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Root Peroxidase Isoenzymes as an Aid in *Citrus* Breeding and Taxonomy

J. Button, A. Vardi and P. Spiegel-Roy[1,2], Agricultural Research Organization, Volcani Center, Bet Dagan (Israel)

Summary. A rapid method for differentiating between *Citrus* clones as well as between zygotic and nucellar plants has been developed. It is based on determination of peroxidase isoenzyme patterns in small root samples. Ten to 14 bands could be distinguished, in contrast to the small number of bands present in leaves. Tetraploid seedlings gave uniform patterns closely resembling the diploid, while triploids showed segregation in isozyme pattern.

Introduction

Enzyme polymorphism has been demonstrated in many species and used in plant taxonomy and genetics (Shannon 1968; Pierce and Brewbaker 1973). It appears to be under monogenic control (Pierce and Brewbaker 1973). A number of closely related clones have been distinguished by this method in potato (Desborough and Peloquin 1967, 1968), pineapple (Brewbaker 1971), carnation (McCown *et al.* 1969), beans (Matthews and Williams 1973) and *Citrus* (Iglesias *et al.* 1974).

In *Citrus* breeding one of the major problems is to distinguish between zygotic and nucellar progeny at an early stage. Unless the seed parent is sexual, the breeder has to depend on the very unreliable selection of seedlings by vegetative characters only or, more often, has to carry an unnecessarily large number of progeny to fruiting in order to identify hybrids. Some approaches to the problem have been concerned with embryo position (Iwamasa et al. 1967), leaf morphology (Teich and Spiegel-Roy 1972), analysis of essential oils (Pieringer and Edwards 1965; Cameron and Scora 1973) and, more recently, flavonoids (Tatum *et al.* 1974). An isoenzyme study of leaf extracts in *Citrus* and *Poncirus* seedlings revealed a maximum of three bands with peroxidase and nine bands with esterase. No distinction could be made between zygotic and seedlings on the basis of leaf peroxidase but, on the basis of esterase isozymes, some interspecific hybrids could be distinguished from their parents (Iglesias *et al.* 1974). Age of organ and environmental factors are known to modify isozyme patterns in plants (Pierce and Brewbaker 1973). Photoperiod has also been found to affect differentially the activity of certain isoenzymes in *Citrus* and *Poncirus* (Warner and Upadhya 1968).

There is a danger in confounding environmental and age effects with genetic ones when leaves are used (Hart and Bhatia 1967) and total peroxidase activity has been found to be higher in roots than in leaves (Goren and Goldschmidt 1966). We decided to examine isozyme activity in both leaf and root, with greater emphasis on the peroxidase content of the root.

This paper deals with the method finally evolved for the characterization of some *Citrus* clones and hybrids used in our breeding program.

Materials and Methods

Plant Material

Five *Citrus* clones currently used in a breeding program at Bet Dagan, Israel, were selected for this study. In addition we examined a number of polyploids as well as suspected hybrids. The five clones were: 'King' mandarin (*C. reticulata* Blanco); 'Wilking' mandarin (*C. reticulata* Blanco); 'Temple' tangor (*C. reticulata* x *C. sinensis*); 'Clementine' mandarin (*C. reticulata* Blanco); and 'Mikhal' (an open-pollinated seedling of 'Clementine').

Leafy stem cuttings from five clones were treated with 100 mg/l IBA for 12 hours, then rooted with bottom heat (26° C) in sand, with intermittent mist. Ten cuttings were used per clone. Roots from each cutting were kept separately. Root samples were also taken from young seedlings of polyploids ('King' 4x, 'Wilking' 3x and 'Temple' 3x), as well as from young seedlings of supposedly hybrid nature and from nucellar seedlings (the latter were obtained from controlled pollination with *Poncirus trifoliata*).

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[1] Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan (Israel).

[2] Please address reprint requests to Dr. Aliza Vardi.

Sample preparation and determination of isoenzyme pattern

Roots were washed in de-ionized water and dried with blotting paper. Determination of peroxidase isoenzymes was made in a) fresh, b) frozen, and c) frozen and subsequently freeze-dried, materials. A sample of 'King' mandarin leaves was taken from two separate trees and treated as in c).

