

## GENETIC PROOF OF THE OCCURRENCE OF MONO AND DIZYGOTIC HYBRID TWINS IN *Citrus* ROOTSTOCKS

Herculano Penna Medina Filho, Rita Bordignon, Rosa Maria Lizana Ballve and  
Walter José Siqueira

### ABSTRACT

Nucellar cells which develop within the female gametophyte give rise to adventitious embryos [Strassburger (*Jenaische Ztschr. fur Naturw.* 12: 647-667, 1878)]. Monozygotic polyembryony by cleavage of the sexual embryo was the explanation of Frost (*Current Sci.*, Special Number, *Genetics*: 24-27, 1938) for 10 pairs of phenotypically identical twin hybrids from single seeds. A third type of polyembryony was suggested by Bacchi (*Bot. Gazet.* 105: 221-225, 1943) in *Citrus paradise* after cytological observations of two gametophytes in a single ovule that would lead to dizygotic, non-identical twins.

This investigation presents genetic proof of the occurrence of the three types of polyembryony by genetic analysis of *Pgi-1*, *Pgm-1*, *Prxa-1*, *Got-1* and *Got-2* isozyme loci in single seed plants derived from controlled crosses.

Nearly 1000 seedlings were genotyped revealing 633 hybrids. Among them, 12 twin pairs and one triplet were identified: the *Citrus sunki* x *Citrus aurantium* triplet and, of the twins (*C. sunki* x *Citrus limonia*, *C. sunki* x *C. aurantium*, *C. sunki* x *Poncirus trifoliata*), 10 monozygotic and two (*C. sunki* x *C. aurantium* and *C. limonia* x *C. aurantium*) dizygotic. By ascertaining the contribution of the female haploid genotype, it was possible to conclude that genetically distinct female gametes participated in the formation of each plant of the two dizygotic twin pairs, thus proving the reproductive realization of Bacchi's earlier cytological observation and ruling out other mechanisms that would generate non-identical twins.

The seed containing the *C. limonia* x *C. aurantium* dizygotics also produced a nucellar plant. This phenomenon, infrequent but nonetheless important, has obvious implications for the evolution of the genus and for citrus breeding.

## INTRODUCTION

Nucellar polyembryony is a well known phenomenon that constitutes the basis of commercial citrus clones budded onto genetically uniform rootstocks. It was first studied in detail by Strassburger (1878). Outside the embryo sac, some nucellar cells in the vicinity of the micropyle undergo successive mitoses and project themselves into the gametophyte where they complete development and give rise to adventitious embryos (Osawa, 1912). After ripening and germination of the seed, they result in several plants, the so-called nucellar clones. Such plants, due to their sporophytic origin, are genetically identical to the mother plant.

Another type of polyembryony in citrus was suggested by Frost (1926, 1938) after the observation, among 1200 hybrids, of 10 cases in which two phenotypically similar hybrids were derived from single seeds. Supposedly, the plants were formed by an early cleavage of the sexual embryo, resulting in monozygotic identical twins.

Yet a third type of polyembryony was cytologically described by Bacchi (1943) in *Citrus paradisi* and Banerji (1954) in *Citrus grandis* where cases of two gametophytes were observed within single ovules. Probably, through normal fertilization, the latter would lead to dizygotic polyembryony and the development of non-identical twins.

Traub and Robinson (1937), Swingle (1943), Brieger and Moreira (1945), Moreira *et al.* (1947) and Ozasan and Cameron (1963) also observed multiple twin hybrids from single seeds. However, it is unknown whether these were cases of mono or dizygotic twins or both. If it is quite difficult in cross progenies to distinguish nucellar from zygotic seedlings, except when *Poncirus trifoliata* is used as male parent, it is even more difficult to determine among zygotics whether twin hybrids are of mono or dizygotic origin. Usual discriminative crosses with trifoliolate pollen are of no value in such cases since mono and dizygotic twins would both be trifoliated.

In this paper we present genetic proof of the occurrence of the three types of polyembryony in citrus: nucellar, mono and dizygotic. Furthermore, it is shown that, in fact, they may result in viable seedlings that can be identified and unequivocally distinguished from each other by analysis of isozyme loci of single seed progenies from controlled crosses.

