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Short communication
MODIFICATION OF THE STAINING METHOD OF POLYPHENOLOXIDASES AND CARBONIC ANHYDRASES IN PLANTS

Comunicación corta
Modificación en el método de tinción de las polifenoloxidasas y anhidrasas carbónica en las plantas

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ABSTRACT. Isozymes have proved to be valuable in genetic diversity studies of different species. Therefore, this study was aimed to describe an efficient histochemical staining modification of carbonic anhydrase and polyphenol oxidase isozymes in plants, which consists of replacing the staining buffers used to develop these isozymes by distilled water. The effectiveness of these proposed adjustments was confirmed when using the "t" test of Student for related data and conducting these tests in different crops. The electrophoretic profiles observed in stainings showed the same pattern of bands without significant differences among treatments, demonstrating that it is possible to replace traditionally used staining buffers in these techniques by distilled water. The high repeatability of such stainings will enable to employ the proposed modifications to develop these isozymes.

Key words: biochemical markers, polymorphism, histochemical staining

RESUMEN. Las isoenzimas han probado ser de gran valor en estudios de diversidad genética de diferentes especies. Por tal motivo se realizó el presente trabajo, con el objetivo de describir una modificación eficiente en la tinción histoquímica de las isoenzimas polifenoloxidasas y anhidrasas carbónica en plantas, la cual consiste en sustituir por agua destilada los tampones de tinción utilizados para revelar estas isoenzimas. La efectividad de estas adaptaciones propuestas se corroboró utilizando la prueba "t" de Student para datos relacionados y realizando estos análisis en diferentes cultivos. Los perfiles electroforéticos observados en las tinciones mostraron igual patrón de bandas y no existieron diferencias significativas entre los tratamientos, lo que demuestra que es posible sustituir los tampones de tinción tradicionalmente empleados en estas técnicas por agua destilada. La alta repetibilidad de estas tinciones permitirá en lo sucesivo emplear las modificaciones propuestas en el revelado de estas isoenzimas.

Palabras clave: marcadores bioquímicos, polimorfismo, tinción histoquímica

INTRODUCTION

Biochemical markers, especially isozymes, are helpful tools in genetic research over the past 30 years and have an important role in studies of genetic markers, to complement the morphological analysis on the correct identification of various taxa levels, accessions and individuals , wherein the detection of genetic homology sometimes more accurate than some genomic DNA markers (1). Isoenzymes have proved valuable in genetic diversity studies of different species (2, 3, 4, 5, 6), mapping and population dynamics, and to estimate the genetic stability in techniques of *in vitro* culture (7 , 8, 9). In addition, they have been used in identifying potentially loci markers, which can be used for assisted selection (10, 11) and in studies related to resistance to biotic and abiotic stresses (12, 13, 14). In fact, it suggests that there is a broad spectrum of isozyme species in different plant tissues (7). Within these isoenzymes is the polyphenoloxidases (PPO) and carbonic anhydrase (CA) have played an important role in research related to plant breeding, due to its high reliability and large number of polymorphic bands present in them (4, 9).

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That is why; taking into account the above, the present work was developed with the aim of describing an efficient modification histochemical staining of polyphenoloxidases and carbonic anhydrase isoenzymes in plants.

MATERIALS AND METOHDS

For the development of this study, samples of 1 g of fresh leaf tissue of ten tomato seedlings which were homogenised in cold with an extraction buffer containing 20 % sucrose (m/v) and 0,02 % dithiothreitol they were taken using a mass/volume ratio of 1/2. The extract was centrifuged at 14 000 rpm at 0 °C for five minutes. The obtained supernatant was subjected to electrophoresis on polyacrylamide gel in a 8,5 % in an electrophoresis vertical chamber *Mighty Small II* from *Pharmacia Biotech* and the protocol described by Laemmli (15) was followed. The running time in each case was determined by the displacement of the Kolrhauch band to about 6 cm startup. An intensity of electric current, constant of 15 mA was used until the band migrated to the separating gel, where the current intensity to 25 mA increased.

