Genetic characterization of new varieties and hybrids of citrus table fruit through isoenzymes

Roberto Pedroso de Oliveira*[1, 2] and Elizete Beatriz Radmann[2]
[1] Embrapa-Centro de Pesquisa Agropecuária de Clima Temperado (CPACT), Caixa Postal 403, CEP 96001-970, Pelotas, RS, Brazil; [2]Bolsista CNPq. (* Corresponding Author. E-mail: rpedroso@cpact.embrapa.br)

ABSTRACT

The aim of this study was to establish isoenzymatic patterns to genetically characterize the orange varieties 'Lane Late', 'Navalete', 'Navelina' and 'Salustiana', mandarin varieties 'Clemenules', 'Marisol' and 'Satsuma Okitsu', and the hybrids 'Nova' and 'Ortanique'. The following isoenzymatic systems were utilized: alcohol dehydrogenase (ADH), esterase (EST), acid phosphatase (APS), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGDH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), peroxidase (PRX), sorbitol dehydrogenase (SDH), superoxidase dismutase (SOD) and shikimate dehydrogenase (SKDH). Proteins were extracted from leaves and the isoenzymatic polymorphisms were analyzed by polyacrylamide gel electrophoresis. The PGM isoenzyme showed the highest differentiation among the genetic materials tested. The banding profiles observed for PGM, SKDH and EST were satisfactory to characterize the mandarin varieties and hybrids studied. However, no polymorphic bands were found in sweet oranges with the 14 isoenzymes tested.

KEY WORDS: Biochemical markers, citrus crop, electrophoresis, genotype identification.

INTRODUCTION

The citriculture has major economic and social importance to the Brazilian agribusiness (Ministério da Agricultura e do Abastecimento, 2000). Even though Brazil is the largest concentrated juice exporter, there is no tradition in the production of citrus for consumption *in natura* (Amaro, 1999). This type of market is open for exploration. The main factors that have affected fruit quality are inadequate crop management, utilization of the same varieties in juice production, with the exception of some mandarins and navel oranges, and the absence of ideal climatic conditions, especially temperature during fruit maturation.

Uruguay has developed a successful citrus table fruit production for consumption and exportation to the European Economic Community. Recently, researchers from Embrapa Clima Temperado, in Pelotas-RS, Brazil, have begun a project to stimulate citrus table fruit production in the south of Rio Grande do Sul, using the same citrus varieties and hybrids. This region has similar soil types and climate conditions to the Uruguayan citrus orchards.

Nowadays, citrus mother trees are maintained by Embrapa. These plants are the source of buds to be distributed to local nurseries as certified grafting material. The original buds were obtained from the Uruguayan certification system. The following varieties were introduced: 'Lane Late, 'Navelate', 'Navelina' and 'Salustiana' (*Citrus sinensis* (L.) Osbeck); mandarin 'Clemenules' (*C. reticulata* Blanco), 'Marisol' (*C. reticulata* Blanco) and 'Satsuma Okitsu' (*C. unshiu*), and the hybrids 'Nova' [*C. clementina* x (*C. paradise* x *C. tangerina*)] and 'Ortanique' (natural hybrid obtained from the crossing between *C. sinensis* (L.) Osbeck x *C. reticulata* Blanco).

The characterization of the varieties and hybrids is an important step in a certification program for the genetic quality control of the propagation material. For this purpose, the morphologic analyses are the most simple and cost-effective; however, they have a limited use in citrus, because the species are phenotypically similar (Frost and Soost, 1968), traits have additive inheritance, are polygenic and highly affected by the environment (Oliveira et al., 2000).

Isoenzymatic markers have been used to characterize varieties and hybrids of citrus (Sawazaki et al., 1992; Luro et al., 1995). These are also known as biochemical markers that can detect an alteration located in the DNA sequence of the gene that codes for an enzyme, and

this change affects the mobility of the protein molecule in the gel. This technique involves : protein extraction, electrophoresis in starch gel (Smithies, 1955) and visualization of the enzymatic products through histochemical methods (Hunter and Market, 1957). These markers are especially important because they are relatively inexpensive, easy to be conducted, show co-dominant inheritance, refer to only one gene copy, allow for the characterization of plants independent of age, and are only marginally affected by the environment (Ferreira and Grattapaglia, 1998). Their main limitations are: low number of loci, variations are restricted to parts of the genome responsible for the production of the enzymes; difficulties with the interpretation of the zymograms; differences in enzyme activity associated with different plant developmental stages and the possibility of environmental influences (Ferreira and Grattapaglia, 1998).