## MATERIAL AND METHODS

Rootstock plants used in this study are maintained in the Centro de Citricultura Sylvio Moreira of Instituto Agronômico de Campinas in Cordeirópolis, SP. *C. sunki* x *C. limonia* and the reciprocal, *C. sunki* x *C. aurantium*, *C. sunki* x *P. trifoliata*, *C. limonia* x *C. aurantium*, *P. trifoliata* x *C. aurantium* and *P. trifoliata* x *C. sunki* crosses were made according to usual procedures of emasculation, pollination and bagging. After fruit

ripening, seeds were collected, washed, surface sterilized, testa and integument removed and germinated in moist Petri dishes at room temperature. At the onset of germination, the embryos were separated, transplanted into speedling flats and taken to a greenhouse to promote rapid growth. Crosses and seed handling procedures adopted are described in Bordignon *et al.* (1990).

Thirty to ninety day old seedlings were subjected to isozyme analysis by horizontal starch gel electrophoresis and scored as hybrids or nucellars, fully detailed in Ballve *et al.* (1991).

The *C. sunki* x *P. trifoliata* progeny were only scored morphologically as uni or trifoliolate, except for the pair of twin plants which were subsequently subjected to isozyme analysis to ascertain their reproductive origin. All seedlings from other progenies were genotyped for five isozyme loci: *Pgi-1*, *Pgm-1*, *Prxa-1*, *Got-1* and *Got-2*. Segregation and recombination of alleles of these loci allow for the identification of hybrids, although a small percentage of them might be confounded with nucellars (see discussion in Ballve *et al.*, 1991) which would be, nevertheless, irrelevant to the results reported here.

## RESULTS AND DISCUSSION

Almost a thousand seedlings were produced and scored from controlled pollinations, and 633 were identified as hybrids. The great majority (91%) emerged from monoembryonic seeds and 4.32% from polyembryonic seeds where the additional embryo(s) was adventitious. The adventitious embryos, in turn, did not always originate from polyembryonic seeds that had a zygotic embryo. In fact, 51% of nucellars were from polyembryonic seeds bearing only nucellars.

A rather rare but interesting class of seedlings, twins and triplet hybrids was also found, evidently originating from single seeds. The controlled crosses and subsequent isozyme analysis of the progenies identified 633 hybrids, including 12 twin pairs and one triplet, as listed in Table I.

There are putatively two ontogenetically different types of twins. The first, according to Frost's theory, is originated by fission of the zygote and the second, following Bacchi's (1943) cytological observations, by the occurrence of two megagametophytes in a single ovule. These would give rise, respectively, to mono (identical) and dizygotic (non-identical) twins.

The underlying reasoning in the identification of hybrids by isozyme analysis lies in the segregation of the parental alleles (Table II) and the ensuing recombination observed in the hybrids, as contrasted with the identical seed parent genotype of the nucellar clones. For example, in the cross *C. sunki* (♀) x *C. limonia* (♂), those seedlings with at least one of the recombinant genotypes FS, MM, FS, and FS/MM, respectively for *Pgi-1*, *Prxa-1*, *Got-1* and *Got-2*, are certainly hybrids.

Table I - Number of genotypically identical and non-identical twin pairs and triplets observed among F<sub>1</sub> hybrid progenies of citrus rootstock crosses.

Cross	Monozygotic twins	Monozygotic triplet	Dizygotic twins	Total hybrids
<i>C. sunki</i> x <i>C. limonia</i>	4	0	0	221
<i>C. sunki</i> x <i>C. aurantium</i>	5	1	1	122
<i>C. sunki</i> x <i>P. trifoliata</i>	1	0	0	88
<i>C. limonia</i> x <i>C. sunki</i>	0	0	0	29
<i>C. limonia</i> x <i>C. aurantium</i>	0	0	1	66
<i>P. trifoliata</i> x <i>C. aurantium</i>	0	0	0	87
<i>P. trifoliata</i> x <i>C. sunki</i>	0	0	0	20
Total	10	1	2	633

Table II - Genotypes of five isozyme loci in the parental citrus rootstocks.

