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Factors affecting *Citrus* tree isozyme-gene expression

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Abstract Ten enzymatic systems of *Citrus* species and cultivars have been evaluated for identification purposes and for genetic variability studies. The following factors that could affect their expression were studied: season of sampling, location, rootstock, position of the branch, infection, and age of the tree. Differences involving the presence-absence of the Cu/Zn SOD within the same tree were found. This difference is mainly related to the position of the leaf relative to the sunlight. No change was observed at any of the ten enzymatic systems assayed regarding the location, the rootstock, the growing condition, the season, or the infection with most virus and virus-like pathogens. Viroids induced noticeable changes on 6PG and PRXa zymograms in *C. medica.* A new peroxidase (not present in healthy plants) was detected that could be related to appearance of symptoms. This may induce errors when trees without sanitary control are characterized by this enzymatic system. On the other hand, it provides a new possibility for studying the plant response to the presence of viroids. An effect of age, from 3 months up to 12 years, was observed on citrange Troyer and mandarin Cleopatra PRX, MDH and 6PG patterns. An important change occurs around the first year, most likely related to the end of the seedling stage. This is followed by a long transition phase, the end of which (around 9 years later) coincides with a change in the PRX pattern. These age-related changes seem to involve post-translational modifications of pre-existing isozymes.

Key words *Citrus Tree architecture* · Viroid infection · Tree maturation · Isozymes

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Introduction

World production of *Citrus* in 1988–1989 was estimated to be 64.4 million tonnes, exceeding that of bananas and plantains (*Musa*), grapes (*Vitis*) and apples (*Malus*) (FAO 1990). Citrus species are trees with persistent leaves, hesperidium fruits, and seeds without endosperm, and often with two or more nucellar embroys.

Sizable capital investment in horticultural enterprises requires that nurserymen, growers, and breeders have confidence in the identification of their material. Furthermore, unambiguous identification is a fundamental step in the certification and registration of new cultivars in order to protect breeder's rights. Morphological traits have traditionally been used for identification, but in many cases a morphological assessment of flowering and fruiting material is not possible because of season, immaturity of material, or because the material of interest is a rootstock. An early and common use of isozymes has been in the characterization and identification of cultivars (Torres 1989). Moreover, they have been used as markers of important agronomic traits such as nematode resistance (Rick and Fobes 1974) and salt tolerance (Bretó et al. 1994) in tomato, gain weight and ear number in corn (Edwards et al. 1987), or kernel weight in almond (Asíns et al. 1994). Recently, the peroxidase activity of *Citrus* species has been suggested as a marker for the assessment of tolerance and susceptibility in the greening disease (Lelyveld and van Vuuren 1988) and the bacterial canker disease (Jiao et al. 1992).

There are many reasons for the popularity of starch-gel electrophoresis as a biochemical technique (Hamrick 1989), but foremost among these is the fact that isozymes provide a series of readily scored, single-gene markers. However, care must be taken because several common factors may alter gene expression and, hence, change zymograms. Most of these factors, such as development, light, herbicide, salt, ageing or pathogens, have been studied in corn, tomato and other annual species

(Matters and Scandalios 1986; Scandalios 1987; White et al. 1990; Asíns et al. 1993). Few studies deal with the effect of these factors in trees where development and contact with the biotic and abiotic environment lasts so long. Moreover, Protopapadakis (1987) has pointed out the existence of changes in the enzymatic patterns of *Citrus medica* cultivars as a consequence of the citrus species used as a rootstock.

Our aim in the present paper is to use isozyme systems to study the genetic variability and relationships between the 54 species, 14 genera and 31 hybrids that constitute the IVIA Citrus Germplasm Bank; therefore, this paper has two main objectives: first, to test the reliability of zymograms in the characterization of *Citrus* species and cultivars for identification and genetic variablity studies; and second, to study the changes in enzymatic patterns that can be observed in relation to the season of sampling, location, rootstock, infection with pathogens, and age of the tree.

