as rice, barley, maize, bean, potato, wheat, soybean, among others (5, 6, 8).

The present work was developed, taking the formerly expressed into account, with the objective of detecting genetic variability and/or stability in economically important crops, seeded under *in vitro* conditions.

MATERIALS AND METHODS

For developing this work, isoenzyme electrophoretic analyses were applied to leaf tissue from micropropagated seedlings of tomato (*Lycopersicon esculentum* Mill), soybean (*Glycine max*, L Merrill) and coffee (*Coffea*, spp). The same procedure was applied to potato (*Solanum tuberosum* L) embryogenic calluses, extracted from *in vitro* materials seeded in MS medium (9) and supplemented with different hormonal combinations, according to the analyzed crop (1, 2, 10).

For preparing extracts, 0,5 sized samplings from such materials were collected and homogenized in cold medium, using the established extraction buffer for each crop. The extract was centrifuged at 12 000 rpm and a temperature of 0^0C during 10 minutes. Afterwards, the supernatant was submitted to electrophoresis, which was run in polyacrilamide gels at different concentrations, according to isoenzyme nature for the separating gel, using a concentrating gel at 4% in all cases. Tris-HCl 0.375M pH8.9 and Tris-Glycin 0.025M-0.019M pH8.3 buffers were used in the compartments (11).

In each case, time for running electrophoresis depended on the movement of Kohlhauch band until near 6 cm from where separating gels started. The procedure was performed at a steady intensity of 25 mA, in a vertical electrophoresis Mighty Small II chamber from "Pharmacia Biotech". After separation was performed, specific stainings for each evaluated system were carried out.

Vegetable material and extraction buffer used in each analyzed crop

Tomato. Vegetable material: *Amalia* variety ternate leaves from *in vitro* regenerated seedlings, and vitroseedlings from regenerated seedlings starting from cotyledonal segments, which were cultivated in MS medium. In this case, 16 regenerating plants were used and compared to *Amalia* variety (starting material). Extraction buffer: Tris-HCL 0.05M to pH = 7.2, 10 % sucrose and 14 uM dithriothreitol at 1:2 (2).

Soybean. Vegetable material: *William 82* variety leaf samples from *in vitro* regenerated seedlings, and vitroseedlings from regenerated seedlings starting from hypocotyl segments, which were cultivated in MS medium using 0.5 $mg.L^{-1}$ ANA and 1 $mg.L^{-1}$ BAP. In this case, 18 regenerating plants were used and compared to *William 82* variety (starting material). Extraction buffer: Phosphate 0.2M at pH = 7.0, 10 % sucrose and 14 uM dithriothreitol at 1:2 (12).

Coffee. Vegetable material: *Robusta* variety leaf samples from *in vitro* regenerated seedlings starting from embryogenic calluses of this variety. In this case, 10 regenerating plants were used and compared to *Robusta* variety (starting material). Extraction buffer: Tris-HCL 0.05M at pH = 7.2, 10 % sucrose, 5 % PVP, 20 % sulfite-bisulfite solution at 2 % and 14 uM dithriothreitol at 1:2 (6).

Potato. Vegetable material: *Desirée* variety calluses cultivated in media with different hormonal combinations of 2,4D and kinetine, as well as in those where kinetine was substituted for MH5 and BB-16 (Cuban bioregulators). Extraction buffer: Tris-HCL 0.05M at pH = 7.2, 10 % sucrose, 5 % PVP and 14 uM mercaptoethanol at 2:1 (10).

Table I shows the different isoenzymatic staining systems used for each crop: peroxidases (Prx), esterases (Est) and polyphenol oxidases (Ppo) (11), amino transferase aspartate (Got), acid phosphatases (Aps) and malate dehydrogenase (Mdh) (13); as well as carbonic anhydrase (AC) (14).

Table I. Gel percentages according to each isoenzymatic system in different crops

Tomato		Soybean		Coffee		Potato	
8.5 %	10 %	8.5 %	10 %	7.5 %	10 %	8.5 %	10 %
Prx	Est	Prx	Est	Prx	Est	Prx	Est
Got	AC	Ppo	AC			Got	AC
Mdh			Aps			Ppo	Aps

RESULTS AND DISCUSSION

Tomato. Figures 1, 2, 3, 4 and 5 show the isoenzymatic electrophoretograms obtained after revealing gels, using the specific staining for AC, Mdh, Pox, Est and Got systems.

Enzymatic activity was observed in AC system (Figure 1), where both regenerating and donor plants presented two thick bands. Even though there is no evidence of its characterization in tomato, it is known that this is one of the most prevailing soluble proteins and that, in C3 plants, it is mainly found in chloroplasts, its average weight being from 140 to 250 kDA (15).