Histochemical stains for PPO and CA enzymes according to the methods described by Weeden and Wendel (16) and respectively González[A] were performed; then incubated in 5 % of acetic acid (v/v). The band patterns of each accession were visualized under white light according to the number and position of each band. Three repetitions were performed in each analyzed electrophoretic system. Enzymatic staining patterns obtained in the Tris-HCl 0,1 M buffer, pH 7,2 and Tris-HCl 0,5 M, pH 7,19 for PPO and CA systems, respectively, were used as controls in the analysis of the results (T1). Different treatments that sustitute these buffers by replacing distilled water with pH values between 6,63 and 6,89, and ionic conductivity below 3 μScm^{-1}, which are set forth in Table I were used.

From the average values of the relative position of the bands (Rf) present in zymogrammes in each of the treatments, the "t" Student for data related test was performed using SPSS 11.5 for Windows . These electrophoretic analyses were also conducted in young leaves of potato (*Solanum tuberosum* L.), and strawberry (*Fragaria ananassa* Duch) to verify the effectiveness of the proposed stains.

RESULTS AND DISCUSSION

Multiple forms of PPO and CA activity found, confirm the presence of these isoenzymes in leaf tissue of tomato seedlings. These isozyme systems showed no alterations in banding patterns when tissue revealed with traditional buffers Tris-HCl 0,1M, pH 7,2 and Tris-HCl0,5 M, pH 7,19 for PPO and CA, respectively, since it was observed that the 10 evaluated tomato seedlings showed equal amount and relative position of bands, regardless of the source of water or buffer used (Figure). These results correspond to the points made by other authors, who point out that pH values between 6 and 8 are the most stable of the CA enzyme (17). Likewise, research has been conducted to determine in PPO, the greater stability pH in pear *(Pyrus communis* L.) and apple (*Pyrus malus* L.), informing that this enzyme was most stable at pH 6,5 and between 6,5 and 7,5 respectively (18).

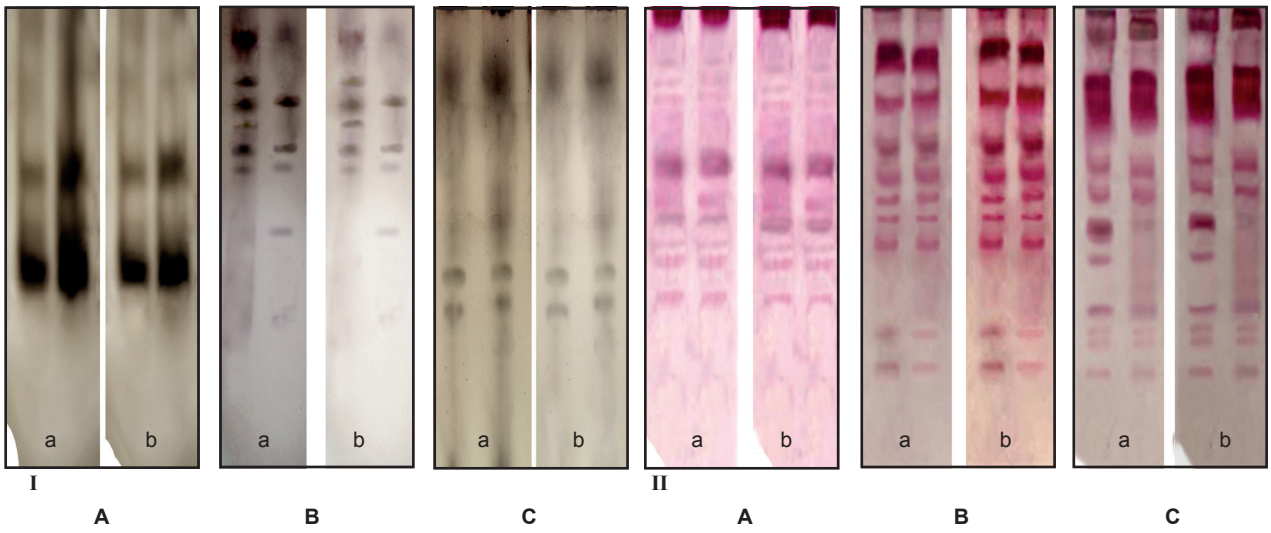
By using these staining procedures for potato and strawberry, interspecific variations were found, since differences in electrophoretic mobilities in different crops were observed, these being higher among potatoes and strawberry for CA and between tomato and potato in PPO. It should be noted the presence of three and four common bands in the two cultures of the Solanum genus. However, also identical band patterns were obtained in both isoenzymatic systems using distilled water (T2, T3, T4 and T5) and traditional systems buffers (T1) (Figure, Table II).