The objective of this study was to establish isoenzymatic patterns for the genetic characterization of the orange varieties such as 'Lane Late', 'Navelate', 'Navelina' and 'Salustiana'; the mandarin varieties 'Clemenules', 'Marisol' and 'Satsuma Okitsu', and the hybrids 'Nova' and 'Ortanique'.

MATERIAL AND METHODS

The plant material evaluated was composed of orange varieties such as 'Lane Late', 'Navelate', 'Navelina' and 'Salustiana' (*Citrus sinensis* (L.) Osbeck); of mandarin 'Clemenules' (*C. reticulata* Blanco), 'Marisol' (*C. reticulata* Blanco) and 'Satsuma Okitsu' (*C. unshiu*) and of the hybrids 'Nova' [*C. clementina* x (*C. paradise* x *C. tangerina*)] and 'Ortanique' (natural hybrid probably obtained from the crossing *C. sinensis* (L.) Osbeck x *C. reticulata* Blanco).

The genetic characterization of the varieties and hybrids was conducted utilizing 14 isoenzymatic systems: alcohol dehydrogenase (ADH), esterase (EST), acid phosphatase (APS), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGDH), glutamate oxaloacetate transaminase (GOT), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate desidrogenase (MDH), peroxidase (PRX), sorbitol dehydrogenase (SDH), superoxidase dismutase (SOD), and shikimato dehydrogenase (SKDH). Mature leaves from three-year-old mother plants cultivated in plastic pots (20 L) under greenhouse conditions were analyzed. After being collected, the leaves were rinsed in tap water, soaked in distilled water and dried on paper towels.

Samples (10 mg of leaf) were transferred to porcelain plates with round grooves, where they were kept over ice cubes. Afterwards, the samples were ground with a glass pestle in the presence of specific buffers. The protein extraction buffer used for the enzymes ADH, APS, GOT, 6-PGDH, IDH, LAP, MDH, PGI, PGM, PRX, SDH, SOD and SKDH had histidine pH 6.5 with 0.15% ß-mercaptoethanol (1:2), while for EST, the buffer contained histidine pH 8.3 with 0.15% ß-mercaptoethanol (1:1) according to Scandalios (1969).

The enzymatic extract was filtered to Wattmann paper 3MM and loaded into (1.5 x 4.0 mm) wells on either 5% or 6% polyacrylamide gel utilizing a stain steel comb.

The electrophoresis was conducted in a horizontal system using the running buffer according to Shields et al. (1983) in 5% polyacrylamide gels for the enzymes ADH, 6-PGDH, IDH, MDH, PGI, PGM, SDH, SOD and SKDH. The running buffer, according to Scandalios, (1969) was used for LAP, APS and GOT in 6% polyacrylamide gels, and for EST and PRX in 5% polyacrylamide gels. The electrophoretic migrations were conducted at 4°C and the differential potential was kept at around 10 V cm^{-1}.

The histochemical development of the gel was carried out using the protocols, according to Scandalios (1969) , for the enzymes APS, EST, LAP and PRX; Vallejos (1983) for IDH, 6-PGDH, PGI, PGM and SKDH, and Ayala et al. (1972) for ADH, GOT, MDH, SDH and SOD. The gels were kept at 37°C until bands were formed. Afterwards, the gels were fixed in distilled water, methanol and acetic acid (5:5:1).

Results were evaluated three times based upon the relative migration of the bands, and only those consistent and stable were graded.

RESULTS AND DISCUSSION

A total of 30 alleles were obtained from the analyses of four varieties of sweet orange, three varieties of mandarins, and two hybrids of citrus table fruit utilizing 14 isoenzymes. Among them, 16 were polymorphic between two genetic materials, while the others were monomorphic. The polymorphic alleles were revealed through the isoenzymatic analysis of EST, IDH, LAP, PGM and SKDH (Figure 1), while APS, GOT, MDH, PRX and SOD were monomorphic (Figure 2). The enzymes ADH, PGI, 6-PGDH and SDH did not show any isoenzymatic products under the conditions tested.