Table 1 Cultivars assayed in autum 1991 and spring 1992. All material used was free of pathogens and grown in containers in a screenhouse (Navarro et al. 1988)

Species	Cultivar
Mandarin[a]	Kinow
C. bergamia Risso and Poit	Burjasot
C. temple Hort. ex. Y. Tan.	
C, latifolia Tan.	Bearss
C. madurensis Lour.	Comun
C. meyeri Y. Tan.	
Glycosmis pentaphylla (Retz.) Correa	
C. excelsa Wester	
C. medica L. var. ethrog	Arizona 861-S-1
Atalantia ceylanica (Arn.) Oliv.	
C. aurantium L.	Guo-kuo-cheng
C. sinensis (L.) Osb.	Pineapple
C. clementina Hort. ex. Y. Tan.	SRA-91
Fortunella margarita (Lour.) Swing	
C. deliciosa Ten	Tardivo di ciaculli
Tangelo[a]	Mapo
C. limon (L.) Burm f.	Messina
C. nobilis Lour	Campeona
Pummelo[a]	Chandler
Poncirus trifoliata (L.) Raf	Rubidoux
C. unshiu (Mak.) Marc.	Wakiyama
Tangor[a]	Ortanique
C. grandis (L.) Osb.	Deep red
C. paradisi Macf.	Henderson

[a] Artificial hybrid between Citrus species

Materials and methods

Four separate experiments were conducted to test the hypothesis of the consistency of patterns of ten isozyme systems for: (1) the effect of the season (autumn or spring) on the plant material tested (Table 1). (2) the effects of the locations (Valencia or Castellón), the rootstock and the growing conditions (the field or a screen-greenhouse) (Table 2); (3) the effect of infections with pathogens (see Table 3); and (4) the age of the plant (see Table 4). At least four plants per species and affecting factor were analyzed to discard genetic segregation arising from zygotic seedlings. All material used for experiments 1, 2, and 4 was free of pathogens. Except for experiment 2 (fields in Valencia and Castellón), all other plants were grown in containers in a screenhouse (Navarro et al. 1988). All infections tested in experiment 3 were performed with pure isolates from the IVIA collection of citrus virus and virus-like diseases.

Leaf tissue was always used to obtain crude extracts for electrophoresis. When several types of leaves were tested they correspond to circular twig (approximately 2-years old), angular twig (approximately 1-year old) and the expanded leaf of new shoots; otherwise, mature leaves from circular twigs were used. After cutting them into small pieces, 100 mg of leaf tissue was transferred to a microcentrifuge tube with 100 µl of 1% glutation in 0.1 M TrisHCl ph 8 buffer. Samples were extracted by crushing with a glass rod. Whatman 3-MM wicks were soaked in the homogenate and then inserted into a 12% starch gel. Electrophoresis was carried out following the general methods described in Wendel and Weeden (1989). The enzymatic systems examined were: phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6PG), aconitase (ACO), malic acid dehydrogenase (MDH), glutamate oxaloacetate transaminase (GOT), superoxide dismutase (SOD), peroxidase (PRX), isocitric acid dehydrogenase (IDH) and leucine aminopeptidase (LAP). The staining methods for PGI, PGM, 6PG, ACO, MDH, GOT and SOD are referenced in Bretó et al. (1993), for PRX in Asíns et al. (1983), and for IDH and LAP in Vallejos (1983). The differentiation of the Mn, Fe and Cu/Zn forms of SOD was performed following Almansa et al. (1989). The Pineapple cultivar of sweet orange was used as a control in every electrophoresis.

Results

No change was observed at any of the ten isozyme systems assayed regarding location, rootstock, growing condition and season, although the staining intensity was higher in autumn.

When sampling leaves of different branches of *C. limon* (L.) Burm f. var. Messina and *C. latifolia* Tan. var. Bears, a change in SOD patterns concerning the presence-absence of a Cu/Zn isozyme (Rf: 0.65) was observed (Fig. 1). One possibility that we explored to explain this was the effect of two nutrient solutions (Nutriargos Zn, Mn, 0.5% P/V and Nutriargos Mg, 1% V/V) and the two phytosanitary treatments (Neoron,

Table 2 Cultivars assayed in Castellón and Valencia on mandarin Cleopatra (Cl), *C. macrophylla* (Ma), *C. aurantium* (Am) and Carrizo (Ca) and Troyer (Tr) citranges