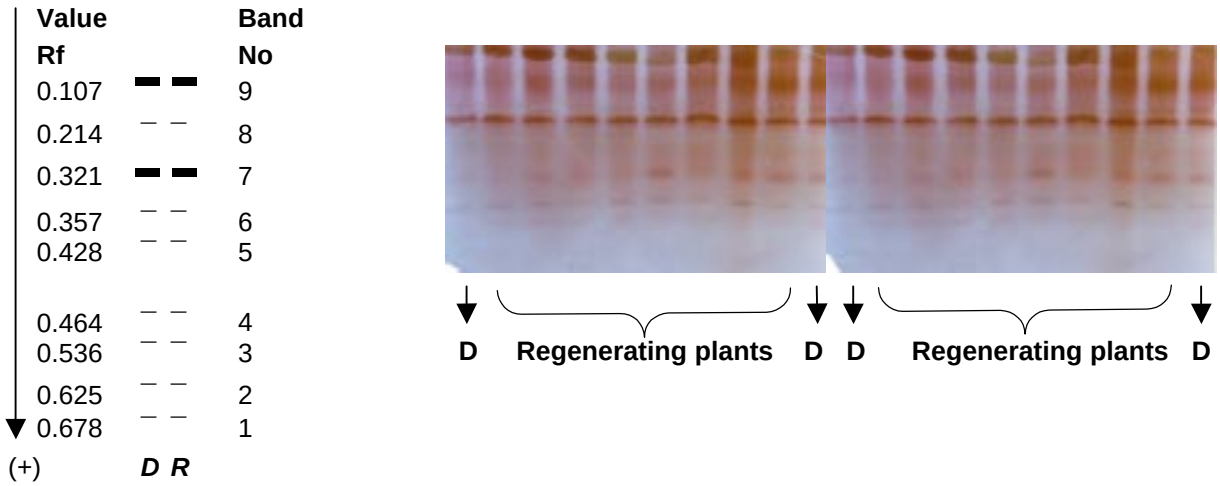


Figure 1. AC zymogram of regenerating plants (R) and Amalia cultivar (D)

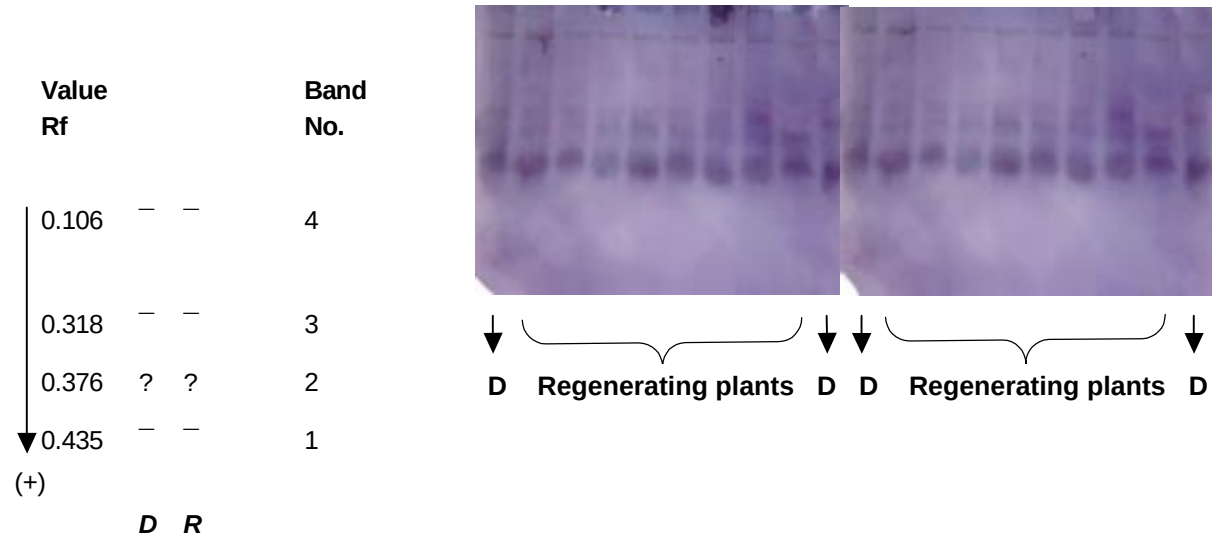


Figure 2. Mdh zymogram of regenerating plants (R) and Amalia cultivar (D)

