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COMPARISON OF DIFFERENT METHODS FOR IDENTIFYING ZYGOTIC AND NUCELLAR SEEDLING IN CITRUS

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Abstract. Different biochemical methods were compared for their ability to differentiate zygotic from nucellar seedlings in three-year-old sour orange progenies. These progenies were derived from open pollination of 10 sour orange selections, from selfing of one sour orange accession and from crossing of the same accession with *P. trifoliata*. Polyacrylamide gel electrophoresis was used to detect isoenzyme polymorphism. Young shoot homogenates were tested for coagulation and browning. Results from the techniques are compared.

Most varieties of *Citrus* are apomictic and polyembryonic. This implies that nucellar embryos develop together with, or instead of, zygotic embryos. As a result, progenies from selfing or crossing of polyembryonic taxa are mixtures of nucellar and zygotic seedlings in varying proportions in the different taxa.

It is very difficult to identify zygotic and nucellar individuals with certainty until after fruiting, unless parents have markedly different vegetative characteristics. It is, therefore, of great interest to develop methods that distinguish zygotic from nucellar plants at an early stage of seedling development. This implies the identification of reliable genetic markers. The dominant character "trifoliolate leaf" of *Poncirus trifoliata* has been employed for this purpose for many years; this marker is obviously not useful for crosses within the genus *Citrus*.

Several approaches have been tried to identify biochemical characters for use as genetic markers in the screening of *Citrus* progenies at early stages of development; most methods involve analysis of biochemical or enzymatic compounds of leaf, shoot or root.^{11,18,19,24,25} Because of the complex requirements of characters for use in discriminating the uniform genomes of nucellar plants from the recombinant sexual individuals (little affected by the environment, not subject to ontogenetic variations, possibly determined by single codominant genes, etc), it has not been possible to determine genetic markers of general use in *Citrus*, whose genetics is rather complex and little known.

The present work is a comparative study of different biochemical methods with the aim of identifying genetic markers that can be used to discriminate between nucellar and zygotic seedlings of sour orange (*C. aurantium* L.). In particular, we were interested in developing both a relatively quick method for screening a large number of seedlings and in more refined techniques for genetic studies. Moreover, the aim was to be able to discriminate zygotic and nucellar individuals in populations derived, besides from controlled crosses, from selfing and from open pollination.

Materials and Methods

Plant material. The populations screened in this study were three year old seedlings grown under nursery conditions. In detail, progenies from ten accessions of sour orange (*C. aurantium* L.) were derived as follows: AA CNR 23 x *P. trifoliata* (L.) Raf. (var.

Rubidoux); AA CNR 23 (selfed); AA CNR 8, 14, 16, 18, 23, 24, 26, 28, and 29 (open pollinated). In tables and figures, the individual seedlings will be identified by Arabic numerals, preceded by the number indicating the mother accession (e.g., 8/1, 8/2, 8/3 will indicate three seedlings of the progeny of the accession AA CNR 8). Progeny from selfing experiment will be denoted AA x AA.

The biochemical methods used in this study were polyphenol-oxidase browning and coagulation of young shoot homogenates whose genetic control has been previously studied^{4,5,7,8,9,11,12,14} and electrophoretic separation of isoenzymes,^{21,22} which also has been widely used in *Citrus*.^{1,6,10,16,17,23,24,26} The dominant character "trifoliolate leaf" was used as a control marker in the controlled pollination experiment.

Enzymatic browning and coagulation. The terminal 1 to 3 cm of growing shoots from the mentioned populations were collected and homogenized in 0.05 M K phosphate buffer solution (pH 7.2) at room temperature; the ratio of buffer solution to fresh weight was 3/1. The homogenates were poured on white blotting paper and immediately scored for coagulation or non-coagulation. After about half an hour they were scored for browning or its absence. When coagulation alone was scored, 10 mM K metabisulfite was added to the buffer solution.

Isoenzyme separation. Root extracts were prepared from 200 mg of homogeneous 1 mm diameter roots, according to the procedure described by Spiegel-Roy.²⁴ Leaf extracts were prepared by grinding in a chilled mortar 1.5 g of leaves (approximately 3 months old) with 1.5 ml of 20% sucrose in the presence of acid-purified sea sand. Homogenates were then centrifuged in the cold at 6000 x g for 15 minutes.

Polyacrylamide gel electrophoresis was performed in a vertical gel slab apparatus (Pharmacia GE-2/4). Four 8 x 8 cm slabs were run at a time under refrigerated conditions. Bromophenol blue and pyronin G were used as markers for anodic and cathodic runs, respectively.