Species	Cultivar	Castellon (field)	Valencia (field)		Valencia (screenhouse)
C. sinensis	Pineapple	–	Tr	Cl	Tr
C. sinensis	Washington Foyos	Cl	Tr	Cl	Tr
C. clementina	Fina JA-1-158	Ca	Tr	Cl	Tr
C. clementina	Nules AM	Ca	Tr	Cl	Tr
C. limon	Fino 74-L-08	Ma	Ma	Am	Ma
C. unshiu	Frost	Cl	Tr	Cl	Tr
C. paradisi	Marsh	Cl	Tr	–	Tr

Table 3 Plant material and infections assayed

Species	Cultivar	Propagation	Infection[a]	Type of pathogen	Time from infection
C. aurantifolia (Christm.) Swing	Mejicana	Seedling	CVT T-304	Virus	3 months
			CTV T-388	Virus	6 months
			VEV VE-209	Virus	6 months
C. medica var. ethrog	Arizona 861-S-1	Clonal	CVd-Ia	Viroid	2 years
			CVd-IIa	Viroid	2 years
			CVd-IIIa	Viroid	2 years
			CEVd	Viroid	2 years
C. sinensis	Pineapple	Seedling	PSB	Virus?	1 year
C. sinensis	Madame Vinous	Seedling	Stubborn	Mycoplasm	10 years
Tangor	Dweet	Seedling	CG 204	Virus?	6 months
			PSB	Virus?	2 months
C. limon	Eureka Allen	Clonal	CL 900	Virus	8 years

[a] CTV: Citrus Tristeza virus isolates
VEV: Vein enation virus
PSB: Psorosis
CG: Concave gum-causing agent CL: Crinkly leaf virus
CVd: Citrus viroids
CEVd: Citrus exocortis viroid

Table 4 Plant material assayed during development

Species	Cultivar	Propagation	Age
C. excelsa		Seedling	3 months
			6 months
			1 year
			10 years
C. medica var. ethrog	Arizona 861	Clonal	4 months
			5 years
C. sinensis	Pineapple	Seedling	3 months
		Seedling	6 months
		Seedling	15 months
		Clonal	3 years
		Clonal	12 years
Citrange[a]	Troyer	Seedling	3 months
			6 months
			1 year
			3–4 years
			12 years
C. reshnii Hort. ex Tan.	Cleopatra	Seedling	3 months
		Seedling	6 months
		Seedling	15 months
		Clonal	8 years
		Clonal	12 years

[a] Artificial *Citrus* hybrid

0.15% V/V and Actellic, 0.25% V/V) which are usually sprayed over plants at the IVIA Citrus Germplasm Bank. Four leaves per branch were separately painted with each solution and another with distilled water. Three branches of each cultivar were used. No association between the presence-absence of the Cu/Zu isozyme with any treatment was observed. Then, the effect of the position of the branch in relation to light and to the presence of flowers on the branch were tested. Four branches at the top of the lemon cultivar (two with flowers and two without) and four branches from the lower part of the tree where the sunlight hardly reaches (two with flowers and two without) were used to sample three leaves of each. All 12 leaves from the top showed the Cu/Zn SOD while only two from the lower part of the tree showed a light-stained Cu/Zn SOD.

Viroids were the only pathogens (Table 3) that induced noticeable changes on zymograms (6PG and PRX). 6PG patterns showed a higher staining intensity in infected plants (Fig. 2). A more striking difference was found for anodic peroxidases where a new isozyme, 2, with an Rf: 0.61, not present in healthy plants, was detected in the three types of leaves from plants infected with CVd-IIIa and CEVd and leaves 1-year old, or older, from plants infected with CVd-Ia and CVd -IIa (Fig. 3).

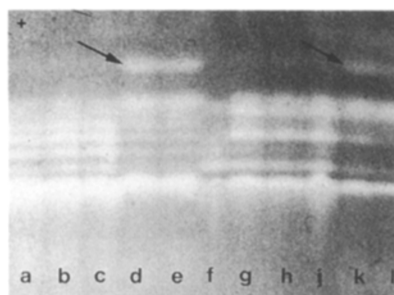
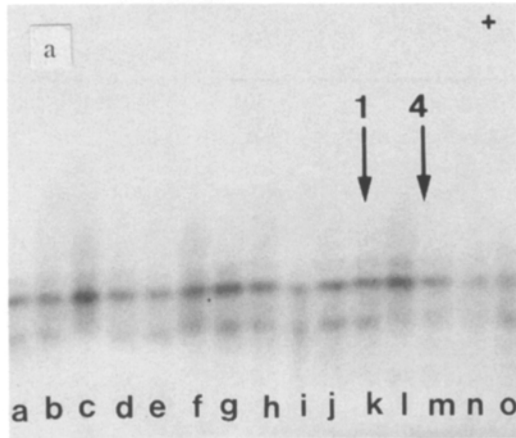