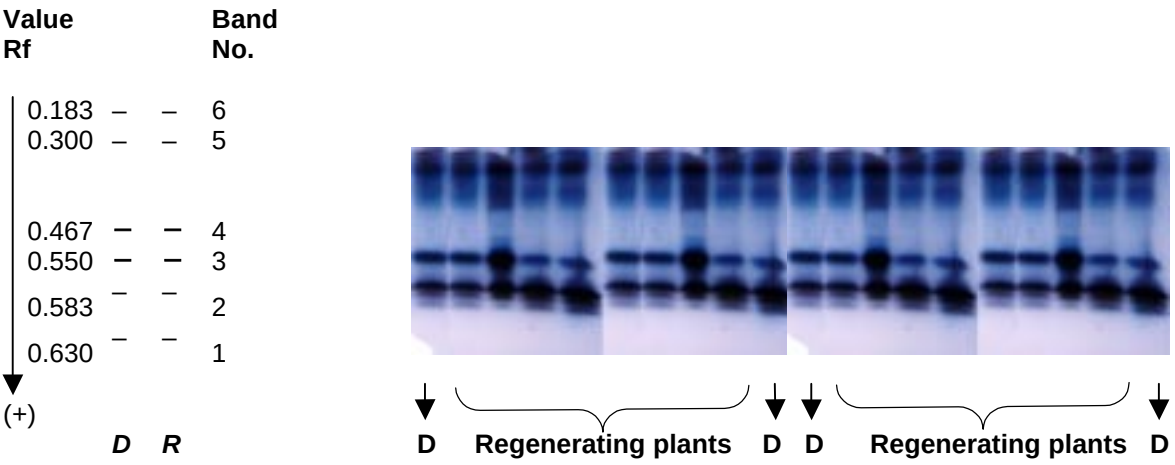


Figure 3. Prx zymogram of regenerating plants (R) and Amalia cultivar (D)

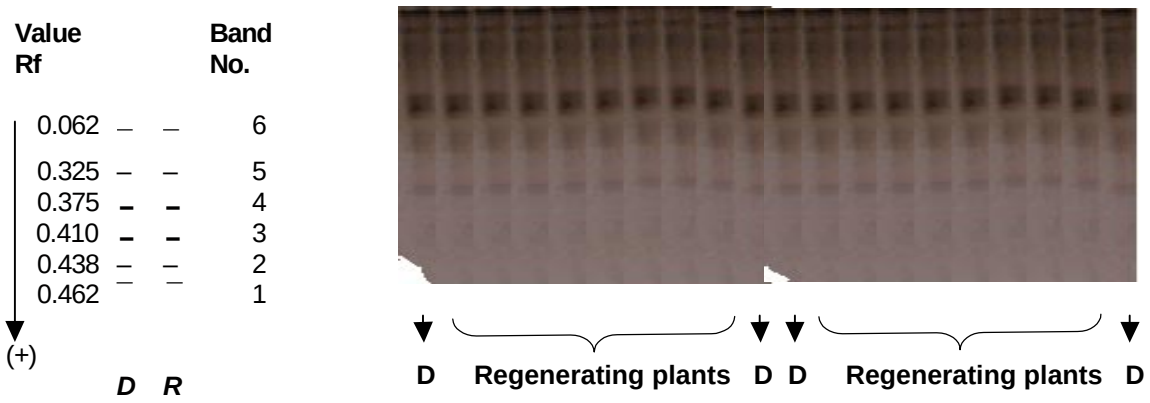


Figure 4. Est zymogram of regenerating plants (R) and Amalia cultivar (D)

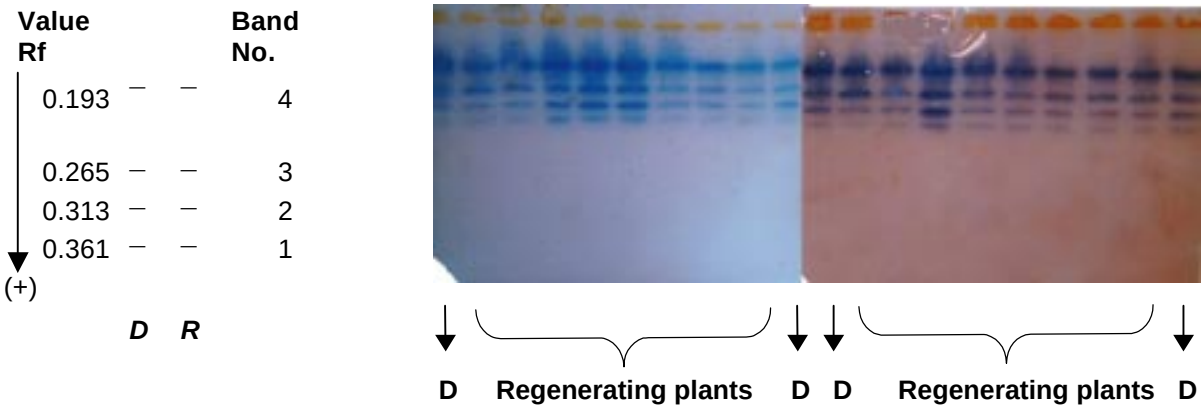


Figure 5. Got zymogram of regenerating plantes (R) and Amalia cultivar (D)

In the same way, the evaluated material presented electrophoretic similarity in Mdh system (Figure 2), which is included within crop genetic maps (16, 17), as well as characterized by presenting four well-defined fractions (0.106, 0.318, 0.376 and 0.435). It is known that this system could be monomorphic or dymeric in tomato and presents four polymorphic isoforms, from which only Mdh-1, Mdh-3 and Mdh-4 have been mapped and placed in chromosomes 3, 7 and 12, respectively (18).