The largest difference among the genetic materials

evaluated was revealed by the enzyme PGM. Among the five alleles obtained, the three polymorphic separated the varieties and hybrids of table fruit citrus in four groups: 1) 'Lanelate', 'Navelate', 'Navelina' and 'Salustiana'; 2) 'Clemenules', 'Marisol' and 'Nova'; 3) 'Satsuma Okitsu', and 4) 'Ortanique'. The enzyme SKDH produced seven polymorphic alleles that formed three groups: 1) 'Lanelate', 'Navelate', 'Navelina' and 'Salustiana'; 2) 'Clemenules', 'Marisol' and 'Ortanique', and 3) 'Satsuma Okitsu' and 'Nova'. The EST showed three alleles, two of which were polymorphic, separating the citrus into three groups: 1) 'Lanelate', 'Navelate', 'Navelina', 'Salustiana' and 'Satsuma Okitsu'; 2) 'Clemenules' and 'Ortanique', and 3) 'Marisol' and 'Nova'. Three alleles were obtained from IDH, two polymorphic and one monomorphic, thus forming two groups: 1) 'Lanelate', 'Navelate', 'Navelina', 'Salustiana', 'Clemenules', 'Marisol' and 'Nova', and 2) 'Satsuma Okitsu' and 'Ortanique'. LAP produced four alleles, and two were polymorphic, separating two groups: 1) 'Satsuma Okitsu', and 2) the other varieties and hybrids studied (Figure 1).

Therefore, the characterization of the mandarin variety 'Satsuma Okitsu' and the hybrid 'Ortanique', when compared to other genetic materials evaluated,