Fig. 1 SOD zymograms from leaves of *C. limon* var. Messina (*a, b, c, d, e*), *C. sinensis* var. Pineapple (*f*) and *C. latifolia* var. Bears (*g, h, j, k, l*). Lanes *a, b, c, g, h* and *j* are from leaves taken from two inner branches, and lanes *d, e, k* and *l* belong to leaves from two outer branches. *Arrows* show the differential Cu/Zn SOD isozyme

An effect of age, from 3 months up to 12 years (Table 4), was noticed for PRX, MDH and 6PG patterns on citrange Troyer and mandarin Cleopatra. After both cultivars reach 1-year old, the staining intensity of several isozymes increases strongly. Leaves from mature citrange and mandarin plants (more than 8 years) show a different PRX pattern which can be used to distinguish them from juvenile plants (Fig. 4). MDH zymograms also changed after the age of 1-year (Fig. 5), although no difference was found between 4- and 12-year old plants. The subtle difference in the migration of the slowest 6PG isozyme found between 5 months- and 1-year old mandarins is shown in Fig. 6.

Discussion

Higher enzymatic activity may be related to intense physiological activity; in fact the fertirrigation of plants



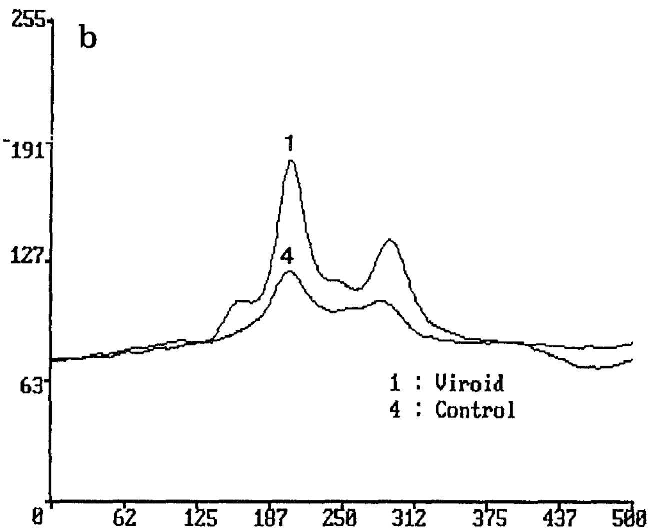


Fig. 2 **a** 6PG zymograms from leaves of *C. medica* var. Arizona infected with CVd-Ia (*lanes a, b, c*), CVd-IIa (*d, e, f*), CVd-IIIa (*g, h, i*) or CEVd (*j, k, l*) viroids and healthy plants (*m, n, o*). *Lanes c, f, i, l* and *o* are from expanded leaves of new shoots. *Lanes b, e, h, k* and *n* from angular twigs, and *lanes a, d, g, j* and *m* from circular twigs. **b** Comparison of staining intensity of bands by densitometer scanning from *lanes k* (infected) and *m* (healthy)

Fig. 3 Anodal peroxidases from leaves of *C. medica* var. Arizona infected with viroid isolates or from healthy plants. *Lanes* as in Fig. 2. *Arrows* show the differential isozymes after viroid infection

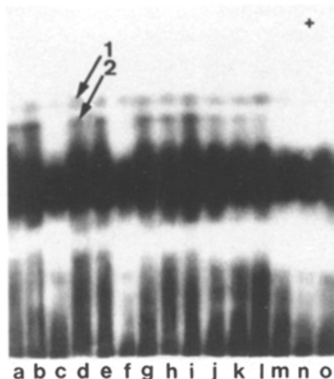


Fig. 4 Anodal and cathodal peroxidases from leaves of mandarin Cleopatra (*a, b, c, d, e*) and Citrange Troyer (*f, g, h, i, j*) plants of different age. *lanes j* and *e* are from plants 3-months old; *lanes i* and *d*, from 6 months; *h*, from 12 months; *c*, 15 months; *g*, from 3–4 years; *b*, from 8 years, and *f and a*, from 12 years. *Arrows* show isozymatic differences for mature plants (older than 10 years)