Anodic isoperoxidases from root extracts were separated on 8% acrylamide gels, pH 7.5, as previously described¹⁷; cathodic isoenzymes were analyzed on 15% acrylamide gels, pH 4.1, according to the method of Reisfield²⁰; no stacking gel was used. Zymograms for peroxidase activity were obtained by incubating gel slabs at room temperature for 20 minutes in 10 mM guaiacol, 10 mM H₂O₂, in 0.2 M K phosphate pH 5.8; gels were subsequently fixed and stored in 7% acetic acid. In some experiments, bands were revealed with 0.3 mM *o*-dianisidine and 3.3 mM H₂O₂ according to the method of Gardiner and Cleland.¹³

Esterase isoenzymes from leaf extracts were analyzed on 7.5% acrylamide gels, pH 8.9, according to the method of Davis²; no stacking gel was used. Slabs were then incubated with α -naphthyl-acetate and Fast Blue RR Salt under the conditions described by Torres and Tisserat²⁷ to evidence esterase activity. After fixing in 50% methanol, gel slabs were stored in 7% acetic acid.

Results and Discussion

Citrus aurantium x *Poncirus trifoliata*

A very good correspondence between biochemical methods and phenotypic genetic marker (leaf shape) was observed in the progeny originated from controlled pollination of sour orange (AA CNR 23) by *P. trifoliata*.

As far as the enzymatic browning-coagulation method is concerned, the mother plant was browning and non-coagulating; *P. trifoliata* was non-browning and non-coagulating.

Ninety one seedlings of this cross were examined by this method. Out of these, 56 monofoliolate seedlings were non-coagu-

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lating and browning, identical to the mother plant: they were therefore classified as nucellar. Only 2 monofoliate seedlings appeared to be different for the color of the spot and were consequently rated as zygotic.

The 33 trifoliate seedlings examined presented differences in respect to the "nucellar" spot. One seedling was brown and coagulated; 2 were not brown and coagulated; 7 not coagulated and brown (with browning clearly different from the monofoliate plants) and 23 not coagulated and not brown.

Although satisfactory for the aim of the screening, these results appear rather puzzling from the genetic point of view. In fact, the proportions of different phenotypes were not in agreement with the inheritance of dominant browning and non-coagulating genes, as they have been previously described.^{5,9}

Heterozygosity of both parents for the recessive coagulation character would suggest a higher proportion of progeny with coagulating phenotype (25%), while only 3 individuals out of 33 were observed. Moreover, a possible heterozygosity of the browning parent does not account for the high proportion of non-browning seedlings (25 out of 33). A similar excess of non-browning in a progeny of sweet orange [*C. sinensis* (L.) Osbeck: browning] × *P. trifoliata* has been described by Esen *et al.*⁷ who hypothesize anomalies in the complementation of the *Poncirus* genome with that of *Citrus*, modifying enzyme and substrate synthesis.

Electrophoretic analysis of peroxidases from roots evidenced, as already described,¹⁷ that the two parents were characterized by distinct anodic isoperoxidase banding patterns. The 123 trifoliate zygotic offspring examined presented characteristically varied isoperoxidase zymograms, in which both "maternal" and "paternal" bands were diversely combined; 136 unifoliate offspring on the contrary showed identical "nucellar" zymograms (coinciding with the "maternal" banding pattern); the presence of two zygotic individuals, exceptionally unifoliate, was confirmed by the browning method. In Fig. 1, anodic isoperoxidase patterns of nucellar and zygotic individuals, as well as of parent plants, are reported.

As a conclusion, the two methods revealed sensitive and precise tools in the discrimination of nucellar and zygotic progenies derived from genetically "distant" parents. The problem of genetic identification becomes more complex when the zygotic progeny originates from very "close" gametes, as in the case of selfing which may be a considerable component of open pollination.

