Variety purity assessment of fifteen citrus rootstocks by isozymes prior to field trial implementation

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Abstract — Introduction. Citrus rootstocks are usually propagated commercially by seed because facultative apomixis through nucellar embryony favors high levels of genetic uniformity. In each seed, one zygotic embryo usually coexists with several somatic embryos of nucellar origin. Dependent on the genotype and environment, the probability of development of zygotic embryos determines the rate of apomixis characteristic of each rootstock cultivar. Commercial varieties grafted on zygotic rootstocks would show lower growth and yield than those on nucellar individuals of the same variety. Selection against zygotic rootstocks is partially accomplished in nurseries by routinely discarding off-type seedlings after visual evaluation. However, for comparative field trials, seedlings need to be more accurately evaluated and individuals of sexual origin rigorously eliminated. Isozyme analysis readily distinguishes zygotic and somatic seedlings. **Materials and methods**. Forty plants from each of 15 rootstock cultivars, to be assayed under Algarve agricultural conditions, underwent isozyme analysis. Extracts for analysis were obtained by homogenizing leaf pieces in liquid nitrogen and resuspending the homogenate in an appropriate buffer. After starch gel electrophoresis of the leaf extracts, five isozyme systems were revealed: Pgi, Pgm, Idh, Mdh and Got. **Results and discussion**. Several plants, identified as originating from self-pollination or cross-pollination, were discarded. For the rootstocks analysed, apomixis rates varied from 88% to 100%. Morphological traits, as plant height or stem diameter, of zygotics and nucellar seedlings showed no significant difference. (© Elsevier, Paris)

Portugal / *Citrus* / variety trials / selection / rootstocks / electrophoresis

Vérification, par la technique des isozymes, de la pureté variétale de 15 porte-greffes d'agrumes, en préalable à une expérimentation en champ.

Résumé — Introduction. Les porte-greffes d'agrumes sont habituellement multipliés par graines car l'apomixie facultative due à l'embryonnie nucellaire favorise la production d'une descendance homogène. Dans une graine, un embryon zygotique coexiste, en général, avec plusieurs embryons somatiques d'origine nucellaire. Selon le génotype et l'environnement, la probabilité du développement des embryons zygotiques détermine le taux d'apomixie caractéristique d'un cultivar de porte-greffe donné. Des variétés commerciales greffées sur porte-greffes issus d'embryons zygotiques auraient une croissance et un rendement inférieurs à celles greffées sur embryons nucellaires de la même variété. Un tri permettant d'éliminer les embryons zygotiques par évaluation visuelle des hors types est couramment pratiqué en pépinière. Cependant, pour des essais de comparaisons variétales en champ, les plantules doivent être contrôlées avec soin et les individus d'origine sexuée doivent être systématiquement éliminés. **Matériel et méthodes**. Pour chacun de 15 cultivars de porte-greffes, 40 plants cultivés à Algarve (Portugal) ont été étudiés par électrophorèse. Les extraits analysés provenaient d'homogénats de broyat de feuilles placés sous azote liquide, puis remis en suspension dans un tampon approprié. Après électrophorèse sur gel d'amidon, cinq systèmes enzymatiques ont été observés : Pgi, Pgm, Idh, Mdh et Got. **Résultats et discussion**. Plusieurs plants, révélés comme provenant d'auto- ou d'interfécondation, ont été éliminés. Pour les porte-greffes analysés, le taux d'apomixie a varié de 88 à 100 %. Les caractères morphologiques des plantules d'origines sexuée ou nucellaire – tels que hauteur du plant ou diamètre du tronc – n'ont pas montré de différences significatives. (© Elsevier, Paris)

Portugal / *Citrus* / essai de variété / sélection / porte-greffe / electrophorèse

1. introduction

The majority of commercially important citrus cultivars are polyembryonic. Polyembryony is usually accompanied by facultative apomixis, characterised by simultaneous growth in the same seed of sexual embryos resulting either from self-pollination or cross-pollination, together with multiple embryos of somatic (nucellar) origin. Apomixis by nucellar embryony, impeding genetic recombination, is a major obstacle to genetic improvement of citrus. On the other hand, apomixis is an important requirement for the citrus rootstock industry, since it allows rootstocks to be clonally propagated by seeds, assuring high genetic and phenotypic homogeneity among progeny. However, depending on genotype and environment, a certain number of zygotic plants can develop from rootstock seeds. In commercial nurseries, phenotypic uniformity among plants is obtained by rouging off-type plants. This practice is also expected to increase genetic uniformity, reducing the number of zygotic plants. Visual selection, however, has been proved to be almost ineffective for that purpose.