The electrophoretic diagram of Prx isoenzymes (Figure 3) showed a high enzymatic activity in the analyzed material. Such system presented six well-defined bands, as well as features of *Lycopersicon* genus (5).

Different studies on crop mapping (17) have allowed knowing that var *Amalia* presents eight Prx isoforms, which codify a band complex. That is why this is one of the most used crops in biochemical studies, both in *Lycopersicon* and in other plant genera, for being within the most polymorphic ones, as well as due to stability and easy reproduction of its bands. This also includes the important role played by Prx isoenzymes in cell differentiation and their relationship with resistance to adverse biotic and abiotic factors (19, 20).

Likewise, it has been informed (21) that, due to its marked polymorphism, Est was one of the most appropriate systems for somaclonal isoenzymatic characterization of tomato. No polymorphism was found when the isoenzyme profile of such system was analyzed in vitroseedlings, compared to *Amalia* cultivar (Figure 4). This confirms that no genetic changes are associated to this system, due to the regeneration procedure used.

In regard to Est enzymes, it should be highlighted that they are conformed by a complex group of proteins, associated with specific intracellular proteins[1] (11, 14, 17), which present a total of eight polymorphic sites in the crop (17).

No appreciable changes in the electrophoretic pattern of Got system (Figure 5) were revealed by biochemical results in the enzymatic analysis. This system has been used for detecting hybrid variability of tomato seeds (5), also with the purpose of detecting genetic relationships in *Citrus* genus.

Soybean. This crop showed a significant electrophoretic homogeneity in all studied isoenzymatic systems (Figures 6, 7, 8, 9, 10).

Figure 6 shows the electrophoretic pattern of Prx system, which is characterized by presenting four well-defined monomorphic bands, with electrophoretic mobility of 0.39, 0.42, 0.46 and 0.48.

[1]Fernández, 1999, personal communication

Value Rf			Band No.
0.39	–	–	4
0.42	–	–	3
0.46	–	–	2
0.48	–	–	1

(+)

Regenerating W-82

Figure 6. Prx zymogram

Value Rf			Band No.
0.31	–	–	4
0.44	–	–	3
0.51	–	–	2
0.63	–	–	1

(+)

Regenerating W-82

Figure 7. Est zymogram

Value Rf			Band No.
0.08	–	–	2
0.12	–	–	1

(+)

Regenerating W-82

Figure 8. Ppo zymogram

Value Rf			Band No.
0.16	–	–	4
0.21	–	–	3
0.31	–	–	2
0.39	–	–	1

(+)

Regenerating W-82

Figure 9. AC zymogram

Value Rf			Band No.
0.127	?	?	2
0.445	–	–	1

(+)

Regenerating W-82

Figure 10. Aps zymogram

In this regard, it should be pointed out the fact that such system has been widely studied in soybean, since it presents a marked polymorphism, which seems to be a direct result of genetic differentiation (11). For this reason, electrophoretic similarity in this system suggests the existence of genetic homogeneity between donors and regenerating plants.

Electrophoretic analysis of Est in soybean regenerating plants revealed the existence of high homogeneous enzymatic activity in the analyzed material (Figure 7). This way, the presence of four well-defined bands with electrophoretic mobility of 0.31, 0.44, 0.51 and 0.63 could be confirmed.

It could be pointed out the fact that *G. max* presented two isoforms, known as Est 1 and Est 2, which could be identified taking into account their specificity to 1 and 2-naphthyl acetate substrates, where Est 1 produces three anode bands for both substrates. As to Est 2, it only shows its effect on 1-naphtyl acetate and produces three cathode bands, which could be thicker and more active than those detected in Est 1 system. Therefore, it could be said that Est system reveals a substantial activity in soybean (11, 12).

The material used presented two bands with low electrophoretic mobility in soybean regenerating plants for Ppo system (Figure 8) that were similar to *William 82* cultivar, which was the starting material. Some studies on this enzyme indicate that its expression mainly appears in early stages of development, because in adult plants mARN levels in genes are submitted to complex patterns of space and time regulations in vegetative and reproductive organs, generally located in epidermis and trichome cells (22).

Figure 9 shows biochemical results from isoenzymatic AC analysis, where a total of four bands, which are similar in regenerating plants, and the donor genotype appear. Although there is no evidence of the characterization of such enzyme in soybean, it is known that, in general terms, this is one of the most prevailing soluble proteins and in C3 plants it is mainly found in chloroplasts, its average weight being between 140 and 250 kDa (15).

Similar results were found when analyzing Aps system (Figure 10). Such isoenzymatic system is used for monitoring biochemical polymorphism in most vegetable species, it being used in studies on varietal identification and phylogenetic relationship, as well as for detecting response to environmental stress (5, 23). Three sites with acid phosphatase activity were detected in soybean (11). Two of them were invariant and a third one

showed three electrophoretic forms: fast, average and slow, the average mobility band being typical of species belonging to *Glycine* genus and the two remaining ones depending on the geographic area of the variety. It seems that, in this study, bands with fast (0.127) and average (0.445) electrophoretic mobility appeared, the latter one coinciding with that observed in studies of varietal polymorphism in soybean seeds (11).