Protein/enzyme extracts were made from root samples using the following solvents: aqueous sucrose solution (20% w/v); sodium phosphate buffer (pH 6.0); saline solution (0.8% NaCl + 0.2% $NaNO_3$); and saline containing 20% sucrose. After a number of runs, saline was selected as the best solvent since its peroxidase activity, compared with other solvents used, was 2× higher and more bands appeared in the zymogram. The addition of sucrose to the saline prior to extraction had no noticeable effect. Omitting sucrose at this stage reduced variation in the final sucrose concentration and resulted in a greater uniformity in the migration rates of samples during electrophoresis. While isoperoxidase bands of saline extract from fresh, frozen and freeze-dried roots of the same clone were qualitatively indistinguishable from one another, freezing and freeze-drying improved band resolutions.

The extraction procedure finally adopted was as follows. Sampled freeze-dried roots were ground in a small pestle and mortar, transferred to sample tubes, and extracted with 0.5 ml saline overnight at 4° C. The macerate was squeezed, liquid was drawn off and centrifuged at 4000 × g for 10 minutes. Aliquots of 200 µl supernatant were transferred to another sample tube where a drop of tracker dye (1% bromophenol blue or methylene blue in 25% sucrose soln.) was added. Sucrose crystals (0.05 gr) were dissolved in each sample. An aliquot of 25 µl was then layered on top of gels. A minimum of two tubes per sample were run together and at least five separate extractions were made on each sample. Relative peroxidase activity was determined with the aid of a Bausch and Lomb Spectronic 20 colorimeter at 470 nm, based on differences in O.D. readings performed at 10-sec. intervals.

Electrophoresis

Polyacrylamide gel disc electrophoresis was used. A gel concentration of 7%, with a pH of 7.5, proved optimal for the separation of both cathodic and anodic isoenzymes. Sodium borate (pH 8.3) was used as a tank buffer for anodic runs, while a β-alanine/acetic acid buffer, pH 4.5, was used for cathodic runs. The close similarity between different clones for number and position of broad cathodic bands led us to concentrate on anodic isozymes only.

Gels were run at 2 mA per tube for the first two minutes and later at 4 mA per tube until the marker had traversed 80 to 85 mm. The tank buffer was precooled to 4° C and electrophoresis was carried out in a refrigerator at the same temperature. Gels were stained for peroxidases using guaiacol (Siegel and Galston 1967) and subsequently fixed in 2% acetic acid. Peroxidase bands were initially characterized by rF values. A more definite characterization was made when the position of bands was related to a dark band which invariably appeared at a distance of 28 to 30 mm from the origin. A similar technique was used by Siegel and Galston (1967). Zymograms were drawn to scale and the intensity of each band was scored. Photographic records were also kept.

Results and Discussion

Total Peroxidase Activity

Extracts made from freeze-dried root samples (0.1 gr root material + 0.5 ml saline) showed that the total peroxidase activity varied by less than 15% among the five cultivars examined (O.D. difference readings between 60 and 70). Peroxidase activity in mature, freeze-dried leaves of 'King' mandarin was only 5% of that found in root samples. The relatively high and uniform peroxidase concentration in citrus roots offers marked advantages over the use of leaves or shoots in enzyme "fingerprinting." Less plant material is required for each zymogram and no concentration adjustment of samples is required to get an even rate of electrophoresis. The chances of bands appearing or disappearing because of quantitative differences are largely eliminated.

Anodic Peroxidases

Root samples contained 10 to 14 isoenzymebands (Fig. 1) of remarkable consistency. Reproducibility was confined not only to root samples from cuttings of the same tree, but also extended to samples from different trees of the same clone. Isoenzyme patterns from roots of stem cuttings of the old clone and those obtained from roots of nucellar seedlings of the same clone were indistinguishable.

As already mentioned, mature leaves of 'King' showed a much lower peroxidase activity than did roots. This was also reflected in the appearance of only four definite, and one probable, anodic bands, compared with a total of 14 bands in 'King' roots. The two bands from leaf extracts, nearest to the origin, were of low intensity, while the three-band complex near the front closely resembled a corresponding complex obtained from the roots (Fig. 1).

