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## IDENTIFICATION OF ZYGOTIC AND NUCELLAR SEEDLINGS IN *CITRUS* INTERPLOID CROSSES BY MEANS OF ISOZYMES, FLOW CYTOMETRY AND ISSR-PCR

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**Abstract:** ‘Milam’ (a purported hybrid of *Citrus jambhiri* Lush) + ‘Femminello’ lemon (*Citrus limon* L. Burm. f.) allotetraploid somatic hybrids were used as pollen parents in interploid crosses with diploid ‘Femminello’ lemon to achieve *mal secco* tolerance in different populations of seedless triploid lemon types with good fruit quality. A total of 137 plantlets were obtained and subjected to screening experiments, in order to distinguish zygotic embryos from nucellars. Here we report on and discuss the results obtained with three techniques: flow cytometry, isozyme analysis and ISSR-PCR (the inter-simple sequence repeats-polymerase chain reaction). ISSR-PCR resulted to be a very efficient and reliable technique for the identification of zygotic plantlets.

**Key Words:** Somatic Hybrids, Backcrosses, Zygotic Embryos, ‘Femminello’ Lemon

### INTRODUCTION

*Mal secco* is a systemic fungal disease of *Citrus* caused by *Phoma tracheiphila* (Petri) Kantsch & Gik, and it occurs widely in the Mediterranean and Black Sea areas. The development of improved lemon cultivars with tolerance or resistance to *mal secco* is an important breeding objective in this area. We tested the offspring obtained from a backcross between the allotetraploid somatic hybrid ‘Milam’ (a purported hybrid of *Citrus jambhiri* Lush) + ‘Femminello’ lemon (*Citrus limon* L. Burm. f.) and diploid ‘Femminello’ lemon. Our goal was to generate seedless triploid lemon types tolerant to the *mal secco* disease with good fruit quality.

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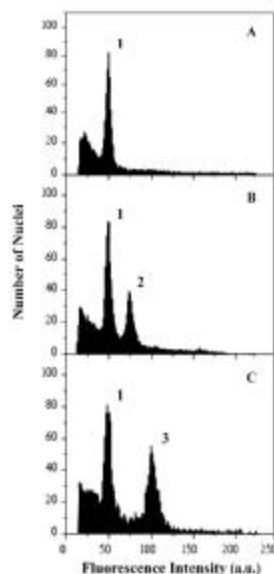


Fig. 1. Flow cytometry analysis of PI stained leaf nuclei in suspension; A) DNA fluorescence histogram of diploid (2C) “Femminello” showing only one peak (1) corresponding to G1 nuclei; B) a triploid cross (3C) of Femminello X (Milam+Femminello): two peaks are visible as a diploid reference (channel 50) and a triploid hybrid, which are numbered as (1) and (2), respectively; C) DNA fluorescence histogram of the parental somatic hybrid (M+F): two peaks are shown as a diploid internal reference (channel 50 approx.) and a tetraploid somatic hybrid (channel 100 approx.), numbered as (1) and (3), respectively.

Many *Citrus* species reproduce apomictically by seed, through nucellar embryony: these embryos are genetically identical to the mother plant, and develop within the same embryo-sac together with the zygotic embryo. Therefore, in order to distinguish zygotic seedlings from the nucellars, specific methods are needed. In order to analyze a total of 137 seedlings regenerated from ‘Femminello’ x (‘Milam’+‘Femminello’) backcrosses, we tested three techniques: isozyme analysis, flow cytometry and ISSR-PCR.

## MATERIALS AND METHODS

### Plant material

The allotetraploid somatic hybrid ‘Milam’+‘Femminello’ was obtained by protoplast fusion [1], and its pollen was collected from flowers at pre-anthesis stage and vacuum-dried at room temperature. 105 days after pollination, fruits were harvested and the embryo-rescue technique was applied *in vitro* to save zygotic embryos [2]. Plantlets grown on MT agarized medium [3] on magenta

vessels, were transplanted to Jiffy pots and successively transferred to a basal-heating bench to stimulate vegetative growth.

#### **Flow cytometry**

Isolation and staining of nuclei was carried out as previously described [4]. PI stained nuclei suspensions were analyzed with a FACStarPLUS flow cytometer and sorter (Becton Dickinson, San José, USA) equipped with an argon ion laser tuned at  $\lambda=514$  nm adjusted to a 100 mW power output. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei was collected through a 620 nm long-pass filter and converted on 1024 ADC channels. The instrument amplification was adjusted so that the peak corresponding to single CRBC (Chicken Red Blood Cell) nuclei was positioned approximately at channel 400 (corresponding to channel 100 on 256-channel histograms).

