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Characterization of five sour orange clones through molecular markers and leaf essential oils analysis

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Abstract

Five clones of sour orange (*Citrus aurantium* L.) showing significant morphological differences were selected from our germplasm collection and characterized both by genetic and leaf volatiles analysis. The genetic studies were undertaken by the use of molecular markers developed by PCR-based techniques (ISSR and RAPD), while the leaf essential oil patterns were obtained by chromatographic and mass spectrometric determination. Data obtained suggest that reasonably similar information can be achieved from the two techniques, supporting each other in characterization studies.

Keywords: *Citrus aurantium*; Sour orange; ISSR; RAPD; *Citrus* rootstock; Leaf essential oil composition; GC-MS

1. Introduction

Sour orange *(Citrus aurantium* L.) has been and still ranks as one of the world's greatest and most widely used *Citrus* rootstock. Its popularity, mainly due to adequate yields, good fruit quality, tolerance to foot rot, cold and compatibility with the most important *Citrus* species and varieties, has been declining because of citrus tristeza virus (CTV) affecting virtually all scion cultivars, except lemon, on sour orange rootstock. Since 1974 we are collecting sour orange plants showing different morphological and physiological traits, mainly from various parts of Sicily but also from foreign countries (Tusa et al., 1979). To present, a total of 34 sour orange clones have been collected and we are currently selecting the most resistant to biotic and abiotic stress, with particular attention to CTV. Polymerase chain reaction (PCR)-based techniques provide a useful and relatively simple method for cultivar fingerprinting (Luro et al., 1995) and inter-simple sequence repeat (ISSR) amplification appears a technique able to quickly differentiate closely related individuals (Zietkiewics et al., 1994).

ISSR has been previously used to fingerprint trifoliate orange germoplasm accessions (Fang et al., 1997) and other closely related *Citrus* cultivars (Fang and Roose, 1997).

Also PCR methods using arbitrary primers have been widely used as a fingerprint technique. Among these techniques, random amplified polymorphic DNA (RAPD) is the most widely applied (Tinker et al., 1993; Millan et al., 1996) and 10-mer RAPD primers are found to show the best results (Coletta Filho et al., 1998; Elisiario et al., 1999). In *Citrus*, RAPD analysis has been used to identify lemon mutants (Deng et al., 1995), to build genomic maps (Cai et al., 1994), to identify markers linked to relevant agronomic traits (Cheng and Roose, 1995; Gmitter et al., 1996) and for taxonomy studies (Luro et al., 1992).

Five clones from our collection have been selected on the basis of their definite morphological differences and a genetic study has been performed by using genetic markers above described. Investigations have been further extended to the analysis of the leaf essential oils because of the well-recognized genetic nature of the whole pattern (Dugo et al., 1996 and references therein). However, while it appears substantially assessed that a given *Citrus* species is characterized by a

Abbreviations: FCC, flavor and fragance components; ISSRinter-simple sequence repeats; RAPD, random amplified polymorphic DNA; SSR, short sequence repeat

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definite essential oil pattern (Gurib-Fakim and Demarne, 1995), evidence that such pattern could effectively differentiate closely related individuals still needs research efforts. Studies on comparison of the essential oil patterns of various *Citrus* plants, including also the transmission of the genetic traits from the parents to their hybrids both in sexual as well as somatic *Citrus* hybridization experiments suggested a definite correlation between the essential oil pattern and morphological characters, allowing to recognize, in the hybrid plants, the contribution of the genetic traits from the parents (Ruberto et al., 1997a,b; Alonzo et al., 2000a,b).

In the present study five selected *C. aurantium* clones are compared both on the basis of a genetic study as well as of their leaf essential oil patterns. Here, leaf essential oil study has been preferred, instead of flower or peal oils, for comparison purposes, because of the availability of leaves all year round and in the consideration that essential oils from flowers and peels usually results in the prevalence of few components thus making a definite quali/quantitative evaluation of the remaining components more difficult.