It appears that anodic root peroxidases can be used conveniently in the characterization of citrus clones. The three bands nearest the origin were present in all clones examined, but the more mobile bands were characteristic of the different clones (Fig. 1). The three closely spaced, dark stained bands of 'Wilking' allowed it to be easily distinguished from 'King' (maternal parent of 'Wilking') where the three dark bands were spaced further apart. The absence in 'Wilking' of the frontal three-band complex found in 'King' further

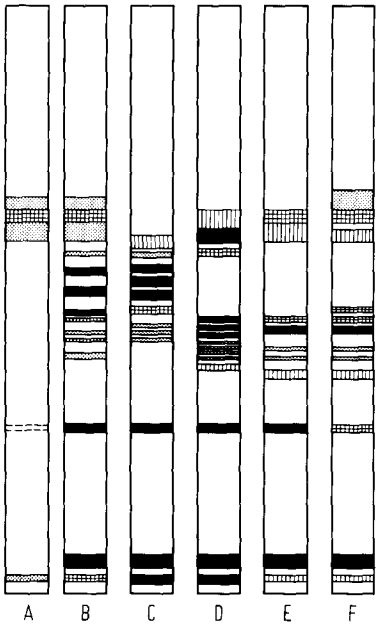

Fig.1. Diagram of isoperoxidases from roots of five *Citrus* clones and leaves of 'King' mandarin (*Citrus reticulata*). A 'King' leaves, B 'King' roots, C 'Wilking' roots, D 'Temple' roots, E 'Clementine' roots, and F 'Mikhal' roots (--- very weak staining; ▦ weak staining; ▥ medium staining; ▤ fairly dark staining; ■ dark staining)

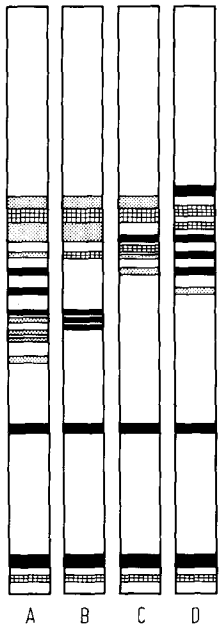

Fig.2. Diagram of isoperoxidases from 'King' mandarin roots and 'King' (♀) × 'Pummelo' (♂) progeny. A 'King' diploid, B-D Three seedlings of 'King' × 'Pummelo' cross

emphasized the difference in isoperoxidase patterns between the two clones. The striking similarity between 'Mikhal' and its maternal parent, 'Clementine', is also evident from Fig.1. However, the regular appearance of two extra bands in 'Mikhal' was sufficient to distinguish it from 'Clementine'.

'Temple' tangor was characterized by the consistent presence of four light-stained, sharp and narrow bands followed by three closely spaced, dark bands. The similarity between 'Temple' tangor and 'Clementine' mandarin has not been demonstrated before and may indicate a common ancestral parentage for these two tangor-type cultivars. A difference in isoenzyme pattern between the mandarins ('King' and 'Wilking') on the one hand, and clones with tangor-type fruit ('Temple', 'Clementine' and 'Mikhal') on the other hand, is evident.

In a population of the 'King' (♀) × 'Pummelo' (♂) cross, some of the offspring carried the winged leaf character associated with the pollen parent. When roots of these plants were assayed for anodic peroxidase, the isozymes were found to differ substantially from the maternal parent ('King') as well as exhibiting different patterns (Fig.2). This supports our assessment, made on the basis of leaf morphology, that these were in fact hybrids and not nucellar 'King' plants. Thin layer chromatography carried out in our laboratory on leaf flavenoids of 'King' nucellar material, and on material obtained by crossing 'King' with 'Pummelo', has further substantiated these results.

A number of polyploid seedlings of 'King' (polyembryonic), 'Wilking' and 'Temple' (monoembryonic) were also examined (Fig.3). The chromosome number of these plants had been established through chromosome counts in root meristems and young leaves. Three different tetraploid 'King' plants all exhibited identical isoenzyme patterns which differed from the diploid in the absence of one weak band. This supports the contention that these plants are autotetraploids, having arisen from a spontaneous doubling of chromosome numbers during the formation of nucellar embryos, and accords with the fact that nearly all citrus tetraploids