After making an integral analysis of zymograms belonging to the different isoenzymatic systems studied, it could be highlighted the fact that 100 % were monomorphic bands, taking into account that many of such systems revealed crop genetic polymorphism (Prx, Est, Aps) (11). A similar degree of importance should be given to the fact that, in general, regenerating plants presented the same morphoagronomical features as *William 82* variety, which was the donor plant. Therefore, these techniques could be used for detecting crop genetic stability.

Coffee. Figures 11 and 12 show the electrophoretic diagrams of Prx and Est isoenzymes for this crop.

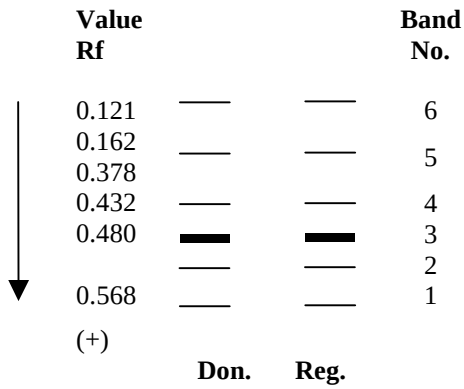


Figure 11. Prx zymogram

As it is seen in Figure 11, Prx system showed a pattern of six common bands in regenerating plants and *Robusta* variety, used as starting material. Similar results were obtained in studies on somatic embryogenesis in this crop (6), and identical band patterns were found between regenerating plants and this variety, using culture medium containing Pectimorf instead of commercial hormones. However, such author found patterns of specific expression on this system, for each development stage of samplings.

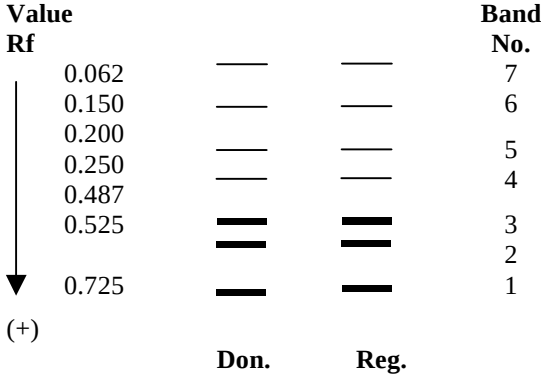


Figure 12. Est zymogram

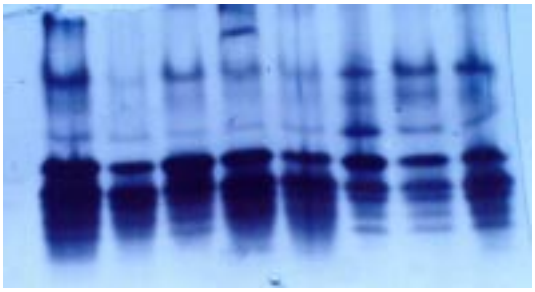
It is important to highlight that Prx isoenzyme has been widely used as genetic marker, since it presents different isoforms in most vegetable tissues (5, 24).

Likewise, Est system showed genetic homogeneity between regenerating plants and *Robusta* variety (Figure 12). This system was characterized by presenting a total of seven well-defined bands in all evaluated material.

The introduction of esterases as genetic indicators has been previously reported (4, 6) in similar studies. Such authors showed the great values of Est system, for carrying out studies on detecting genetic stability in this crop, since they could confirm the efficiency of regeneration on coffee, starting from somatic embryos.

Obtaining a great number of isoforms in this system is possible, since Est plays an important role in plant photosynthetic processes. This fact, in addition to its stability in enzymatic expression, gives it a lot of importance to genetic studies (2, 5, 7).

Potato. Isoenzymatic profiles of Prx and Est systems (Picture 1, Figure 13 and Picture 2, Figure 14) allowed to show their polymorphism on callus level in this crop. The analysis of Prx system in medium I, containing 3 mg.L^{-1} 2,4D, showed lower activity in relation to medium II, which makes significant differences evident mainly in the middle zone, where the number and staining intensity of bands diminished both in the medium containing kinetine and in those where bioregulators were used. Only two bands with poor staining appeared in this zone (6, 7).