#### **Isozyme analysis**

Isozyme banding patterns were analyzed using crude leaf extracts: MDH (malate dehydrogenase), PER (Peroxidase), PGI (phosphoglucose isomerase) and PGM (phosphoglucomutase) were separated using horizontal starch gel electrophoresis on 10% starch gels and a pH 5.7 histidine-citrate buffer [5-7]. Electrophoresis was carried out at 4°C for 3 h at 45 mA constant current. Staining recipes were from the literature [8].

#### **ISSR-PCR**

DNA extraction and ISSR-PCR experiments were carried out as previously described [9] with some modification: a total of 26 primers (Invitrogen, Table 1) [10] were used and DNA amplification was performed in a 96-well GeneAmp PCR System 9700 (Applied Biosystems).

### **RESULTS AND DISCUSSION**

Of a total of 137 plantlets obtained from 'Femminello' x 'Milam+Femminello' crosses, 47 were revealed to be triploid hybrids by flow cytometry (Fig. 1).

Four isozymes were used for early screening: *Mdh*, *Per* and *Pgm* did not reveal locus segregation, and were consequently not useful in the detection of the two classes of embryos. For *Pgi*, 'Milam'+ 'Femminello' somatic hybrid was heterozygous (genotype WSSS) and the 'Femminello' lemon heterozygous (genotype WS) [1]. 15 seedlings turned out to be hybrids (genotype SSS), all the other plantlets showed a banding pattern similar to both parents and therefore it was impossible to distinguish between zygotic (genotypes WWS or WSS) and nucellar plantlets (genotype WS) (Fig. 2).

ISSR-PCR analysis was carried out on 10 plantlets, 8 of which were triploids (zygotic seedlings) and 2 diploids (nucellar seedlings), to test the efficacy of this technique in discriminating zygotic genotypes from nucellars. We scored a total

Tab. 1. ISSR primers used in this experiment and polymorphic fragments obtained.

Primers	Sequence	Polymorphic fragments		Primers	Sequence	Polymorphic fragments	
		M+F*	F*			M+F*	F*
#1	HVH(TG) <sub>7</sub> T	--	--	#14	DBD(AC) <sub>7</sub>	1	1
#2	HVH(TCC) <sub>5</sub>	1	2	#15	(TCC) <sub>5</sub> RY	4	1
#3	HVH(CA) <sub>7</sub> T	1	1	#16	(CA) <sub>8</sub> RG	1	--
#4	BDB(CA) <sub>7</sub> C	--	--	#17	(CA) <sub>8</sub> RY	--	--
#5	BDB(TCC) <sub>5</sub>	1	3	#18	(GA) <sub>8</sub> YT	2	1
#6	(GA) <sub>8</sub> YG	1	1	#19	(GA) <sub>8</sub> YC	2	1
#7	(GT) <sub>8</sub> YC	1	2	#20	(GT) <sub>8</sub> YG	1	--
#8	(AG) <sub>8</sub> YT	1	--	#21	VAV(GT) <sub>7</sub> G	2	2
#9	(AG) <sub>8</sub> YC	3	3	#22	(AC) <sub>8</sub> YT	2	3
#10	(AG) <sub>8</sub> YG	1	--	#23	(AG) <sub>8</sub> T	2	2
#11	(AC) <sub>8</sub> YA	3	2	#24	(AG) <sub>8</sub> G	--	--
#12	(AC) <sub>8</sub> YG	2	--	#25	(GA) <sub>8</sub> T	1	--
#13	DBD(AC) <sub>7</sub> A	--	--	#26	(GA) <sub>8</sub> C	1	1

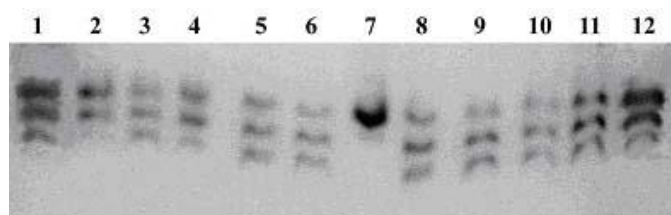


Fig. 2. PGI isozyme banding pattern of 'Milam + Femminello' (M+F), 'Femminello' (F) and their tested offsprings [Fx(M+F)]. Lane 1: F, lane 12: M+F, lanes 2-11: Fx(M+F) tested offsprings. Lane 7: zygotic hybrid (genotype SSS).