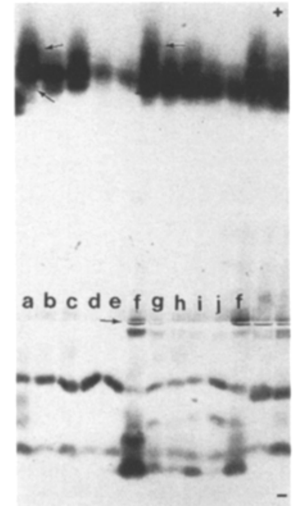
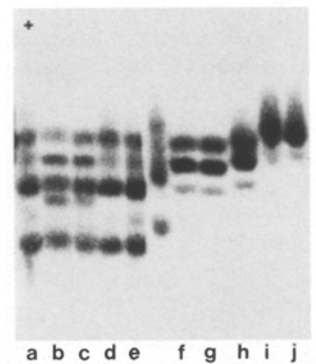


Fig. 5 MDH zymograms from leaves of Citrange Troyer (*a, b, c, d, e*) and mandarin Cleopatra (*f, g, h, i, j*) plants of different age. *Lanes b* and *f*, are from 3-month-old plants; *lanes c* and *g*, from 6 months; *d*, from 12 months; *h*, 15 months; *e*, 3–4 years, *i*, 8 years; and *a* and *j*, 12 years



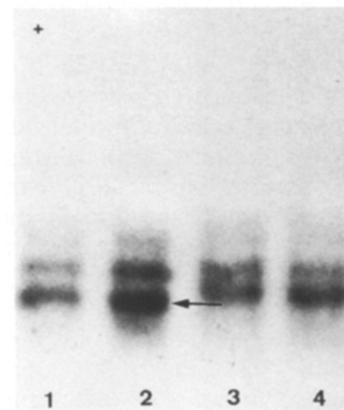


Fig. 6 6PG zymograms from leaves taken from 5-month old (*1, 2*) and 1-year old (*3, 4*) mandarin Cleopatra plants

1 day before isozyme analysis is not an uncommon trick to obtain higher staining intensities in zymograms. The fact that citrus show its maximum vegetative activity in spring, which is related to the content of mineral nutri-

ents and their distribution through the tree (Nadir 1974), leads to a greater content in phenolic compounds and, therefore, less clear zymograms are obtained in spring versus those of autumn.

Unexpectedly, no absolute homogeneity for enzymatic patterns through the tree was found in respect of the behaviour of the Cu/Zn SOD. This is not due to the age of the leaf, to the phytosanitary or nutritive solutions, or to the irregular distribution of a virus. Moreover, this change has been obvious only in *C. limon* and *C. latifolia*, its close relative (Herrero et al. 1992).

Monk et al. (1989) and Perl et al. (1993) reviewed evidence in plants that correlated high SOD levels with resistance to drought, ozone, sulphur dioxide, calcium deficiency, hypoxia, ethylene application, wounding, and the herbicid paraquat. Consequently, genetic-engineering experiments involving the overexpression of Cu/Zn SOD and Mn SOD have been recently carried out in model plant species (Bowler et al. 1992; Perl et al. 1993). Contrasting with our results on differential Cu/Zn SOD expression in *C. limon* and *C. latifolia*, the maize SOD isozymes, a well known gene-enzyme system, do not exhibit significant tissue specificities or developmental shifts in their expression (Baum and Scandalios 1979). In general, superoxide radicals are more likely to be formed during periods of high photosynthetic activity, and disturbance of normal photosynthetic reactions increases this likelihood even further (Bowler et al. 1992). Thus, when plants of *Nicotiana plumbaginifolia* were kept in the dark for 3 days prior to illumination, FeSOD mRNA levels increased dramatically in response to light but were not significantly affected by diurnal fluctuations of light and dark (Tsang et al. 1991). In a tree, the exposure of leaves to sunlight is very different depending on the position of the branches where they are located. We think that the non-constitutive expression of the Cu/Zn SOD observed in these closely related and highly vigorous citrus trees may be related to physiological differences of the branches, mainly due to their position in relation to light, i.e., depending on the position of the branches, not all the leaves are equally active photosynthetically. This would be a possible explanation for the benefits of proper pruning. Whether or not this fine tuning of Cu/Zn SOD activity, reported here for the first time confers some advantage to the tree needs further experimentation. Unfortunately, this finding means that the characterization of citrus species and cultivars by means of enzymatic systems must avoid the use of Cu/Zn SOD, even when using healthy plants.