Picture 1. Prx systems in potato calluses

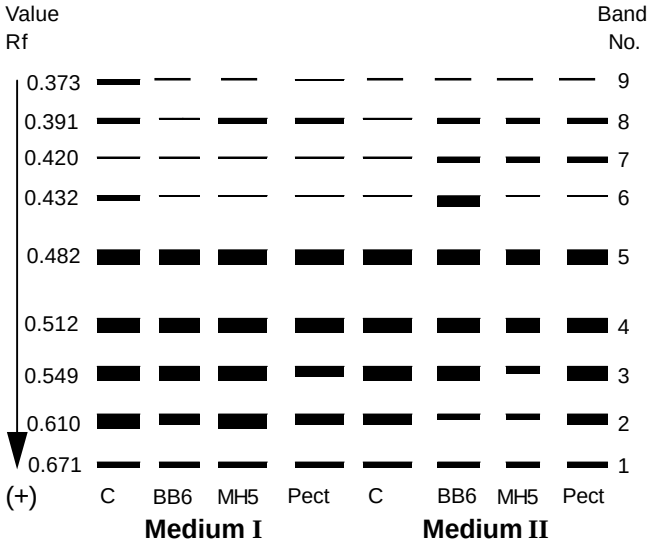
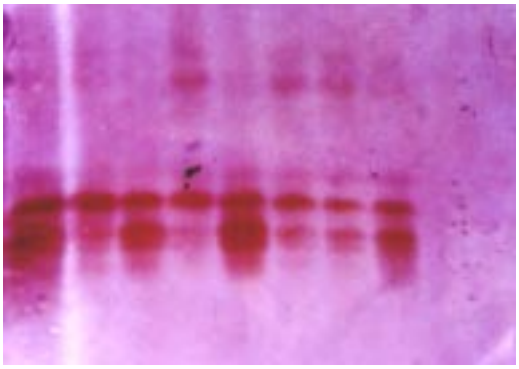


Figure 13. Peroxidase zymogram in potato calluses



Picture 2. Est systems in potato calluses

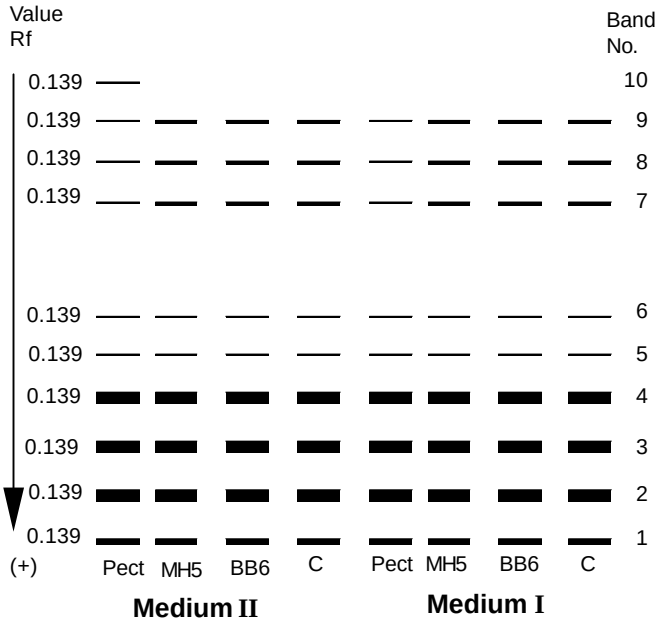


Figure 14. Esterase zymogram in potato calluses

Regarding medium II, containing 0.5 mg.L^{-1} 2,4D, it presented a higher enzymatic activity that was in correspondence with a higher callus growth. There were three zones of activity, the middle zone standing out, presenting three well-defined bands both in this medium and in those supplemented with MH5 and BB6 as a substitute for kinetine. In this sense, the effect of BB6 is to be highlighted, since there was an increase on the staining activity of band 6, in relation to the previous ones. In general, nine bands appeared in this medium.

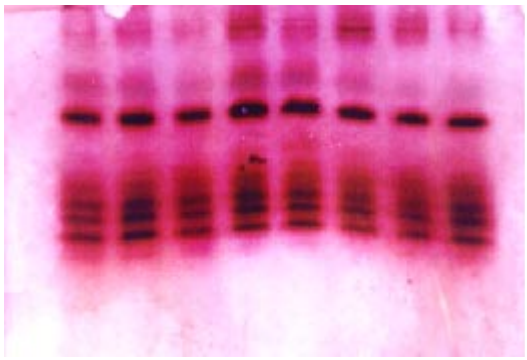
A relationship between the results for this system and the appropriate callus morphological characteristics for both media was shown, in correspondence with a higher enzymatic staining activity.

On the other hand, it has been pointed out the importance of Prx isoenzymes for biosynthesis of cell wall components, as well as cellular differentiation (19). Likewise, the existence of marked differences in patterns of Prx bands was confirmed after analyzing sampling of leaf tissue, callus and root of *Desiree* variety (11), which suggests using this enzymatic system as a complement for breeding studies on this crop.

A similar enzymatic polymorphism was recorded in Est system for I and II media, where the fact that a distinctive band (10) appeared in the medium containing 10 mg.L^{-1} Pectimorf should be highlighted. The remaining bands were common to all media, even though in the rest of the combinations, band-staining activity increased in the middle zone (bands 6, 7, 8 and 9), which is typical of an active metabolic function. There appeared a total of 10 bands, from which nine were common to all the used media.

Some authors (1) proved this enzymatic system sensibility in barley for detecting esterases at embryogenic callus stage, before embryos were totally formed; and this issue was confirmed in the present work when using the same system during the process of callus formation in potato (var *Desiree*). Such system was also used (25) for characterizing commercial potato varieties, establishing a key for crop genetic identification.