number of polymorphic bands of 34 for the 'Milam'+ 'Femminello' somatic hybrid and 26 for 'Femminello' lemon (Tab. 1). The 2 diploid genotypes (nucellar seedlings) showed a banding pattern equal to that of the mother plant ('Femminello' lemon); among the 8 triploid hybrids tested, 2 genotypes showed a banding pattern equal to that of 'Femminello' lemon, while 6 genotypes displayed locus segregation, confirming their hybridity. Fig. 3 shows the banding pattern obtained from primer #12, in which only 4 hybrids show locus segregation (lanes 3, 6, 10, and 12). The other four hybrids (lanes 4, 5, 7 and 11) showed locus segregation with other primers.

Flow cytometry was able to very easily and accurately distinguish triploid genotypes (zygotic seedlings) from diploids (nucellars), and it is doubtless a very successful and reliable technique for detecting zygotic hybrids in interploid crosses, in which the different ploidy helps the identification of the true hybrids.

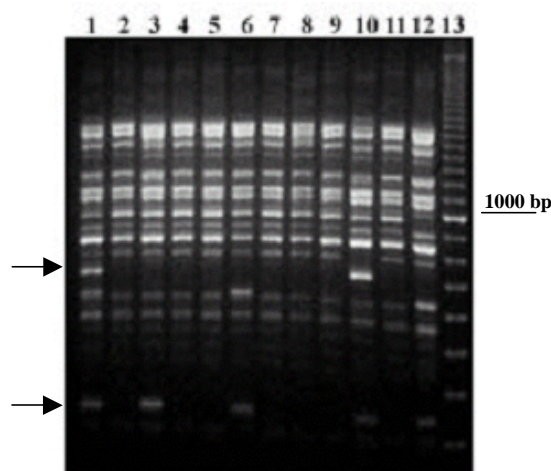


Fig. 3. Specific markers generated by ISSR-PCR (arrows), primer #12 (Table 1). Lane 1: Milam+ Femminello (M+F), lane 2: Femminello (F), lanes 3-12: Fx(M+F) tested offspring, lane 13: 100 bp PCR molecular ruler (Biorad). Lanes 3-7 and 10-12: 3n genotypes, lanes 8-9: 2n genotypes.

The isozymes analysis also has some limitations. In fact their expression can be influenced by environmental conditions and the developmental stage of the tissue type, and the number of polymorphic loci in the genome is very small. In fact, as mentioned above, from a total of 47 triploids (detected with flow cytometry), we could distinguish only 15 hybrids with isozyme analysis.

ISSR-PCR requires a PCR amplification of genomic DNA using a single primer composed of a microsatellite sequence anchored at the 5' or 3' end by one to three arbitrary nucleotides [11]. It is a useful technique, highly reproducible and requires only 10-30 ng of DNA template, allowing a very early screening of the progeny. The resolution of PCR products can be performed on agarose gel and results are available within 9 hours (starting with isolated DNA). ISSR-PCR has been successfully employed in fingerprinting analyses of many crop species, *Citrus* included [10], and in the characterization of *Citrus* somatic hybrids [9]. It is known that this technique is not very informative because of its dominant nature (i.e. heterozygous and homozygous genotypes cannot be distinguished), which requires a high number of loci to be scored, and therefore a great number of primers must be tested. However, this limitation arises in the case of crosses between phylogenetically very close *Citrus* genotypes, where it is difficult to distinguish between zygotic and nucellar embryos. In our experiments, we crossed two genotypes genetically very close to each other: in fact, the pollen parent (the 'Milam'+ 'Femminello' somatic hybrid) shares a gene pool very similar to that of the mother parent ('Femminello' lemon). This may explain

why the ISSR-PCR procedure was unable to reveal the hybrid nature of 2 of the tested triploid hybrids.

Considering that flow cytometry is an efficient method for detecting zygotic genotypes only in interploidy crosses and taking into account the great limitations of isozyme analysis, the ISSR-PCR technique, despite its above-cited disadvantages, could be a useful method for discriminating zygotic embryos from nucellars in the genetic improvement of *Citrus*. In our hands, ISSR-PCR showed to be speedy, highly reproducible and requiring low quantities of DNA, making it very useful for the analysis of *Citrus* hybrids.

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