Parents	<i>Pgi-1</i>	<i>Pgm-1</i>	<i>Prxa-1</i>	<i>Got-1</i>	<i>Got-2</i>
<i>C. sunki</i>	FF	FF	FM	SS	FF/MM
<i>C. limonia</i>	FS	FF	MM	FS	FS/MM
<i>C. aurantium</i>	WS	FS	FS	SS	FM/MM
<i>P. trifoliata</i>	FS	PM	FF	PM	MT/SS

It is evident that hybrids of different genotypes can be produced from a single cross. This fact constitutes the basis for distinguishing identical from non-identical twins. If twin hybrid seedlings are different from each other with respect to one or more scored loci, they are certainly non-identical, dizygotic twins. On the other hand, if they have the same overall genotype at the segregating loci, it is very likely that they were identical, monozygotic twins. The probability of dizygotic twins having the same genotype and thus being erroneously considered monozygotic is quite small if one considers the number of discriminating hybrid genotypes for each cross. For instance, in the previously mentioned example of *C. sunki* x *C. limonia* there are four segregating loci pairs (*Pgi-1*, *Prxa-1*, *Got-1*, *Got-2*), three with two different alleles. In such a cross, 15 of 16 are

different hybrid discriminating genotypes (one is the same as the maternal and would ordinarily be disregarded). Thus, in this case, if a pair of twins presents the same genotype over these four loci, they are very likely monozygotic, the probability of being similar by chance segregation being  $15/(15)^2 = 6.6\%$ , that is, quite small.

As presented in Table I, ten pairs of monozygotic twins, one triplet and two dizygotic twin pairs were observed among 633 hybrids. Their genotypes are indicated in Table III and Figure 1 shows some of them, their leaves and isozyme gels. It is not clear if the pollen parent can influence the occurrence of such monozygotic twins. The frequency of hybrid zygotes of *C. sunki* involving *C. limonia*, *C. aurantium* and *P.*

Table III - Isozyme genotypes of identical (i) and non-identical (ni) twin (2) and triplet (3) hybrids observed in single polyembryonic seeds with (+) or without (-) an additional nucellar embryo.

Cross	<i>Pgi-1</i>	<i>Pgm-1</i>	<i>Prxa-1</i>	<i>Got-1</i>	<i>Got-2</i>
<i>C. sunki</i> x <i>C. limonia</i>					
(i) (2) (-)	FF	FF	FM	FS	FS/MM
(i) (2) (-)	FF	FF	MM	SS	FF/MM
(i) (2) (-)	FF	FF	MM	SS	FS/MM
(i) (2) (-)	FF	FF	FM	FS	FF/MM
<i>C. sunki</i> x <i>C. aurantium</i>					
(ni) (2) (-)	FS	FF	FM	SS	FF/MM
	WF	FS	FS	SS	FM/MM
(i) (3) (-)	WF	FS	MS	SS	FF/MM
(i) (2) (-)	FS	FF	FF	SS	FM/MM
(i) (2) (-)	FS	FS	MS	SS	FM/MM
(i) (2) (-)	WF	FS	FM	SS	FM/MM
(i) (2) (-)	FS	FS	MS	SS	FM/MM
(i) (2) (-)	FS	FF	FF	SS	FF/MM
<i>C. sunki</i> x <i>P. trifoliata</i>					
(i) (2) (-)	FS	FP	FF	PS	FT/MS
<i>C. limonia</i> x <i>C. aurantium</i>					
(ni) (2) (+)	WS	FF	MS	FS	FS/MM
	FS	FF	MS	SS	FF/MM