Since Torres et al. [1, 2] successfully used the isozyme analysis to distinguish nucellar from zygotic plants, this method has become widely accepted to determine the sexual or somatic origin of citrus plants [3, 4]. The main aim of this study was to determine the apomixis rate of 15 citrus rootstock cultivars, and to confirm the somatic origin and variety authenticity of young plants of these rootstocks, by isozyme analysis. Selected plants will be tested under field trials in Faro, Portugal, for tolerance to soil salinity, calcareous soils and virus diseases (e.g., citrus tristeza virus) which are major limiting factors to the citrus industry in the Algarve region.

2. materials and methods

2.1. plant material

Forty young plants each of 15 citrus rootstock cultivars were grown under greenhouse conditions. These cultivars were Changsha mandarin, Cleopatra mandarin, Sunki mandarin, Sunki mandarin × *Poncirus trifoliata* 30590, Cleopatra mandarin × *P. trifoliata* 30583, 30584 and 30585, Cleopatra mandarin × Carrizo citrange 30575 and 30576, citrandarin 31443, sour orange Gou Tou B7, sour orange B6C-T1, Troyer citrange B2 FAO 31655, Troyer citrange 4AS and Bowman citrange 33821.

2.2. plant extract preparation

After washing, 0.3 to 0.4 g of leaf tissue was ground to a fine powder in a mortar under liquid nitrogen and resuspended in extraction buffer: 0.2 M Tris-HCl, pH 7.2; 20 mM cysteine, 0.2% Triton X-100 (10%), 5 mM Dithiothreitol, and 1 mM PMSF. Polyvinylpyrrolidone (PVP) was added up to 2% (w/v) final concentration, to complex polyphenols. After 20 min centrifugation, at 4 °C in a microfuge (13 000 rpm), supernatants were recovered and immediately analysed.

2.3. electrophoresis

Extracts were analysed by starch gel electrophoresis. Tris-citrate buffer, pH 7, was used, both as gel and electrode buffer for separation of all isozyme systems. Electrophoresis was performed at 180 V for 5 h at 4 °C. Bromophenol blue in 30% glycerol was used both to signalize sample well and to follow gel front progression.

2.4. staining and analysis of gels

Gels were cut into six 1 mm thick slices. Five slices were stained for five enzymatic systems: malate dehydrogenase (Mdh; EC 1.1.1.37); isocitrate dehydrogenase (Idh; EC 1.1.1.42); glutamate oxaloacetate transaminase (Got; EC 2.6.1.1); phosphoglucomutase (Pgm; EC 2.7.5.1) and phosphoglucoisomerase (Pgi; EC 5.3.1.9). Staining procedures were carried out, with minimal modifications, according to Lebrun and Chevalier [5]. Stained gels were fixed in 13% acetic acid glacial and photographed.

2.5. morphological study

With the aim of determining the degree of effectiveness of selecting zygotics by morphological characteristics, the stem height and width (at the fourth internode) of the 600 seedlings analized were measured. Average values of plant height or stem diameter of zygotics were then compared with the average values of nucellar seedlings using a statistical *t* test.

3. results and discussion

The rationale of using isozyme analysis to distinguish between nucellar and zygotic plants was described by Torres et al. [2]. The probability (P) of identifying plants resulting from self pollination is obtained by the formula: $P = 1\text{-}1/2^n$; (n = number of analysed heterozygous loci). This equation makes evident that discrimination between zygotic (arising from self pollination) and somatic embryos by isozyme analysis depend on the number of revealed heterozygous loci. Zygotic individuals which arise by selfing can be easily detected provided they are homozygous at least at one locus at which the maternal progenitor is heterozygous. Plants originated by outcrossing can be identified by the occurrence of new alleles not present in the seed parent.

3.1. isozyme loci

A total of seven heterozygous loci were detected from the analysed rootstock cultivars: Pgi-1, Pgm-1, Pgm-2, Mdh-1, Mdh-2, Idh and Got-1 (*table I*). Eighteen alleles were scored for the five isozyme systems (*figures 1, 2*; *table I*). Pgm-1 was scored only for Sunki mandarin. For the remaining rootstock varieties, enzymes corresponding to this locus were often lightly stained and as a result an accurate analysis was almost impossible. At the Pgm-2 locus, only four alleles were

Table I.
Genotypes of seven loci analysed, in 15 citrus rootstock cultivars and probability of identification of zygotics resulting from self pollination (P). Rootstock genotypes.