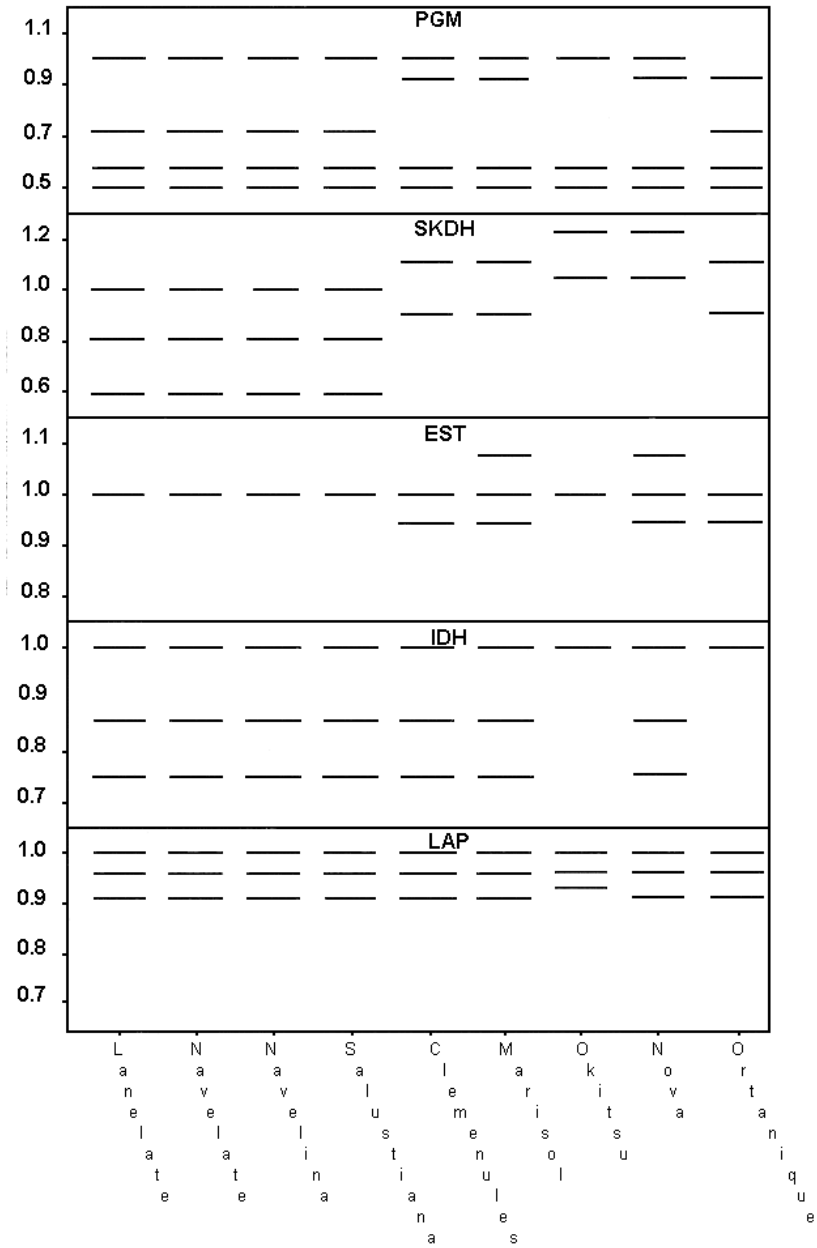

Figure 1. Representation of the isoenzymatic standard profiles produced by the analysis of PGM, SKDH, EST, IDH and LAP in varieties and hybrids of citrus table fruit.

can be done only through the analysis of PGM. For the hybrid 'Nova', it was necessary to combine the results obtained from the analysis of PGM and SKDH, and for the mandarin 'Clemenules' the results from PGM and EST must be combined. The differentiation among the mandarin varieties 'Clemenules', 'Marisol' and 'Satsuma Okitsu' and the hybrids 'Nova' and 'Ortanique', was possible by associating the results of PGM, SKDH and EST.

The genetic characterization of mandarin varieties and hybrids of citrus can be done easily through biochemical and/or molecular methods due to the diversity of these plants, due to crossings (Sawazaki et al., 1992). Therefore, the present study provides a very simple, efficient, and inexpensive methodology, established with only three isoenzymatic markers (PGM, SKDH and EST). This approach allows for the genetic characterization of the mandarin varieties 'Clemenules', 'Marisol' and 'Satsuma Okitsu', and the hybrids 'Nova' and 'Ortanique'.

The isoenzymatic standards are being used on a routine basis to characterize plants from the citrus mother trees that are being kept at Embrapa Clima Temperado to control distribution of genetic materials. This methodology can be used by other laboratories as the use of these varieties and hybrids progress to other Brazilian regions.