The presence of viroids may qualitatively affect the PRX patterns of some species. This may lead to error when trees without sanitary control are characterized using this enzymatic system. However, on the other hand, it opens up new possibilities for studying the plant response (sensitive versus tolerant responses) to infections with viroids. Viroids are single-stranded, highly base-paired, covalently closed circular RNA molecules of between 246 and 388 bases. Many are important pathogens of agricultural crops, including grapevine, citrus, coconut, avocado, potato, tomato and cucumber (Symons 1991). Sensitive citron (*C. medica*) clones, such as USDCS 60-13, Arizona 861 and Arizone 861-S1, are always used as biological indicators for viroid diagnosis by budwood grafting. If individual viroid strains are inoculated in any of these clones, different symptoms are observed (Durán-Vila et al. 1988). Doubtless, CV-I and CV-IIa viroid strains produce milder and/or less generalized responses. Abanto et al. (1991) also observed that the type of viroid markedly affected the protoplast yield from infected mesophyll tissues.

In many plants, peroxidase has frequently been correlated with disease resistance (Arora and Wagle 1985; Reuveni and Bothma 1985; Madamanchi and Kuc 1991). In *Citrus* species, peroxidase activity has been suggested as a marker in both the greening disease (Lelyveld and van Vuuren 1988) and the bacterial canker disease (Jiao et al. 1992) for the assessment of tolerance and susceptibility. However, since the new peroxidase we see after viroid infection occurs in a sensitive genotype (which is the reason why it is used as a biological indicator) it cannot be related to a resistance response but rather to the appearance of symptoms (sensitive response). Thus, the sooner the new peroxidase appears (at the recently expanded leaf), the more severe and generalized are the symptoms (CEV and CV-IIIa viroid strains). Symptoms in citrons are not related to the multiplication of the viroid strain (Durán-Vila, personal communication) because all of them replicate and become distributed very well in citrons; rather, they must be the result of an altered metabolism where the new expressed peroxidase may be at least part of the cause of the symptoms. Thus, it seems a good gene-enzyme system to study plant-microbe (citrus-viroid) interaction.

Regarding the increased 6PG activity found a viroid-infected plants, a higher activity of this enzyme in plants resistant to citrus bacterial canker disease was found recently by Jiao et al. (1992) who relate this, and other key regulatory enzymes involved in the pentose-phosphate pathway and the glycolysis-tricarboxylic acid cycle, to disease resistance through the biosynthesis of phenolics (Friend 1985; Jambunathan et al. 1986). Given that *C. medica* is susceptible to citrus canker (Gottwald et al. 1993), it would be interesting to test its susceptibility once 6PG staining intensity increases after a viroid infection.

Our results concerning the lack of change of zymograms due to the rootstock is in disagreement with those of Protopapadakis (1987). He found that most significant change in zymograms concerns the peroxidase system of *C. medica* L. cultivars grafted on four rootstocks grown in an experimental field. When all the cultivars were grafted on *C. karna* Raf. a new isoperoxidase (named C) appears. Our results relating to the effect of viroids on *C. medica* suggest that when these varieties were grafted on *C. karna* some accidental infection with viroids could have occurred provoking the

expression of isoperoxidase C. It is well-known that these microorganisms can be easily transmitted from plant to plant; for instance, by the grafting or pruning tools (Durán-Vila et al. 1988).