Rootstocks	Loci							P (%)
	Pgi	Pgm1	Pgm2	Mdh1	Mdh2	Idh	Got	
Changsha mandarin	FF	–	5.5	FF	FF	MM	SS	0
Cleopatra mandarin	FF	–	5.5	FF	FF	MM	SS	0
Sunki mandarin	FF	2.3	5.5	FF	FF	MM	SS	50
Sunki mandarin × *Poncirus trifoliata* 30590	FF	–	3.5	FS	FF	FM	SF	94
Cleopatra mandarin × *Poncirus trifoliata* 30583	FF	–	3.5	FS	FF	FM	SF	94
Cleopatra mandarin × *Poncirus trifoliata* 30584	FF	–	3.5	FS	FS	FM	SF	97
Cleopatra mandarin × *Poncirus trifoliata* 30585	FF	–	3.5	FS	FS	FM	SF	97
Cleopatra mandarin × *Carrizo citrange* 30575	FS	–	3.5	FS	FS	MI	SM	98
Cleopatra mandarin × *Citrange carrizo* 30576	FS	–	3.5	FS	FF	FM	SM	97
31443 Citrandarin	FS	–	2.3	FS	FS	FM	SM	98
Sour orange Gou Tou B7	FS	–	1.5	FF	FF	MM	SS	75
Sour orange B6C - T1	FS	–	2.5	FF	FF	MM	SM	88
Troyer citrange B2 FAO 31655	SS	–	2.3	FS	FS	FI	SM	97
Troyer citrange 4 AS	SS	–	2.3	FS	FS	FI	SM	97
Bowman citrange 33821	FF	–	2.3	FS	FF	FM	SF	94

Alleles are designated by letters or numbers [2, 4]. Pgi: phosphoglucoisomerase; Pgm: phosphoglucomutase; Mdh: malate dehydrogenase; Idh: isocitrate dehydrogenase; Got: glutamate oxaloacetate transaminase.