Even though the isoenzymatic analyses are very

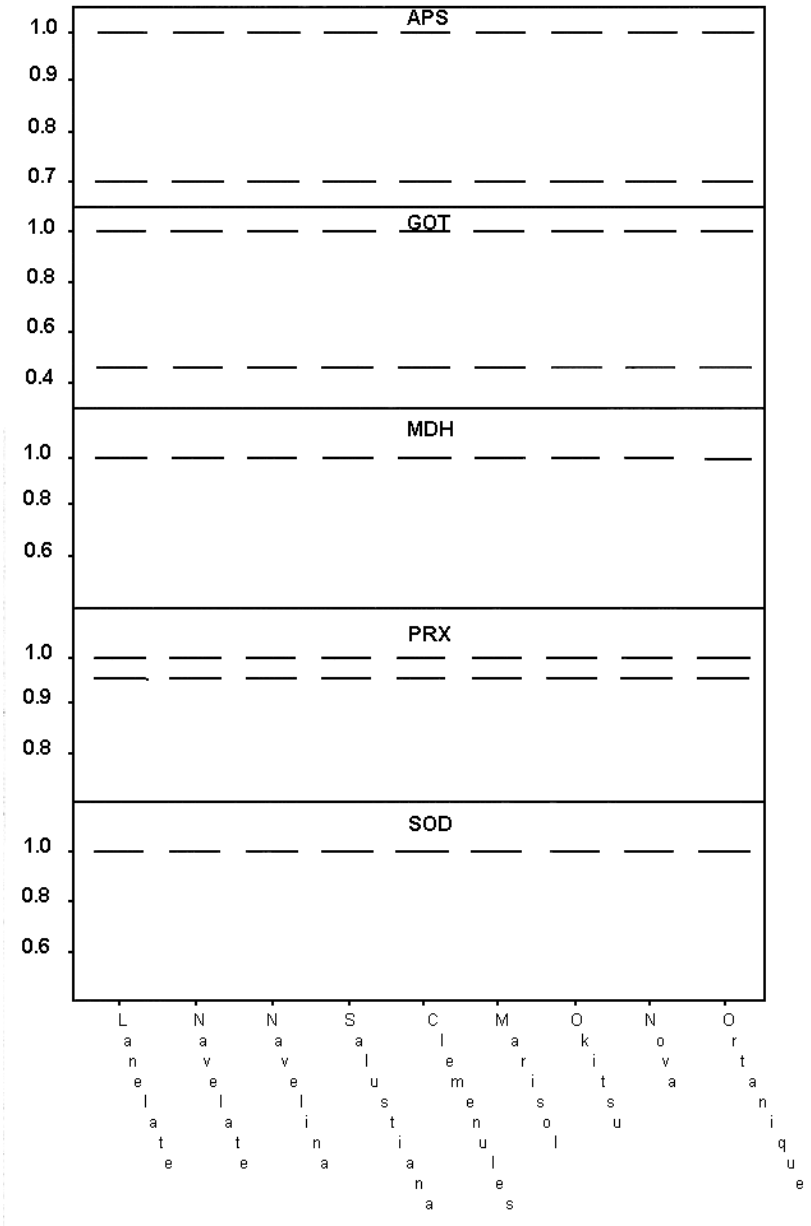

Figure 2. Representation of the isoenzymatic standard profiles produced by the analysis of APS, GOT, MDH, PRX and SOD in varieties and hybrids of citrus table fruit.

informative in identifying mandarins and citrus hybrids, the same does not occur with sweet oranges. None of the 14 isoenzymes evaluated showed polymorphic bands among the varieties 'Lanelate', 'Navelate', 'Navelina' and 'Salustiana'. This was expected since the different varieties of sweet oranges were the results of alterations, such as mutation that occurred in only a few genes (Scora, 1975). These are difficult to be detected through biochemical and molecular markers, even though most of the time there are strong phenotypic differences among the plants. The absence of polymorphism among sweet oranges was also reported by Sawazaki et al. (1992) with isoenzymes; by Roose (1988) with RFLP (Restriction Fragment Length Polymorphism); by Targon et al. (2000) with RAPD (Random Amplified Polymorphic DNA), and by Novelli et al. (2000) with microsatellites. According to Oliveira et al. (2000), the characterization of sweet orange varieties can be carried out by the use of morphological characters.

RESUMO

Caracterização genética de novas variedades e híbridos de laranja utilizando isoenzimas

Este trabalho teve por objetivo estabelecer padrões isoenzimáticos para caraterização genética das variedades de laranja 'Lane Late', 'Navelate', 'Navelina' e 'Salustiana', de tangerina 'Clemenules', 'Marisol' e 'Satsuma Okitsu', e dos híbridos 'Nova' e 'Ortanique'. Os sistemas utilizados foram: álcool desidrogenase (ADH), esterase (EST), fosfatase ácida (APS), fosfoglucoisomerase (PGI), fosfoglucomutase (PGM), 6-fosfogluconato desidrogenase (6-PGDH), glutamato oxaloacetato transaminase (GOT), isocitrato desidrogenase (IDH), leucina aminopeptidase (LAP), malato desidrogenase (MDH), peroxidase (PRX), sorbitol desidrogenase (SDH), superoxidase dismutase (SOD) e xikimato desidrogenase (SKDH). Folhas foram utilizadas para a extração de proteínas, sendo o polimorfismo isoenzimático analisado por eletroforese em gel de poliacrilamida. O sistema PGM proporcionou a maior diferenciação entre os materiais genéticos. Padrões de bandas produzidos pelos sistemas isoenzimáticos PGM, SKDH e EST foram suficientes para caracterizar as variedades de tangerina e híbridos estudados. Porém, não se obteve polimorfismo entre as laranjas doces com os 14 sistemas isoenzimáticos.

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