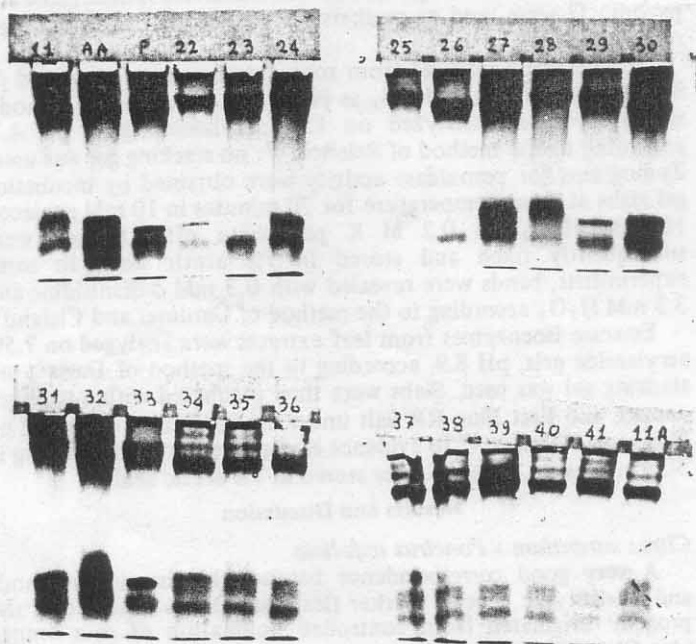


Fig. 1. Anodic isoperoxidase banding patterns from root extracts of sour orange AA CNR 23 (AA), *P. trifoliata* (P) and their offspring. Seedlings No. 11, 22 to 33 were trifoliate; No. 11A and 34 to 41 had unifoliate leaves.

The sour orange accessions

In the first approach in determining the degree of genetic variation among the populations studied, all the sour orange accessions were compared on the basis of different biochemical methods.

The polyphenol oxidase browning method showed small but clear color differences among the spots of the different accessions. No differences in the coagulation character, on the contrary, were observed. As both characters present in sour orange phenotypes (browning and non-coagulating) are dominant,^{5,9} the genetic differences that can be expected in sour orange populations are limited to quantitative expressions of the browning phenomenon, except for recombination of recessive alleles.

As far as isoenzyme separation is concerned, root peroxidase anodic isoenzyme banding patterns appear to be very similar in the different sour orange accessions (Fig. 2). Additional bands were not detected with different electrophoretic or staining methods.

Results on cathodal peroxidases from root tissue were promising but were not continued because of high cost and variability. If variability can be eliminated, it may be a useful technique in critical cases.

Among several different enzymes from sour orange leaves whose electrophoretic separation has been attempted, esterases, which have been used for genetic characterization in different species including *Citrus*,^{3,15,16,28} gave the best results.

Esterase banding patterns from sour orange leaf extracts were relatively complex, but could be well separated by long runs (20 minutes after bromophenol blue had reached the bottom of gel slabs). As shown in Fig. 3, accessions AA CNR 14, 16, 18, 24, 26, and 27 show very similar banding patterns, with minor quantitative differences. Clearly different patterns characterize AA CNR 8, 23, and 29. The homogeneity of the patterns of the different accessions was checked on a series of offspring which were rated nucellar according to the enzymatic browning method. Both the browning method and esterase zymograms showed the existence, in the progeny of AA CNR 28, of two distinct phenotypes, neither of which corresponded to the mother plant. The progeny was eliminated because of evident error in the labeling of the seedlings.

The zygotics

The discrimination of nucellar and zygotic seedlings is based on the possibility of distinction between individuals genetically identical to the mother plant (nucellar seedlings) and individuals presenting genetic differences (zygotic seedlings); in this latter case, differences arise from complementation of genomes of distinct parents in the case of crosses, or from segregation of

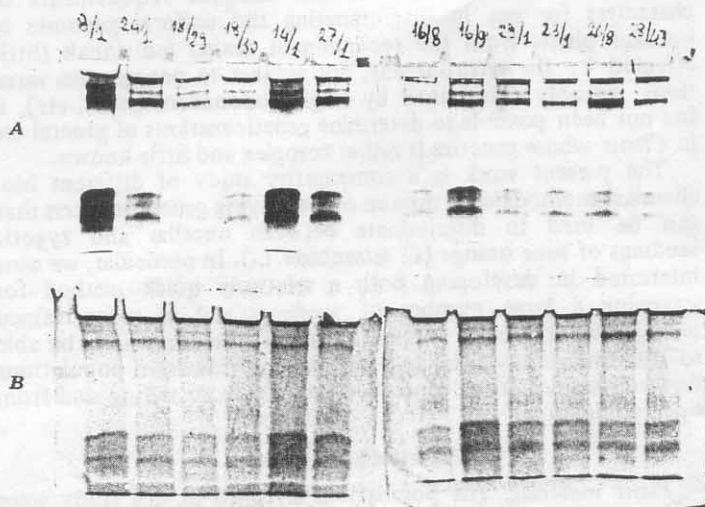


Fig. 2. Anodic isoperoxidase banding patterns from root extracts of seedlings from different sour orange accessions (open pollinated). The upper slabs (A) were stained with guaiacol - H₂O₂; the lower slabs (B), containing the same samples and run in identical conditions, were stained with *o*-dianisidine (as described in Materials and Methods).