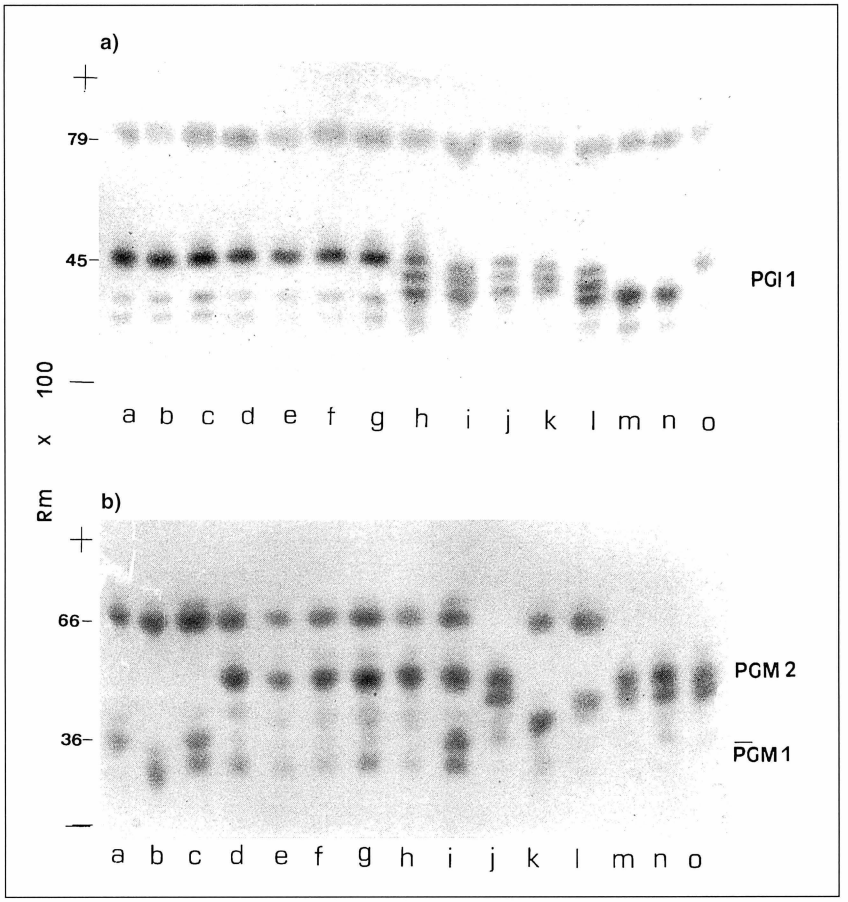

Figure 1.
Zymograms of two isozymatic systems
a) phosphoglucoisomerase or PGI, and
b) phosphoglucomutase or PGM, of 15 rootstock cultivars:
a. Changsha mandarin;
b. Cleopatra mandarin;
c. Sunki mandarin;
d. Sunki mandarin × *Poncirus trifoliata* 30590;
e, f, g. Cleopatra mandarin × *Poncirus trifoliata* 30583, 30584 and 30585;
h and i. Cleopatra mandarin × Carrizo citrange 30575 and 30576;
j. 31443 citrandarin;
k and l. Sour oranges Gou Tou B7 and B6C-T1;
m and n. Troyer citranges B2 FAO 31655 and 4AS;
o. Bowman citrange 33821.

detected, however the faster allele is referred to as number 5. Allele number 4 can be found in some mandarins, such as the common Mediterranean mandarin (Willowleaf; *Citrus deliciosa* Tenore) or the Portuguese mandarin 'Carvalhais'. Locus Got-2 is only revealed in some cultivars. Got-3 was not scored because all the 15 rootstock varieties at this gel zone of activity displayed a monomorphic three-banded pattern. This monomorphic pattern can be observed also among several *Citrus* × *Poncirus* hybrids and scion *Citrus* cultivars (not shown). Such a constant pattern can be explained by the presence of two independent homozygous loci with which enzyme products can interact forming a third, intermediate band.

The probability of identifying zygotic individuals arising from selfing (*table I*) ranged from zero for highly homozygous mandarins: Changsha and Cleopatra, to 98% for hybrid Cleopatra mandarin x Carrizo citrange 30575 and citrandarin 31443 which are heterozygous at all analysed loci.

Independent of the low probability of distinguishing zygotic plants resulting from self pollination among seedlings of the almost homozygous Sunki mandarin, we could detect one zygotic plant which was generated by selfing. In addition, four zygotic plants, which resulted from outcrossing, were also detected among its progeny.

3.2. apomixis rate and selection of nucellars

In the first step of our study, 40 seedlings of each rootstock cultivar, randomly chosen, were analysed for their isozyme patterns and their apomixis rates were computed. In genotypes characterised by facultative apomixis, the ratio zygotic / somatic embryos depends on environmental conditions [6]. However, a characteristic variation for such parameters should be expected for each genotype. The apomixis rate ranged from 88% to 100% among the 15 rootstock cultivars studied (*table II*). Among 600 seedlings analysed, 15 had zygotic origin. Five of them resulted from selfing and ten from outcrossing. Zygotics were detected only in five rootstock cultivars: Sunki mandarin, Cleopatra mandarin × *P. trifoliata* 30585, Cleopatra mandarin × Carrizo citrange 30575, Sour orange B6C-T1 and Bowman citrange 33821 (*table II*).

3.3. morphological selection of zygotics

Comparison between average values of plant height or stem diameter of zygotics and nucellar seedlings showed no significant differences (*figure 3*). Thirteen out of the identified 15 zygotic plants have stem diameter values lying within the frame of its variation on nucellar plants. Only one zygotic plant exhibits a stem height out of

the range of the variation of this parameter in nucellars. These results agree with those previously obtained by Khan and Roose [6] and Moore and Castle [7] who have not found any consistent correlation between genetic origin (zygotic or nucellar) of rootstock seedlings and morphological traits. Despite their lower mean height, the zygotic plants identified by the above authors were similar to nucellars in height, leaf size, thorn length, petiole length and stem diameter. Our analysis confirmed that the identification of zygotic seedlings using morphological traits is highly unsuccessful. Isozyme analysis remains a reliable method for this purpose.

We selected 30 plants per rootstock cultivar from a sample of plants previously submitted to true-to-type selection by morphological characteristics, and all were confirmed as nucellars by isozyme analysis. After budding with three different scion cultivars, these selected rootstock plants will be assayed for their agronomic performances and their adaptability to ecological conditions in Algarve, Portugal.