The study of changes in some enzyme-gene systems, specially PRX, in the common citrus rootstock citrange Troyer constitutes a good model to approach an understanding of the tree maturation process. One of the limiting factors in this kind of study is to start with genetically identical seedlings; otherwise genetic variability due to heterozygosity of the mother plant, and/or cross pollination, masks any differences in gene expression through time. Most *Citrus* species are apomictic showing a variable percentage of zygotic seedlings. After isozyme analysis of around 100 seedlings of Troyer (heterozygous for almost 50% of their enzymatic loci) no zygotic seedlings was found. As Meier-Dinkel and Kleinschmit (1990) point out, investigations on the physiological and biochemical basis of ageing of woody plants are rare and, up to now, no general marker(s) has been found to define either the juvenile or the adult growth phase of trees. The changes in isozyme patterns we have observed modify the definition of maturation offered by Meier-Dinkel and Kleinschmit (1990) as the genetically programmed process of phase change resulting in different phases of development: the embryogenic phase, the juvenile or seedling phase, the transition phase and the mature phase. Our results suggest that the seedling phase lasts up to the first year in citrus given that the MDH, 6PG and PRX patterns change significantly at that time; this must reflect an important physiological change, which is then followed by a long transition phase, the end of which (9-years later) is not coincident with any change in 6PG or MDH, but only in the PRX zymorgram. Several functions of peroxidases, related to phytohormones (IAA and ethylene) and lignification, must be logically involved in maturation. 6PG plays a role in the oxidative pentose phosphate pathway which has three main functions in plants. Firstly, it generates NADPH which is used as a reductant in biosynthetic processes (e.g. fatty acid biosynthesis); therefore is particularly important in non-photosynthetic tissues, such as differentiating tissues and germinating seeds. Secondly it generates D-ribose-5-phosphate which is required for the synthesis of nucleotides and nucleic acids. Thirdly, it generates D-erythrose-4-phosphate which is required for the synthesis of shikimic acid, the precursor of aromatic rings. MDH is a more complex enzymatic system because of its subcellular compartmentalization and its involvement in several important metabolic processes including gluconeogenesis from fatty acids and proteins (isozymes localized in glyoxysome and mitochondrion), respiration (isozymes in chloroplast and cytoplasm) and control of the stomatal aperture in guard cells (Goodwin and Mercer 1986). In fact, MDH (and also PRX) has also been shown to change under salt stress where the plant developmental process is dramatically modified mainly in terms of precocity, internode length and height (Asíns et al. 1993).

Concerning the nature of these enzymatic changes, they could stem a number of causes ranging from differential gene transcription to post-translational modification. Most often, gene expression regulation is at the level of transcription (Kuhlemeier 1992). However, we do not believe that all the changes we have observed are the consequence of the expression of new structural genes coding for PRX, MDH or 6PG isozymes but rather result mostly from modifications of pre-existing gene systems. Many of these situations have been reported for MDH and PRX such as: specific cleavage or the addition of various small molecules, including glucose, phosphate residues or carbohydrate moieties, by the action of newly expressed regulatory genes or modifiers (Weeden and Wendel 1989). Another type of modification that could explain the migrational change of 6PG may be similar to the different forms in which the plastid-specific glyceraldehyde-3-phosphate dehydrogenase can exist depending on the NAD/NADP ratio in the cell (de Looze and Wagner 1983). Therefore we are most likely dealing with changes occurring during the process of maturation rather than changes that initiate maturation. The length of the juvenile period strongly affects the breeding efficiency of woody perennials. A long juvenile period is considered to be a major obstacle in breeding improved cultivars (Hansche 1983; Lavi et al. 1992). A better understanding of the molecular basis of the differences between the juvenile and the mature phase may allow for the development of specific treatment to induce artificial rejuvenation or to shorten the juvenile period in woody plants. The changes here reported can be used as markers within maturation, where the morphological and agronomic changes associated with it are quantitative rather than qualitative (Hutchinson and Greenwood 1991). On the other hand, the use of PRX, MDH and 6PG systems for the identification of citrus cultivars should be avoided because of the possible effect of the age of the citrus plants on their zymograms.

From our results it is concluded that for identification and genetic variability studies of *Citrus* species, Cu/Zn SOD should not be used when few branches per tree are sampled. This recommendation should be extended to PRX, MDH and 6PG when there is no sanitary or age control. So far as we are aware this is the first time that isozyme markers have been related to the architecture of a tree, and to viroid infections and different phases occurring during the maturation of a woody perennial plant.

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