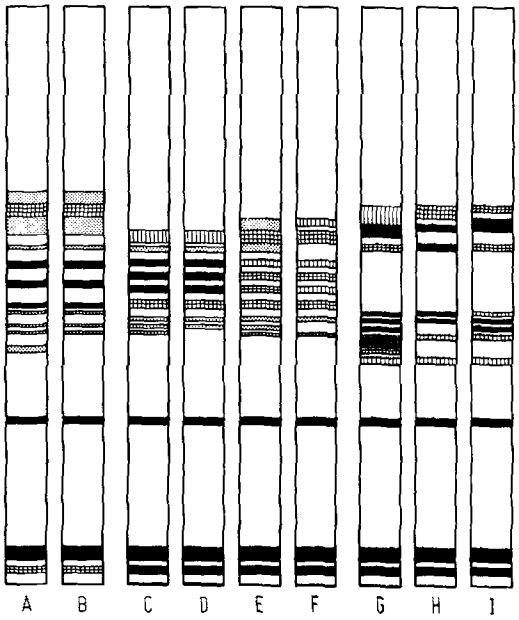

Fig.3. Diagram of isoperoxidases from roots of *Citrus* diploids, triploids and tetraploids. A 'King' diploid, B Pattern characteristic of three different 'King' tetraploids, C 'Wilking' diploid, D-F Patterns of three different triploid seedlings of 'Wilking', G 'Temple' diploid, H-I Patterns of two different triploid seedlings of 'Temple'

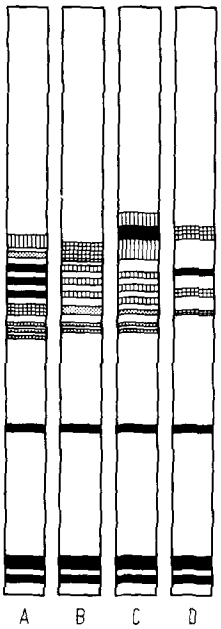

Fig.4. Diagram of isoperoxidases from roots of diploid 'Wilking' and from open-pollinated 'Wilking' progeny. A 'Wilking' diploid clone, B-D Open-pollinated diploid seedlings of 'Wilking'

have been found in varieties forming nucellar embryos (Iwamasa 1966; Cameron and Soost 1969).

Triploids obtained from both 'Wilking' and 'Temple' seed were examined. Of the three 'Wilking' triploids, one (Fig.3D) differed from the diploid by a single band only, while two others (Fig.3E and F) differed somewhat more in pattern and the lower intensity of the three characteristic bands. The two 'Temple' triploids were easily distinguishable from one another and from the diploid (Fig.3G-I). Since 'Wilking' and 'Temple' are both monoembryonic, the spontaneous triploids examined might have arisen by fertilization of an unreduced gamete (possibly the female one) by haploid pollen (Cameron and Soost 1969).

The observed isoenzyme patterns confirm the expected segregation among monoembryonic triploid offspring, in contrast to the constancy of pattern in the three tetraploid 'King' seedlings.

Roots from diploid 'Wilking' seedlings arising from open pollination were also assayed for anodic peroxidases (Fig.4). While all the patterns differed from those of the maternal parent, one seedling showed a closer similarity than the two others (Fig.4B). One of the latter (Fig.4C) bore some resemblance to 'King' (maternal parent of 'Wilking').

In conclusion, the method developed allows the differentiation of many more bands than previously reported in *Citrus*. It should facilitate a satisfactory distinction between clones as well as between nucellar and zygotic offspring and might prove a useful tool in taxonomic studies. Because of the high peroxidase content of roots, young plants as well as small samples can be used for isoenzyme determination. Comparable results were obtained whether fresh, frozen samples or freeze-dried material had been employed and results with nucellar material, old clone, and cuttings were consistently uniform. Recently, we began to investigate the use of roots from cultured embryos in agar, for isozyme determination. Such a method, when finally standardized, might allow nucellar and zygotic offspring to be distinguished at a very early stage, as well as the recovery of a large number of zygotes in *Citrus* breeding material.

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J. Button
Plant Tissue Culture Research Unit
Dept. of Botany
University of Natal
Pietermaritzburg (S. Africa)

Dr. Aliza Vardi
Dr. P. Spiegel-Roy
Agricultural Research Organization
Volcani Center
Bet Dagan (Israel)