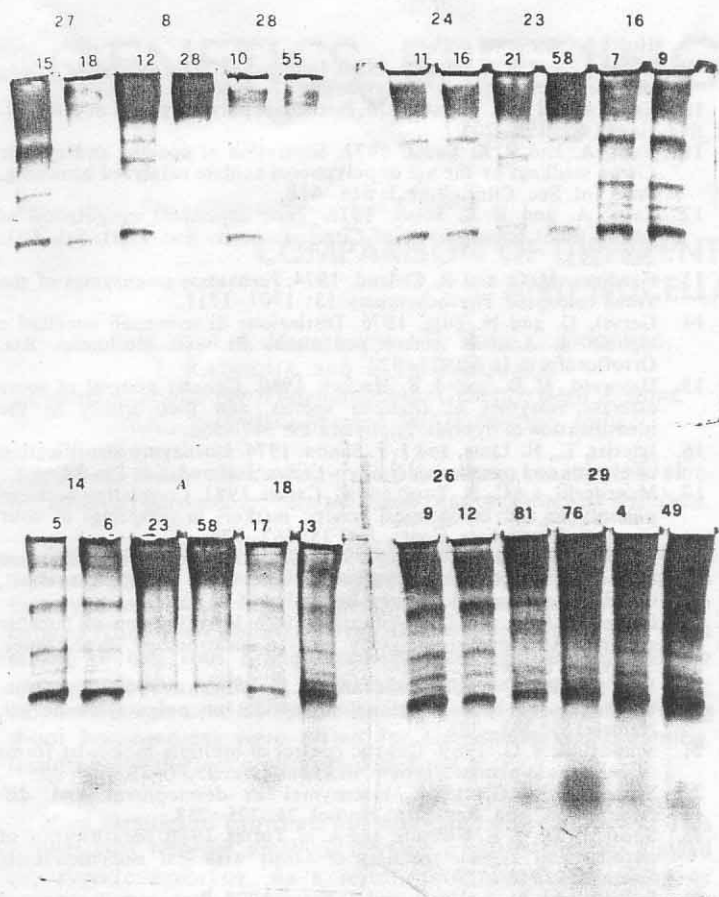


Fig. 3. Esterase isoenzyme banding patterns from leaf extracts of seedlings from different sour orange accessions. All progenies derived from open pollination, with the exception of A sample (AA CNR 23 selfed). All seedlings (except 29/4 and 29/49, zygotic) were nucellar; differences between samples 28/10 and 28/55 are explained in the text.

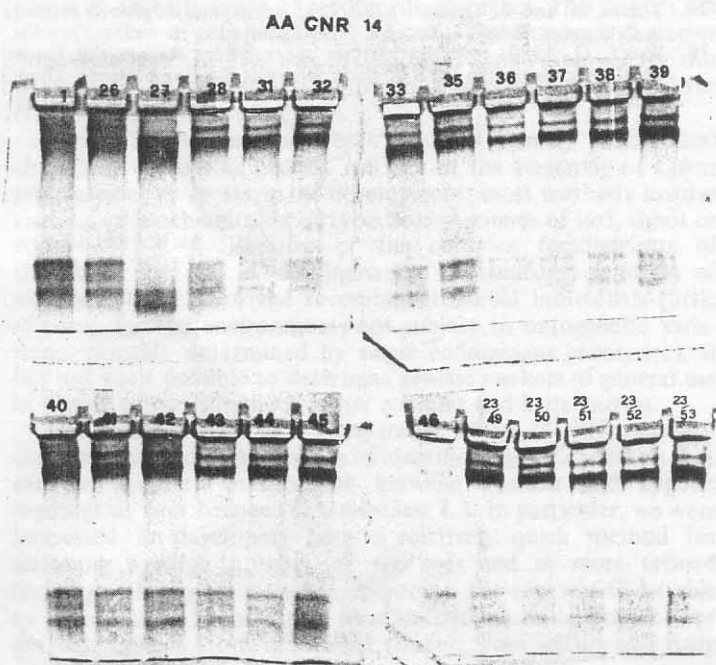


Fig. 4. Anodic isoperoxidase banding patterns from root extracts of progenies from open pollination of sour orange accessions AA CNR 14 and 23 (marked separately).

genes through the gametes produced by the same plant in the case of selfing. Both possibilities are obviously present in the case of open pollination.

When the problem is limited to the analysis of progenies from a controlled cross between genetically distant parents (see C.

aurantium × *P. trifoliata* experiment), all the biochemical methods employed have easily evidenced clear genetic markers expressing the genome complementation in the zygotic progenies. It is evident that in the case of genetically close entities, as the sour orange accessions in our study, all of Sicilian origin, identification of precise genetic markers is more difficult. With the biochemical methods it has nevertheless been possible to identify zygotic individuals, with a satisfactory coincidence among the different methods used.