Pictures 3 (Figure 15) and 4 (Figure 16) show AC and Apc isoenzymatic systems, where AC is markedly monomorphic, presenting a total of seven bands in two well-defined zones and all bands were common to every system.



Picture 3. AC systems in potato calluses

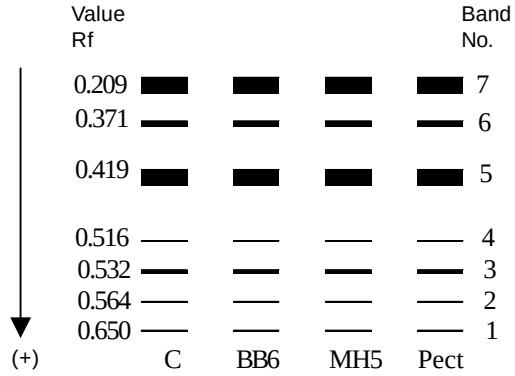
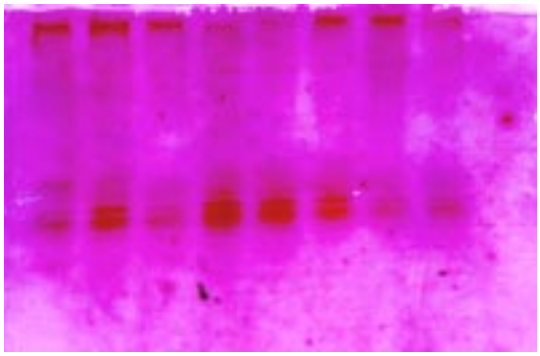


Figure 15. Carbonic anhydrase zymogram

As to Aps system, it showed a marked monomorphism, presenting a total of 10 bands in all treatments, distributed in three zones of activity (Aps1, Aps2 and Aps3). The zone of major migration was that presenting the highest number of bands.



Picture 4. Aps systems in potato calluses

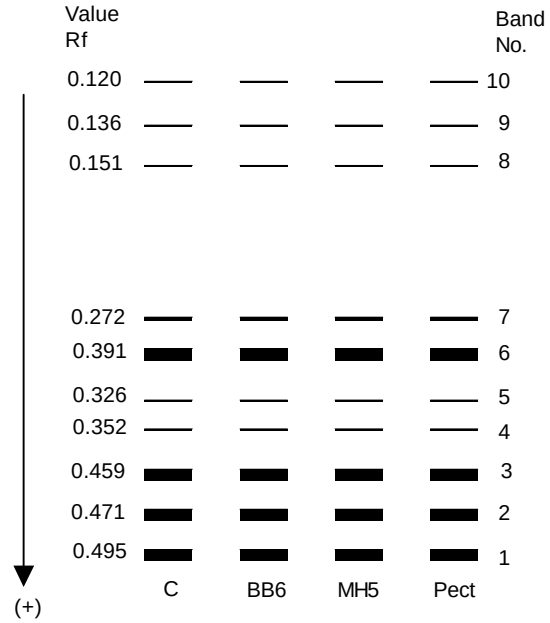


Figure 16. Acid phosphatase zymogram in potato calluses

In general terms, results showed the existence of a marked enzymatic activity in calluses which were 35 days of age, developed in media I and II, indicating that, at this stage, calluses already presented a metabolic function, which could be associated with their callus tissue differentiation.

Several authors (1) have reported the use of these systems on potato, when working on somaclones (7); an estimation of genetic variability was made in hybrid populations and a study (26), using accessions belonging to a germplasm bank on *Solanum* genus.

CONCLUSIONS

In general, results from the different enzymatic systems, studied in these crops, corroborated the existence of *in vitro* culture genetic stability in tomato, soybean and coffee crops, which implies the existence of identical band patterns between donor and regenerating plant phenotypes. Likewise, these techniques allowed detecting potato somaclonal variation, which was seen through changes in peroxidase and esterase patterns of potato calluses, cultivated in different culture media supplemented with Pectimorf. This confirms that, by using such medium, a genetic variability can be detected.

Results also evidenced the benefits of using isoenzymes as markers for monitoring genetic stability and/or variability, caused by *in vitro* cultures. It should be highlighted that morphoagronomic differences were found among potato somaclones, detected through this method, when they were evaluated under field conditions. Good use of such somaclones is currently being made for developing genetic breeding programs using this crop[2].

