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Identification of citrus varieties using laser-induced fluorescence spectroscopy (LIFS)

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ABSTRACT

The production of sweet orange faces a problem with regard to visually identifying citrus seedling varieties. Despite using analytical techniques such as molecular markers, this kind of classification is not possible due to very subtle genetic variations. This is an important fact given that the cultivation of a variety that differs from the one planned can cause numerous serious problems, such as the treatments applied to the orchards as well as the quality and characteristics of the fruits after harvest. Within this context, we assess the laser-induced fluorescent emission technique (LIFS) as a tool to identify citrus varieties. The results showed that LIFS combined with statistical methods is able to discriminate the different citrus varieties chosen. The rate of success for this classification depends on the combination of canopy and rootstock varieties, and our best result was of around 100%. These results can be used in the development of a new method that is economically viable for seedling certification.

1. Introduction

Citrus fruits are among the most consumed fruits in the world, whether in the form of fresh fruit or as juice, and sweet orange (*Citrus sinensis* L. Osbeck) is the most representative and recognizable species of this group (Novelli et al., 2006). Among sweet orange varieties grown in São Paulo State, the leading orange producer region in Brazil, 55% are late varieties Valencia and Natal, 22% mid-season variety Pera Rio and 23% early varieties Hamlin, Westin, Rubi and Pineapple (Neves, 2010). The sweet orange originated in Asia and its hybrid characteristic seems to come from a cross between mandarin (*Citrus reticulata* Blanco) and pummelo (*Citrus grandis* L. Osbeck) (Davies and Albrigo, 1992; Nicolosi et al., 2000).

The characterization of citrus cultivars is an essential step for certification, breeding and germoplasm conservation programs, as this allows the monitoring of genetic quality (International Board for Plant Genetic Resources, 1988; Zubrzycki, 1997). Citrus varieties show diversity in their morphological traits, such as canopy size and shape, fruit color, size, type, ripening season, and seed number per fruit. These botanical descriptors, heritable, easily visible and measurable, are those most commonly used to characterize and discriminate sweet orange cultivars from those that are not discriminated by biochemical and molecular tools (Scora, 1975; Radmann and Oliveira, 2003). However, research on germoplasm characterization in oranges and other *Citrus* species has been rendered difficult and slower because, in most cases, the morphological characterization of citrus sweet orange cultivars can be only done post fruit bearing and has no practical value to be used in a certified program to produce and control the genetic background of commercialized citrus nursery plants. Therefore, the production of sweet orange faces a problem with regard to the identification of citrus varieties in non-bearing plants in nurseries by simple visual inspection.

It is recognized that sweet oranges have a narrow genetic basis and that most morphological characteristics originated through somatic mutations propagated by their vegetative propagation (Herrero et al., 1996; Golein et al., 2005). Thus, despite the considerable diversity among cultivated genotypes with respect to morphological, physiological and agronomic traits, notwithstanding the use of analytical techniques such as molecular markers, this kind of classification is not possible due to very subtle genetic variations (Spiegel-Roy and Goldschmidt, 1996). Very little DNA variation has been detected using DNA-markers such as variable number of tandem repeats (VNTRs) loci (Orford et al., 1995), inter-simple sequence repeats (ISSRs), markers and restriction fragment length polymorphisms (RFLPs) (Fang and Roose, 1997), random amplified polymorphic DNA (RAPD) (Targon et al., 2000; Bastianel et al., 2001) and isozymes (Novelli et al., 2000).

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Polymorphic microsatellite markers or simple sequence repeats (SSRs) (Golein et al., 2005; Novelli et al., 2006; Zerihun et al., 2009) and single nucleotide polymorphisms (SNPs) (Jiang et al., 2010) for assessing inter and intraspecific variability to identify and characterize sweet orange and mandarins cultivars were developed and characterized. However, the genetic methods for this application are still considered to be inefficient for intraspecific discrimination, time-consuming and expensive.

The cultivation of a variety that differs from the one planned can cause numerous serious problems, such as the different treatments applied to the orchards and the quality and characteristics of the fruits after harvest. Considering that sweet orange is a large-scale cultivation in Brazil and in many parts of the world, with millions of new nursery plants planted every year, the development of specific tools that characterize and discriminate sweet orange cultivars at the nursery stage is necessary.

Spectroscopic methods appear to be promising alternatives for the diagnosis of citrus stresses since they can rapidly measure properties of the samples that are related to their chemical composition (Cardinali et al., 2012; Pereira et al., 2011a,b). Such methods have already been employed to investigate chemical changes in HLB disease trees (Pereira et al., 2010a,b).

Laser Induced Fluorescence Spectroscopy (LIFS) is widely used in a variety of analytical applications, from hepatic steatosis diagnosis to soil analyses (de Oliveira et al., 2009; Milori et al., 2006). This is an interesting method as it allows for quick and low cost measurements practically without sample preparation.

Based on plant leaves having several fluorophores whose concentration depends on genetic characteristics and plant metabolism, we consider that the optical properties of citrus leaves can be different depending on the genetic variety. This paper aims to evaluate the use of LIFS combined with statistical tools in order to discriminate citrus varieties. The application of this study is very interesting for the productive sector, because it can result in a new method that is economically viable for seedling certification.

2. Materials and methods

2.1. Plant material

2.1.1. Production of nursery citrus plants used in the experiments

All citrus scion and rootstock varieties used in this experiment were produced in a commercial citrus nursery according to São Paulo State Certified Citrus Nursery Plants Production Standards (NORMAS, 1998). Scion varieties budwoods and rootstock seeds were originated from registered and certified mother trees by the Centro de Citricultura 'Sylvio Moreira'/Instituto Agronômico de Campinas according to the São Paulo State Program for Mother Citrus Trees Registering (PROGRAMA, 1998). The mother trees were registered based on morphological and agronomics descriptors such as leaf and fruit morphology, fruit maturation and juice quality, number of seeds per fruit and other characteristics (PROGRAMA, 1998). To avoid mistakes and frauds in citrus nursery production, the nursery must have a document that allows the traceability of budwood and seed origin, identifying the species, cultivar and amount of acquired propagation material. Citrus nurseries are periodically inspected by agents of the São Paulo State Agriculture Secretary.

Rootstock seeds were growth in tubettes with granulated 40 fiber coconut substrate (Amafibra). After 3 months the selected seedlings were transplanted to 4-L citruspots with mix 40 fiber coconut substrate. After 3 months when the rootstock stems had a diameter of 0.5 cm, the scion variety bud was grafted. Three to 4 months after grafting, the citrus nursery plant was used for the experiments. From the time of seed planting, the nutritional management was done by fertigation of nutritive solution with final electrical conductivity of 2.0 mS cm^{-1}. The nutritive solution was composed of calcium nitrate (Hydro® special at 0.9 mg L^{-1}), potassium nitrate (0.3 mg L^{-1}), monoammonium phosphate (0.07 mg L^{-1}), magnesium sulfate (0.26 mg L^{-1}), copper EDTA (0.01 mg L^{-1}), zinc EDTA (0.007 mg L^{-1}), manganese EDTA (0.005 mg L^{-1}), boric acid (0.0035 mg L^{-1}), ammonium molybdate (0.0003 mg L^{-1}), iron EDTA (0.05 mg L^{-1}). Fertigation was conducted once or twice a week according to plant development and temperature. Copper hydroxide (Kocide® at 250 g L^{-1}) was sprayed after the seedlings were transplanted and during the plant development after grafting. For pest control, monthly sprays alternating abamectin (Vertimec® at 0.3 mL L^{-1}) + (Talstar® at 0.2 mL L^{-1}) or abamectin (Vertimec® at 0.3 mL L^{-1}) + lambdacyhalothrin (Engeo Pleno® at 0.2 mL L^{-1}) or abametin (Vertimec® at 0.3 mL L^{-1}) + carbosulfan (Marshal 400® at 0.2 mL L^{-1}) and two bimonthly sprays alternating pyridaben (Sanmite® at 0.5 mL L^{-1}) or spirodiclofen (Envidor® at 0.2 mL L^{-1}) and etoxazole (Borneo® at 0.45 mL L^{-1}) were done at 7-day intervals.

For each scion/rootstock combination leaves from 50 citrus nursery trees with 50 cm height were collected.

2.1.2. Samples

The citrus leaves were collected from nursery plants in a commercial citrus nursery in the State of São Paulo, Brazil, and sent to the laboratory for analysis. Two sets of samples were analyzed: one grafted on Cleopatra mandarin (*C. reticulata*) rootstock and

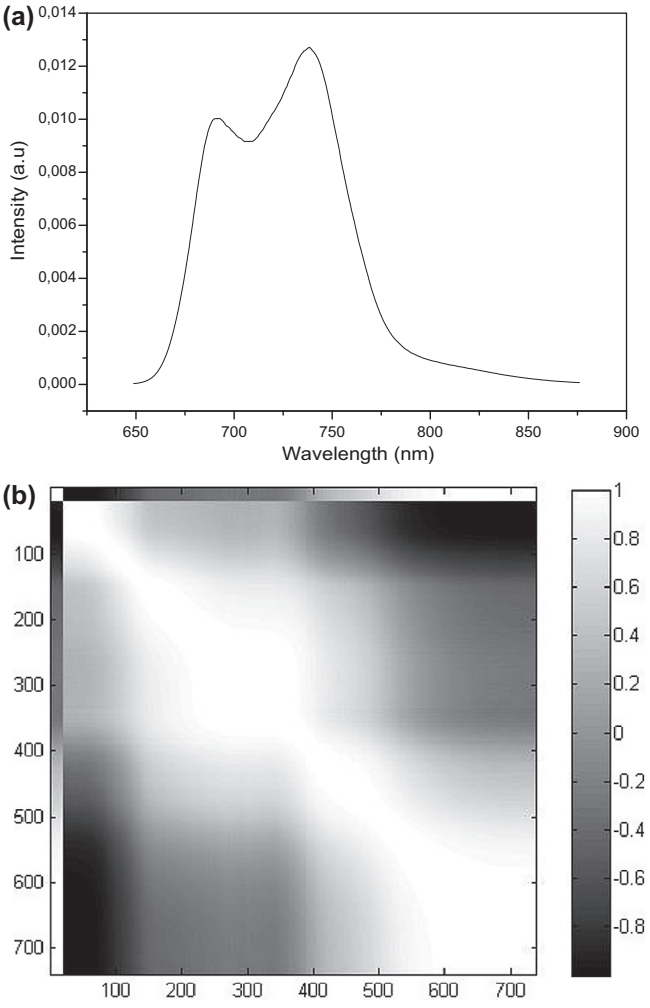

Fig. 1. (a) Typical citrus leaf fluorescence emission. (b) Correlation matrix between wavelength spectra.