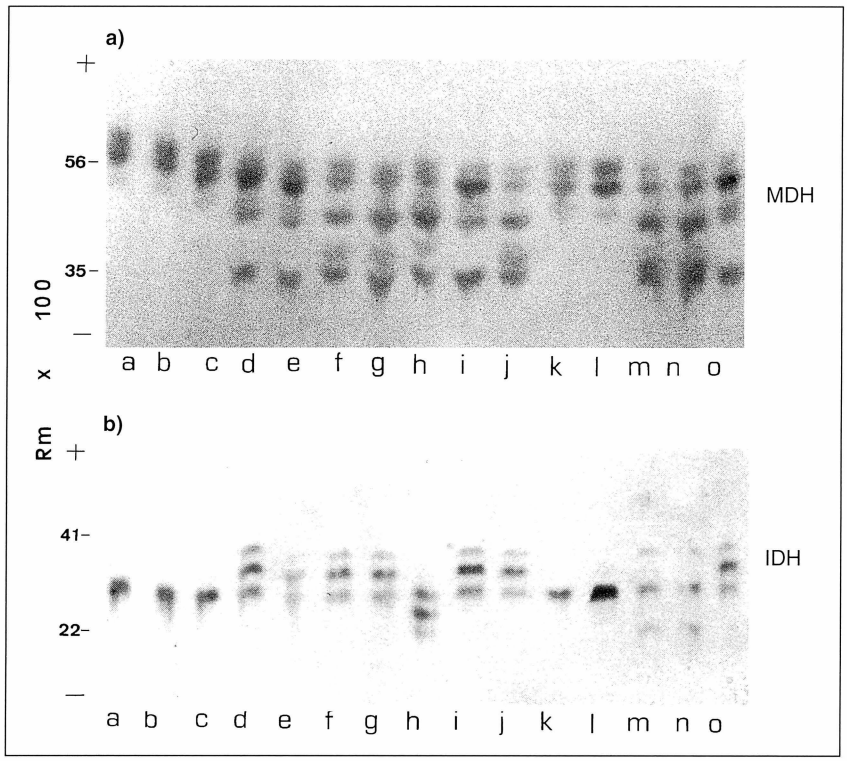

Figure 2.
Zymograms of two isozymatic systems
a) malate dehydrogenase or MDH, and
b) isocitrate dehydrogenase or IDH, of 15 rootstock cultivars:
a. Changsha mandarin;
b. Cleopatra mandarin;
c. Sunki mandarin;
d. Sunki mandarin × *Poncirus trifoliata* 30590;
e, f and g. Cleopatra mandarin × *Poncirus trifoliata* 30583, 30584 and 30585;
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m. and n. Troyer citranges B2 FAO 31655 and 4AS;
o. Bowman citrange 33821.

acknowledgements

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Table II.
Detected zygotic seedlings among 40 plants analysed for each of the 15 citrus rootstock cultivars and corresponding apomixis rates.

Rootstocks	Number of zygotic seedlings	Apomixis rate (%)
Changsha mandarin	0	100
Cleopatra mandarin	0	100
Sunki mandarin	5	88
Sunki mandarin × *Poncirus trifoliata* 30590	0	100
Cleopatra mandarin × *Poncirus trifoliata* 30583	0	100
Cleopatra mandarin × *Poncirus trifoliata* 30584	0	100
Cleopatra mandarin × *Poncirus trifoliata* 30585	1	98
Cleopatra mandarin × Carrizo citrange 30575	1	98
Cleopatra mandarin × Citrange carrizo 30576	0	100
31443 Citrandarin	0	100
Sour orange Gou Tou B7	0	100
Sour orange B6C -T1	4	90
Troyer citrange B2 FAO 31655	0	100
Troyer citrange 4 AS	0	100
Bowman citrange 33821	4	90

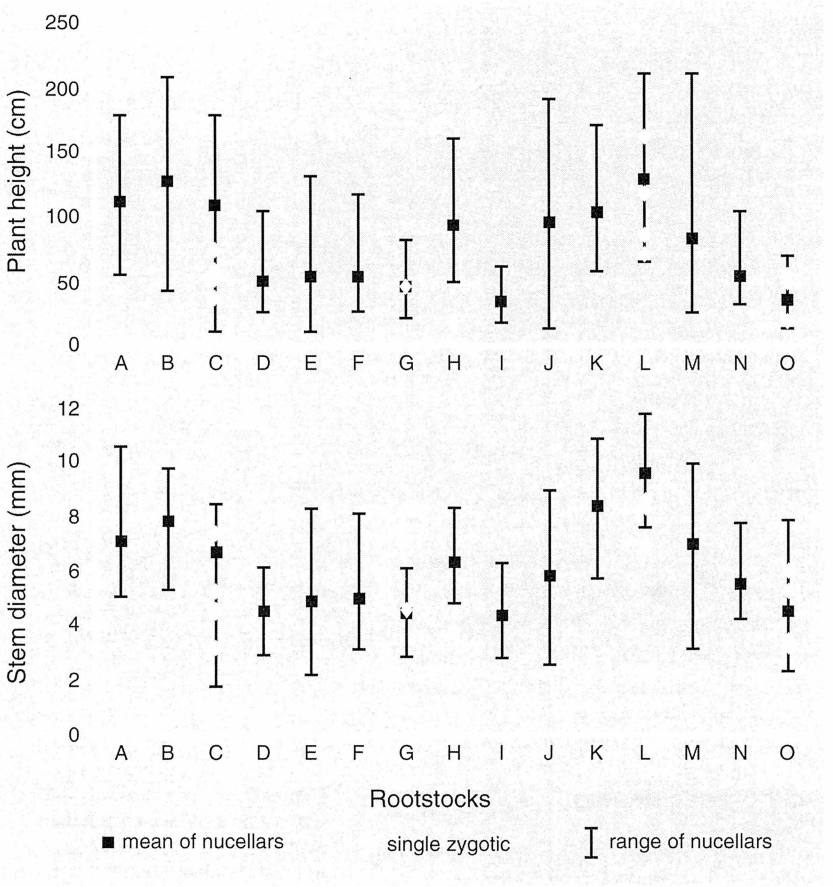

Figure 3.
Mean values and range of plant height and stem diameter (4th internode) of nucellar and zygotic seedlings (18 months old) of 15 rootstock cultivars:
a. Changsha mandarin;
b. Cleopatra mandarin;
c. Sunki mandarin;
d. Sunki mandarin × *Poncirus trifoliata* 30590;
e, f and g. Cleopatra mandarin × *Poncirus trifoliata* 30583, 30584 and 30585;
h and i. Cleopatra mandarin × Carrizo citrange 30575 and 30576;
j. 31443 citrandarin;
k and l. Sour oranges Gou Tou B7 and B6C-T1;
m and n. Troyer citranges B2 FAO 31655 and 4AS;
o. Bowman citrange 33821.

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Verificación, por la técnica de las isozimas, de la pureza varietal de 15 porta injertos de agrios, previamente a una experimentación en campo.

Resumen — Introducción. Se acostumbra multiplicar los porta injertos de agrios por semillas dado que la apomixia facultativa debida a la embrionia nucelar favorece la producción de una descendencia homogénea. En una semilla, un embrión zigótico coexiste, por lo general, con varios embriones somáticos de origen nucelar. Acorde al genotipo y al medio ambiente, la probabilidad del desarrollo de los embriones zigóticos determina la tasa de apomixia característica de un cultivar de porta injerto dado. Variedades comerciales injertadas en porta injertos procedentes de embriones zigóticos tendrían un crecimiento y un rendimiento inferiores a los injertados en embriones nucelares de la misma variedad. En vivero, se suele practicar una selección que permite eliminar los embriones zigóticos mediante evaluación visual de los fuera de tipo. Sin embargo, para ensayos de comparaciones varietales en campo, las plántulas deben controlarse cuidadosamente y deben sistemáticamente eliminarse los individuos de origen sexuado. **Material y métodos**. Para cada uno de los 15 cultivares de porta injertos, se estudiaron 40 plantas cultivadas en Algarve (Portugal) mediante electroforesis. Los extractos analizados provienen de homogenados de triturado de hojas colocadas bajo nitrógeno liquido, y luego en suspensión en un tampón apropiado. Después de electroforesis en gel de almidón, se observaron cinco sistemas enzimáticos: Pgi, Pgm, Idh, Mdh y Got. **Resultados y discusión**. Se eliminaron varias plantas, reveladas como procedentes de auto o de interfecundación. Para los porta injertos analizados, la tasa de apomixia varió de un 88 a un 100%. Los caracteres morfológicos de las plántulas de orígenes sexuado o nucelar – tales como la altura de la planta o diámetro del tronco – no mostraron diferencias significativas. (© Elsevier, Paris)

Portugal / *Citrus* / ensayos de variedades / selección / portainjertos / electroforesis