[2] Moré, 2001, personal communication

REFERENCES

1. More, O. Empleo de nuevos biorreguladores en la obtención de callos embriogénicos de papa. [Tesis de maestría]. UH de La Habana, 2000.
2. Plana, D. Métodos de cultivo y acción de nuevos reguladores del crecimiento en la morfogénesis *in vitro* del tomate (*Lycopersicon esculentum* Mill), cultivar Amalia. [Tesis de Maestría], INCA, 2000.
3. Cabrera, J. C. Obtención de una mezcla de (1-4)-D Oligogalacturónidos reactivos a partir de subproductos de la industria citrícola. [Tesis de grado], INCA, 1999.
4. Khripach, V. A.; Zhabinskii, V. N. and De Groot, A. E. Brassinosteroids: a new class of plant hormones. In: Physiological mode of action of brassinosteroids. New York : Academic Press, 1999, p. 219-245.
5. Florido, M. Caracterización de variedades y especies silvestres de tomate atendiendo a características morfobioquímicas de tolerancia al calor. [Tesis de Maestría], UH, 1999, 87 p.
6. Cevallos, A. M. Establecimiento de una metodología eficiente en el proceso de embriogénesis somática del cafeto (*Coffea* spp.), mediante el uso de marcadores morfohistológicos y moleculares [Tesis de Doctorado], INCA, 2000.
7. Castillo, J. Estimación de la variabilidad genética en poblaciones híbridas de papa (*Solanum tuberosum*, L). [Tesis de Maestria], INCA, 2000. 66 p.
8. Chamberlain, J. R.; Huges, C. E. and Galwey, N. W. Patterns of isozyme variation in the *Leucaena shannonii alliane*. *Silvae Genetica*, 1996, vol. 45, no. 1, p. 1-7.
9. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with the tobacco tissue cultures. *Physiol. Plantarum*, 1962, vol. 15, p. 473-497.
10. Florido, M. Diversidad isoenzimática en papa (*Solanum* spp.).Estimación de la variabilidad genética en poblaciones híbridas de papa (*Solanum tuberosum* L.). En: Resumen Evento ALAP 2000. p 42.
11. Iglesias, L. Estudio de la variabilidad morfoagronómica y bioquímica en soya (*Glycine max* L. Merrill). [Tesis de Grado], INCA, 1986, 232 p.
12. Lara, R. M. Polimorfismo bioquímico en soya (*Glycine max* L. Merrill). Tesis de Grado (Ingeniero Agrónomo). 49 p.
13. Wendel, J. F. and Weeden, N. F. Visualization and interpretation of plant isozymes. In: Isozymes in Plant Biology. Dioscorides Press, 1989. p. 5-34.
14. González, C. Comportamiento genético bioquímico de la lima persa SRA-58 (*Citrus latifolia* Tan) sobre diferentes patrones en Cuba. [Tesis de Grado], UH, 1989.

15. Maleau, N. and Coleman, J. R. Isolation and characterization of a cDNA coding for pea chloroplast carbonic anhydrase. *Plant Physiol*., 1991, vol. 95, p. 264-268.
16. Lan, T. H.; Paterson, A. H. and Wing, R. A. Comparative analysis of tomato RFLP maps. *TGC. Report*, 1993, vol. 43, p. 27-30.
17. Rick, C. M. Download TGRC Stock list (http:// tgrc.ucdavis.edu).
18. Chetelat, R. T. and Verna, J. W. de. Map location of malate dehydrogenase-1 (Mdh-1), a new isozyme marker for 3L. *TGC Report*, 1993, vol. 43, p. 13.
19. Iglesias, L. and González, M. C. Estudios isoenzimáticos asociados con la tolerancia a la salinidad en arroz (*O. sativa* L). *Cultivos Tropicales*, 1995, vol. 16, no. 1, p. 64-69.
20. García, D. B. Actividad biológica de análogos de brasinoesteroides sobre la formación de callos embriogénicos en cafeto (*Coffea canephora* Pierre). [Tesis de Maestría], UH, 1998.
21. Morales, C. M. Caracterización biométrica, citogenética e isoenzimática de somaclones de tomate (*Lycopersicon esculentum*, Mill). [Tesis de Maestría], UH, 1997.
22. Thypyapong, P.; Joel, D. M. and Steffens, J. C. Differential expression and turnover of the tomato polyphenol oxidase gene family during vegetative and reproductive development. *Plant Physiol*., 1997, vol. 113, p. 707-718.
23. Echenique, C. V.; Polci, P. A. Cambios isoenzimáticos inducidos por estrés hídrico y de temperatura en pasto llorón. *Turrialba*, 1994, vol. 44, no. 1, p. 10-17.
24. Cevallos, A.M. (1998). Embriogénesis somática en el cultivo del cafeto (*Coffea* sp). Determinación de marcadores morfohistológicos y bioquímicos. [Tesis de Maestría], UH, 1998.
25. Iglesias, L. Utilización de marcadores bioquimicos y moleculares en el mejoramiento de la papa. *Cultivos Tropicales*, 1994, vol. 15, no. 2, p. 108-121.
26. Cabrita, L.; Elisiário, P.; Leitão, J. and Guerreiro, A. Assessment of the genetic relationship among Citrus species and varieties by isozyme and RAPD markers. *Acta Hort*., 2001, vol. 546, p. 177-181.

Recibido: 19 de julio del 2002
Aceptado: 25 de febrero del 2003