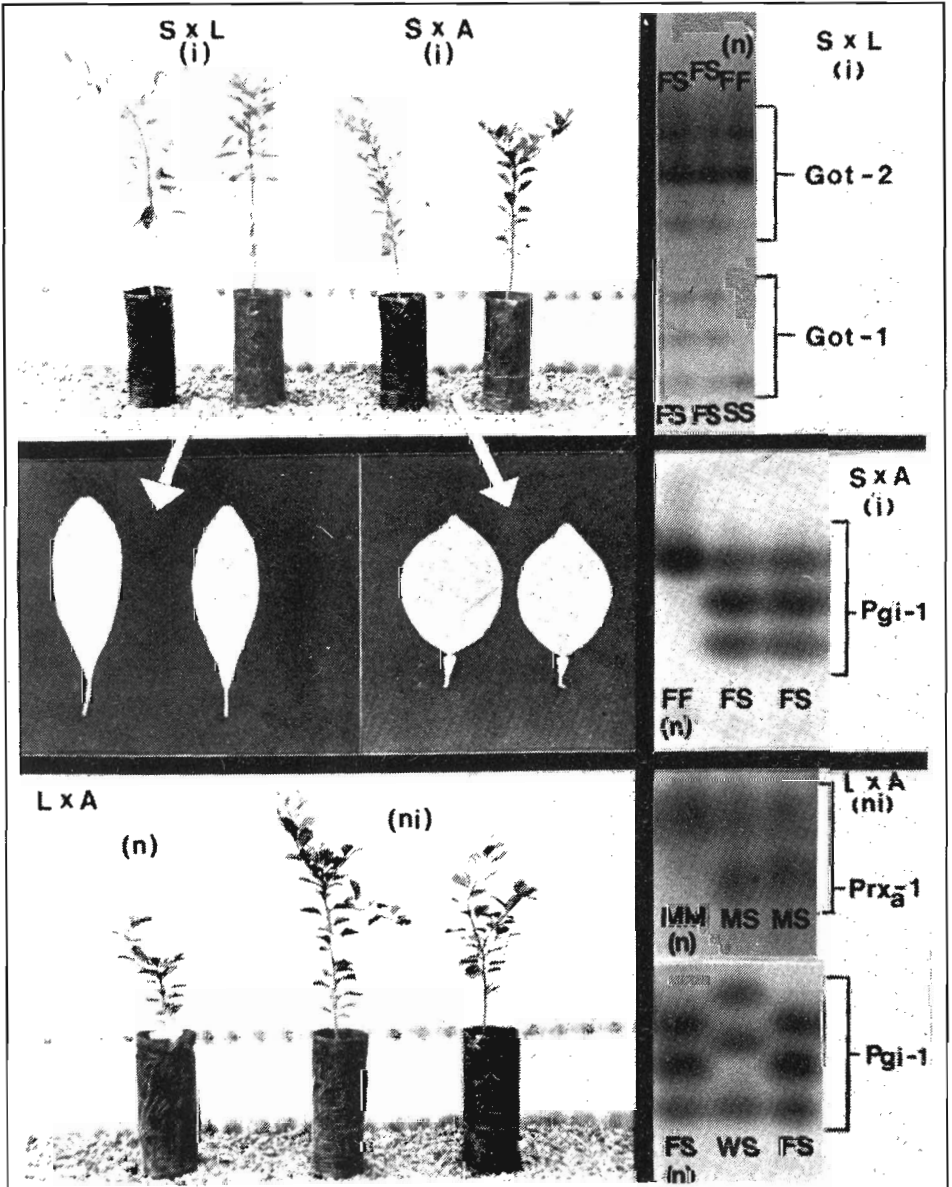


Figure 1 - Plants, leaves and isozyme genotypes of identical (i), non-identical (ni) hybrid twins and nucellar plants (n) of crosses involving *Citrus sunki* (S), *Citrus limonia* (L) and *Citrus aurantium* (A). Plants and leaves of *S x L* (i) and *S x A* (i) are quite similar and have the same genotypes for *Got-1* (FS), *Got-2* (FS/MM though indicated as FS) and *Pgi-1* (FS), respectively. *L x A* single seed plants are shown where one is L nucellar (n) and the other two are ni. Their MS genotypes for *Prxa-1* indicate only that they are hybrid twins. The correspondent WS and FS genotypes for *Pgi-1* prove that they are non-identical.

*trifoliata*, that underwent an early cleavage was 1.8%, 5.3% and 1.2%, respectively (Table I). Since the triplet individuals observed in the progeny of the *C. sunki* x *C. aurantium* cross were genetically identical and thus monozygotic, fission in that zygote must have occurred twice. The monozygotic nature of the triplet supports Bacchi's (1943) belief that more than two hybrid seedlings in the same seed should be ascribed to cleavage alone or to the combination of cleavage with the presence of two gametophytes in the same ovule, since more than two gametophytes in the same ovule would be highly improbable.

The cytologically demonstrated cases of two gametophytes developing in the same ovule does not exclude the possibility that non-identical twins might have their origin by other mechanisms as well. Indeed, in a comprehensive review on polyembryony, Webber (1940) refers to several instances, in other plant genera, in which plural seedlings of gametic progenies may be traced to an additional fertilization of synergids or antipodal cells. Since these cells come originally from a single megaspore, they are, consequently, genetically identical and, therefore, seedling differences necessarily must be allotted to the male gametes.