[A] González, C. *Comportamiento genético bioquímico de la Lima persa SRA-58* (*Citrus*) *sobre diferentes patrones en Cuba*. [Tesis de Doctorado], Cuba, 1989.

Table I. PH values and ionic conductivity of the distilled water used in staining assays to test the proposed methodology

Distilled water used in histochemical stains	pH values	Ionic conductivity ($\mu S\ cm^{-1}$)
T2	6,89	0,95
T3	6,86	0,82
T4	6,66	2,2
T5	6,63	2,6

T2: Distilled water from INCA genetics laboratory
T3: Distilled water from the biology faculty of Havana University
T4: Distilled water from INCA biofertilizers laboratory
T5: Distilled water from CENSA



I: Polyphenoloxidases isoenzymes
A: tomato (*Solanum lycopersicum* L.)
a: traditional buffer

II: carbonic anhydrase isoenzymes
B: potato (*Solanum tuberosum* L.)
b: distilled water

C: strawberry (*Fragaria x Ananassa* Duch.)

Enzyme patterns bands detected in leaf tissue

Table II. Test "t" of samples related to carbonic anhydrase and polyphenoloxidases isoenzymes

	Carbonic anhydrase				Polyphenoloxidases			
Pars	IC	t	gl	Sig.	CI	t	gl	Sig.
T1– T2	-1,49 – 3,64	1,02	6	0,35	-7,24 – 5,36	-0,48	3	0,67
T1–T3	-6,81 – 6,94	-1,99	6	0,09	-3,64 – 1,90	-1,00	3	0,39
T1–T4	-2,46 – 4,94	0,82	6	0,44	-4,29 – 3,77	-0,20	3	0,85
T1–T5	-1,08 – 5,65	1,66	6	0,15	-5,62 – 2,39	-1,28	3	0,29

CI: confidence interval
T1: Buffer Tris – HCL 0,5 M pH 7,19 (control treatment)
T2: distilled water from INCA genetic laboratory
T3: distilled water from the biology faculty in the Havana university
T4: distilled water from INCA biofertilizer laboratory
T5: distilled water from CENSA

The results showed that is possible to replace staining buffers traditionally used in these techniques by distilled water. The high repeatability of these stains allows hereinafter employ the amendments proposed in the developing of these isoenzymes, which will help lower the costs of these techniques by way of reagents.

Note the usefulness of these enzymatic systems, widely distributed in the plant kingdom in genetic and physiological studies in plants. In this regard, it has been postulated PPO participation in defense mechanisms against pathogens, electron transport and oxidation of auxin. The enzyme provides resistance to infection by pathogens (viruses, bacteria, fungi, herbivores) or mechanical damage. Also it indicated that PPOs are involved in the formation and development of roots, in the formation of lignins and biosynthetic processes in plants, such as biosynthesis of betalains and phenolic plant compounds, particularly phenylpropanoid (19, 20, 21). Also, it is noted that the CA, β type, which is found in plants, helps raise the CO_2 concentration within the chloroplast, to increase the rate of carboxylation of *Rubisco* enzyme. This is the reaction that integrates CO_2 into carbohydrates during photosynthesis, being widely used in studies of resistance and tolerance to biotic and abiotic stresses (22, 23, 24).

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