The evident uniformity of anodic isoperoxidase banding patterns of the different accessions suggested a relatively low range of variation of this group of genes within our sour orange accessions. The different progenies were nevertheless scored by this method, and some zygotic individuals were detected. In Fig. 4, samples 14/27 and 14/46 show evident differences from the homogeneous patterns of the nucellar individuals. Unfortunately these two plants could not be examined by other methods because they died before formation of new sprouts. Anodic peroxidases, however, were not useful for the purpose of the experiment. Most plants which were rated zygotic by the other biochemical methods, had isoperoxidase patterns undistinguishable from the nucellar pattern.

As evidenced by the variation in esterase isoenzyme banding within the material scored (Fig. 3), better results were obtained when leaf extracts were analyzed for esterase isoenzymes. Based on clear differences in esterase zymograms, a number of zygotic individuals could be recognized in the progenies of the different accessions. Fig. 5 illustrates some examples of esterase zymograms.

The results were compared with the data obtained by the enzymatic browning method, and a good correspondence between the two techniques was registered (Table 1).

From the above presented data we can conclude that the identification of a number of genotypes different from the mother plant has been possible in sour orange by combining the

Table 1. Comparison between enzymatic browning of young shoots and electrophoretic separation of leaf esterases in the detection of sour orange zygotic seedlings.^z

Progeny	Number of plants	Seedling No.	Phenotypes detected by	
			Browning method	Esterase isoenzyme method
AA CNR 8 (o.p.)	40	8/9	?	Z
		8/15	Z	Z
		8/19	N	?
AA CNR 14 (o.p.) ^y	43	14/2	Z	Z
		14/25	Z	Z
AA CNR 16 (o.p.)	46	16/12	Z	Z
		16/15	Z	Z
		16/40	n.d.	Z
AA CNR 18 (o.p.)	50	—	—	—
AA CNR 23 (o.p.)	58	23/22	Z	Z
		23/35	Z	Z
		23/46	Z	Z
AA CNR 24 (o.p.)	36	24/2	Z	Z
		24/35	Z	Z
AA CNR 26 (o.p.)	69	26/54	?	Z
		27/1	Z	Z
AA CNR 27 (o.p.)	74	27/34	n.d.	Z
		27/49	Z	Z
		27/77	Z	Z
		29/4	Z	Z
		29/11	n.d.	Z
		29/18	Z	Z
		29/42	Z	Z
		29/49	?	Z
		29/58	n.d.	Z
		29/67	Z	Z
29/69	Z	Z		
29/71	Z	Z		
29/72	Z	Z		
29/80	Z	Z		
AA CNR 23 (selfed) ^x	100	AAxAA/46	Z	Z

z: N=nucellar; Z=zygotic; ?=doubtful; n.d.=not determined; o.p.=open pollinated.

y: 14/27 and 14/46, rated as zygotic by isoperoxidases, died.

x: AAxAA/6, rated as zygotic by isoperoxidases, died.

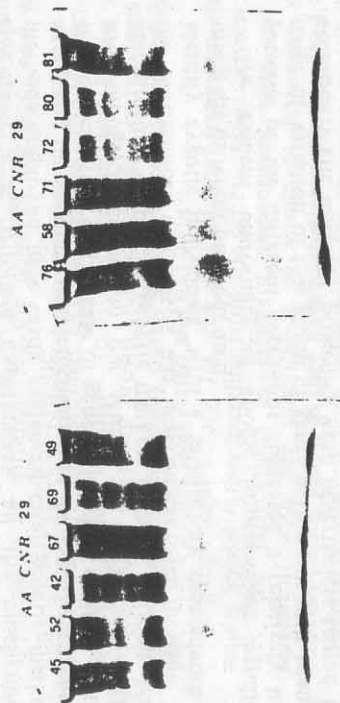


Fig. 5. Esterase isoenzyme banding patterns from leaf extracts of seedlings derived from open pollination of sour orange AA CNR 29. Offspring No. 45, 52, 49, 76 and 81 were rated as nucellar by the enzymatic browning method; the remainder were rated as hybrids by the same method. 29/58, not determined by the browning method, was zygotic.

enzymatic browning method and esterase isoenzyme separation. The failure of isoperoxidases as genetic markers in the screening of open pollinated sour orange populations emphasizes the importance of determining, among several polymorphic enzymes, the more useful markers.

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