In the light of the above considerations, the two pairs of dizygotic twins observed deserve a finer analysis to permit speculation as to whether, in each case, they came from a single megaspore or from two independent ones.

The controlled crosses and the codominance of the isozyme alleles turn this question particularly amenable. Consider, for instance, the cross FS (♀) x WS (♂) for locus *Pgi-1*. In a possible resulting hybrid WS, the S allele had to come from the female side because obviously the W allele was transmitted by the pollen. Following this rationale, one can deduce the whole set of alleles contributed by the seed parent to each individual of the twin pair. It turns out that for the *C. sunki* x *C. aurantium* dizygotic twins (Table III), the female contribution for the first individual was F, F, M, S, F and for the second, F, F, F, S, F, respectively for *Pgi-1*, *Pgm-1*, *Prxa-1*, *Got-1* and *Got-2*. Similarly, for the *C. limonia* x *C. aurantium* case, the haploid female genotypes were S, F, M, F, S and F, F, M, S, F respectively, for the first and second individuals. Thus, it is uncontestable for both twin pairs that in the formation of each of their hybrids genetically distinct female gametes participated. As expected, this also holds true for the male gametes. Hence, such twins constitute genetic proof of the reproductive realization of Bacchi's (1943) and Banerji's (1954) cytological observations.

The seeds containing twin and triplet hybrids, except for one instance, did not have additional nucellar embryos. The exception (*C. limonia* x *C. aurantium*, Table III) is an interesting case for the genetic diversity inwardly hosted by that single ovule: two different embryo sacs, fertilized by two different male gametes plus another distinct embryo, the nucellar one, growing together into viable, normal seedlings. From a practical breeding standpoint, it is important to be aware and to fully understand such events and their genetic implications. Likewise, such elaborate reproductive strategy

assuring not only the preservation of an adapted maternal genotype through the nucellar embryo, but also concurrently allowing the generation of two distinct interspecific hybrids through the dizygotic twins carried in a single seed transcends the status of a mere biological curiosity for should it occur in nature it would have important evolutionary significance.

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### RESUMO

Células nucleares se desenvolvendo no interior do gametófito dão origem a embriões adventícios [Strassburger (*Jenaische Ztschr. für Naturw* 12: 647-667, 1878)]. Poliembriõnia monozigótica por fissão do embrião sexual foi a explicação de Frost (*Current Sci., Special Number, Genetics*: 24-27, 1938) para 10 pares de híbridos gêmeos fenotipicamente idênticos originados de sementes individuais. Um terceiro tipo de poliembriõnia foi sugerido por Bacchi (*Bot. Gazet.* 105: 221-225, 1943) em *Citrus paradisi* após observações citológicas de dois gametófitos em um único óvulo os quais levariam a formação de gêmeos dizigóticos, não idênticos.

Essa investigação apresenta provas genéticas da ocorrência dos três tipos de poliembriõnia através da análise genética dos locos isoenzimicos *Pgi-1*, *Pgm-1*, *Prxa-1*, *Got-1* e *Got-2* em plantas oriundas de sementes individuais derivadas de cruzamentos controlados.

Cerca de 1000 plantas foram analisadas genotipicamente, determinando-se 633 híbridos. Entre esses híbridos, identificaram-se 12 pares de gêmeos e 1 trigêmeo. O trigêmeo (*Citrus sunki* x *Citrus aurantium*) e 10 pares de gêmeos (*C. sunki* x *Citrus limonia*, *C. sunki* x *C. aurantium*, *C. sunki* x *Poncirus trifoliata*) são monozigóticos enquanto que os dois restantes (*C. sunki* x *C. aurantium*, *C. sunki* x *C. limonia*) são dizigóticos. Uma vez determinadas as contribuições alélicas dos gametas femininos foi possível concluir que na formação de cada planta dos dois pares de gêmeos dizigóticos participaram gametas femininos geneticamente distintos. Isso prova que as observações de Bacchi (*Bot. Gazet.* 105: 221-225, 1943) de dois gametófitos dentro de um único óvulo podem realmente resultar em plantas normais. Também, descartam-se outros mecanismos que teoricamente poderiam gerar gêmeos não idênticos.

A semente que originou os gêmeos dizigóticos *C. limonia* x *C. aurantium* produziu também uma planta nucelar. Essa elaborada estratégia reprodutiva, rara, porém importante, tem óbvias implicações na evolução e melhoramento de citros.

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