2. Materials and methods

2.1. Plant material

All our germoplasm collection was grafted on the same standard rootstock sour orange in 1975 in the Lascari field station (38°N, 14°E) and thus grown in the same pedoclimatic and cultural conditions. The selected clones among our sour orange collection were AACNR9A, AACNR19B, AACNR23A, AACNR26A and AACNR32. This latter has been previously reported as ''Gou-Tou'', a Chinese selection CTV-resistant (Castle, 1987; Garnsey, 1992). Some studies indicate that this selection could be of natural hybrid origin (Castle et al., 1989; Müller et al., 1990), as confirmed by a close inspection of its fruits and isozyme profiles (Medina Filho, H.P., Bordignon, R., Müller, G.W., unpublished data). Young leaves (6 months old) were collected from each selected sour orange plant following the cardinal points.

2.2. DNA extraction

The leaves were frozen in liquid nitrogen and stored at −80 °C.

The samples were ground in a mortar with liquid nitrogen and genomic DNA was extracted from the samples using the procedure described from Doyle and Doyle (1987). DNA was quantified by measuring OD 260 as described by Sambrook et al. (1989).

2.3. ISSR analysis

A total of 11 primers – i.e. $(AC)_8YG$, $(AG)_8YC$, $(AC)_8YA$, $(AC)_8YT$, $(AG)_8YT$, $(GT)_8YG$, $(TCC)_5RY$, $(GA)_8YC$, $(CA)_8RG$, $(GA)_8YG$ and $(GT)_8YC$ (reported by Fang and Roose, 1997) – were used to amplify the DNA. The primers were purchased from Life Technologies, Gaithersburg, MD.

Each 25-μL amplification reaction consisted of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 800 μM dNTP, 0.5 μM of each primer, 1 U of Platinum *Taq* polymerase (Life Technologies) and 30 ng of template DNA.

The amplification was performed in a MJ Research thermocycler (Genenco) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 4 min at 94 °C, followed by 36 cycles at 94 °C for 30 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 120 s (extension), followed by a final extension step at 72 °C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel containing 1× TBE (45 mM Tris–borate, 1 mM EDTA) and 0.5 μg/mL aqueous solution of ethidium bromide. About 25 μL of reaction products with an 5 μL of loading buffer (0.25% BFB, 40% w/v sucrose) were loaded and the gel was run for 4 h at 100 V. The gel was then visualized under UV light. Only those bands showing consistent amplification were considered; smeared and weak bands were excluded from the analysis. Polymorphic ISSR markers were scored for the presence or absence of bands. To confirm the obtained polymorphisms, the analysis were tested performing three separate PCR.

2.4. RAPD analysis

Six 10-mer primers – i.e. OPH04, OPAT14, OPH15, OPM04, OPO14 and OPN14 (reported by Coletta Filho et al., 1998) – were used for the RAPD analysis. The primers were purchased from Life Technologies, Gaithersburg, MD.

DNA amplification reactions were performed in a volume of 25 μL with 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 800 μM dNTP, 0.4 μM of each primer, 1 U of Platinum *Taq* polymerase (Life Technologies) and 30 ng of template DNA. The amplification was performed in a MJ Research thermocycler (Genenco) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 90 s at 94 °C, followed by 36 cycles at 94 °C for 1 min (denaturation), 36 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by a final extension step at 72 °C for 10 min. PCR-amplified DNA fragments were visualized as described above. To confirm the obtained polymorphisms, the analysis were tested performing three separate PCR.

2.5. Essential oils extraction and analyses

The leaf essential oils volatile fraction was isolated by steam distillation-extraction on samples of about 0.6 kg using a Clevenger apparatus (AOAC, 1995), dissolved in hexane and analyzed by an HP 6890 GC-MS equipped with the mass selective detector HP 5973. An HP5-MS, 5% diphenyl-95% dimethylpolysiloxane, capillary column (30 m × 0.2 mm, 0.25 μm film thickness) was used as stationary phase. Chromatographic conditions: 1 μL splitless injection, by autosampler, using He as carrier gas at 10^{-3} L/min; injector temperature 250 °C. Oven temperature program: 8 min of 60 °C isotherm followed by a linear temperature increase of 4 °C/min up to 180 °C held for 2 min. MS scan conditions: