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Facultative apomixis and chromosome doubling are sources of heterogeneity in citrus rootstock trials: Impact on clementine production and breeding selection

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ABSTRACT

All commercial citrus rootstocks are polyembryonic and propagated by seeds. Although these seeds produce uniform plant material most of the time, zygotic or polyploid citrus seedlings may arise. The aim of this study was to understand how zygotic or tetraploid rootstock could affect a citrus rootstock selection field trial. A trifoliate orange selection field trial, which was planted in 1974 and grafted with clementine, was re-investigated with respect to the presence of rootstocks that were not true-to-type. Among the 288 trees investigated, flow cytometry identified 2.4% of rootstocks as tetraploid and SSR markers indicated that 6.6% were zygotic. Yield data showed that the presence of tetraploid rootstock dramatically decreased (by about 45%) clementine fruit production. However, zygosity did not always affect fruit production and a range of effects were observed, from a slight increase in production to a 24% decrease. Exclusion of non-true-to-type genotypes from the production analysis indicated that the best candidate for rootstock was a clone previously ranked in the middle of the 32 rootstocks under evaluation. However, the presence of zygotic rootstock did not appear to cause any significant differences in fruit quality during the first 5 years of the investigation, which suggests that non-true-to-type plants cannot be identified by fruit quality parameters. This study indicates that tetraploid and zygotic rootstocks have a strong impact on citrus fruit production in orchards, and removal of off-type seedlings is required prior to planting in any agronomic trial.

1. Introduction

At present, all commercially cultivated citrus trees are grafted onto rootstock. Grafting maintains true-to-type varieties and the use of rootstock can reduce the adverse effects of abiotic stress such as water constraints caused by logging, drought, salinity and alkalinity, as well as conferring tolerance to biotic stresses including *Phytophthora*, nematodes and citrus tristeza virus (CTV) (Soost et al., 1975). Sour orange (*Citrus aurantium* L.) is believed to have been the first citrus rootstock used and although it provides resistance to *Phytophthora*, grafts remain sensitive to CTV (Bar-Joseph and Lee, 1989; Louzada et al., 2008). Worldwide, the damaging effects of CTV have prompted citrus-producing countries to run new rootstock selection trials to replace sour orange.

Scion citrus fruit production and quality is affected by rootstock (Economides and Gregoriou, 1993; Fallahi and Rodney, 1992; Jacquemond et al., 2004; Wheaton et al., 1991; Zekri, 1996; Zekri and Al-Jaleel, 2004), and thus the selection of new rootstock is based on production as well as tolerance to biotic and abiotic stresses. Trifoliate orange (*Poncirus trifoliata*) and its hybrids are commonly selected because of their tolerance to CTV (Garnsey et al., 1987). In Corsica, trifoliate orange has been selected, not only for resistance to CTV, but also because of its capacity to improve clementine yield and quality under acidic soil conditions (Jacquemond et al., 2004).

All citrus rootstocks are polyembryonic and propagated by seeds. A polyembryonic seed produces two or more embryos, one has a sexual origin (zygotic) and the rest are of somatic origin, i.e., nucellar. Thus, a zygotic embryo arises from the fusion of male and female parental gametes, while nucellar seedlings are genetically identical to the maternal parent. In citrus seedlings, the occurrence of zygotic embryos varies with both genotype (Cameron and Frost, 1968; Khan and Roose, 1988) and environmental factors, including tree age and nutrient status, as well as seasonal effects and crop load (Khan and Roose, 1988). Isozyme analyses have shown between 1% and 50% of zygotic plants in polyembryonic citrus seedlings, depending on rootstock genotype (Ashari et al., 1988; Khan and Roose, 1988; Moore and Castle, 1988; Roose and Kupper, 1992; Xiang and Roose, 1988). The frequency of zygotic seedlings in three

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cultivars of trifoliate orange has been shown to range from 0% to 76% (Khan and Roose, 1988), with some plants exhibiting large changes in phenotypical traits. The occurrence of zygotic genotypes was investigated previously in a rootstock trial of 24 trifoliate orange selections (Roose and Traugh, 1988). Isozyme analysis of rootstock bark revealed <4% zygosity and the production of off-type rootstock was generally lower than in nucellar genotypes.

In citrus, chromosome doubling may occur during seed formation and tetraploid seedlings arise spontaneously in nucellar cells (Cameron and Soost, 1968); however, these clones are considered to be genetically identical, since they exhibit the same genome expression profile. The frequency of spontaneous tetraploidy varies from 1% to 11%, depending upon the rootstock analysed (Barrett and Hutchison, 1978; Saleh et al., 2008), and it is considered to be dependent upon the environment (Barrett and Hutchinson, 1982).

Some studies have examined the effects of citrus tetraploid rootstock on fruit production and quality. For example, *P. trifoliata* "tetraploid No. 1" was shown to exhibit more dwarfing and lower fruit quality than diploid forms (Tutberidze and Kalandarishvili, 1975). However, in general, the effects of tetraploid rootstock are poorly documented.

By contrast, the morphological differences between tetraploid and diploid genotypes are well documented. Specifically, tetraploids exhibit thicker and greener leaves, larger fruit and less growth than diploids (Cameron and Frost, 1968; Ollitrault and Jacquemond, 1994). The lower growth rates observed in tetraploid citrus seedlings are associated with reduced rates of whole-plant transpiration (Syvertsen et al., 2000).

In nurseries, citrus seedlings are not 100% true-to-type, despite visual screening to remove off-type seedlings. Unless zygotic and tetraploid rootstocks are identified accurately, non-true-to-type genotypes may lead to errors in performance assessments of varieties and rootstocks. The objective of this study was to determine the impact of off-genotype rootstock on citrus fruit quality and production. Since genetic variants can now be detected via molecular tools, which are easier and much more efficient than visual phenotyping, these methods were used to re-analyse a rootstock selection trial of trifoliate orange cultivars grafted with clementine in 1974. Fruit production was re-assessed with respect to true- and non-true-to-type genotypes.

2. Materials and methods

2.1. Experimental trial

Prior to grafting with clementine "Commune" (*Citrus reticulata* Blanco × *Citrus sinensis* (L.) Osb.) buds of 1-year-old trifoliate orange plants (*P. trifoliata* (L.) Raf.) were selected visually according to homogeneous phenotype. This trial was performed at the INRA-CIRAD Research Station in Corsica, France in 1974. Thirty-two trifoliate orange cultivars were tested (Table 1), with trees planted randomly in nine blocks at a planting distance of 6 m × 4 m. Each bloc contained one tree of each of the 32 rootstock/Clementine combinations. The first fruit production was harvested after 5 years. Harvesting was conducted during the period in which clementine fruits mature, from November to the end of December. The clementine fruit production were harvested and weighed individually for the nine trees of each rootstock/Clementine combination. Measurements of annual yield were taken from 1979 to 1990 (11 years). Harvest date was determined by fruit color and occurred either once or twice, with an interval of 1 or 2 weeks.

Fruit quality was investigated for 5 consecutive years, from 1981 to 1985. In contrast to fruit yield, only four blocks were selected for fruit quality measurements and only 20 fruits per cultivar were assessed from each block. Fruits were chosen which had the same color and medium diameter. Fruits were weighed and hand pressed, and then the juice was weighed to calculate juice percentage (JP) and juice density.

Total soluble solids (TSS in °Brix) were measured using a hand-held ATC-1E ATAGO refractometer. Following decantation, a 5 ml translucent fraction of the juice was weighed to assess titratable acidity (TA) using a DL25 Mettler titrator, according to the AOAC method (0.1 N NaOH, at pH 8.1). Results were expressed in grams of anhydrous citric acid/100 g juice. Maturity was assessed using the ratio of TSS/TA.

Table 1
The 32 trifoliate orange (*Poncirus trifoliata* (L.) Raf.) cultivars in the field trial.

#.	Common name	ICVN[a]	Origin
1	Rubidoux	110126	South Africa
2	Boufarik	110507	Algeria
3	Menager	110116	France
4	Rusk	110437	USA (Texas)
5	Ferme blanche	110435	Algeria
6	Jacobsen	110107	USA (California)
7	English	110097	South Africa
8	SEAB	110438	Algeria
9	Beneke	110436	USA
10	Pomeroy	110081	USA (California)
11	Rusk	110443	USA (Texas)
12	Christian	110338	South Africa
13	Ferme blanche	110087	Algeria
14	Kryder	110446	USA (Florida)
15	Luisi	110088	France
16	Rusk	110082	USA (Texas)
17	Brazil	110510	Brazil
18	Argentine	110505	Argentine
19	Morocco	110511	Morocco
20	Boufarik	110506	Algeria
21	Morocco	–	Morocco
22	Beneke	110083	USA
23	Kryder	110110	South Africa
24	Christian	110084	South Africa
25	Boufarik	110508	Algeria
26	Luisi	110448	France
27	Christian	110085	USA
28	Menager	110449	France
29	Kryder	110108	USA (Florida)
30	Christian	110447	USA
31	Pomeroy	110442	USA (California)
32	Morocco	110512	Morocco

[a] International Citrus Variety Numbering.

2.2. Identification of tetraploid and zygotic seedlings

The ploidy status of trees was determined by flow cytometry using bark or leaf samples obtained from rootstock sprouts, as described by Froelicher et al. (2007). For identification of zygotic seedlings, DNA was extracted from bark (1 cm^2 sample below the graft) or leaves collected from rootstock buds, as described previously (Doyle and Doyle, 1987), and adapted for citrus (Cabasson et al., 2001). DNA samples were extracted from the inner layer of bark tissue, which contains live tissue. DNA was then amplified by PCR using five Single Sequence Repeat (SSR) primers (mCrCIR08A03, mCrCIR07D05, mCrCIR01E02, Ci07C09 and Ci01H05; Froelicher et al., 2007), which exhibit heterozygosity in trifoliate orange. PCR products were separated by vertical denaturing polyacrylamide gel electrophoresis (7 M urea) in 0.5× TBE buffer (Tris 10 mM, 45 mM boric acid and 0.5 M EDTA [pH 8]). PCR products were visualised by silver staining, as described previously (Beidler et al., 1982).