

## Characterisation of multiple embryos in almond

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### Abstract

Multiple embryos within the same tegument occur spontaneously in certain almond [*Prunus dulcis* (Mill.) D.A. Webb] cultivars including 'Nonpareil' and 'Mission'. Seedlings from the same polyembryonic seed are frequently viable, though often one of the seedlings shows weak growth and develops poorly. These dwarf seedlings have been characterised in a previous work as haploid. In this work, we have characterised 450 seedlings from polyembryonic seeds of the Californian cultivar 'Nonpareil', and 100 seedlings from polyembryonic seeds of the Spanish selections 'D97396' and 'D97407', including their germination and later growth. Isozyme and Simple Sequence Repeat markers were used to genetically analyse seedling genetic structure. In addition, individual mitotic karyotypes were determined following staining of root tips. The percentage of twin embryos showing aberrant growth was approximately 25% with mortality rates of about 90%. Results suggested the sexual origin of both embryos. Seedlings from the same seed are genetically identical with a similar growth, though often one of the seedlings from the secondary embryo loses part of the genomic contents and becomes an aneuploid plant and showed a weak development. The use of this material in almond breeding is also discussed.

**Key words:** *Prunus dulcis*, polyembryony, aneuploidy, molecular markers, isozymes, SSRs, karyotype, breeding, germplasm.

### Resumen

#### Caracterización de embriones múltiples en almendro

La aparición de embriones múltiples dentro del mismo tegumento es un fenómeno espontáneo que ocurre en semillas de algunas variedades de almendro [*Prunus dulcis* (Mill.) D.A. Webb] como 'Nonpareil' o 'Mission'. Las plántulas procedentes de estas semillas poliembriónicas son viables, aunque a menudo muestran un crecimiento más débil y retrasado. Estas plántulas enanas han sido caracterizadas como haploides en trabajos previos. En este trabajo hemos estudiado 450 plántulas procedentes de semillas poliembriónicas de la variedad californiana 'Nonpareil' y 100 plántulas procedentes de semillas poliembriónicas de las selecciones españolas 'D97396' y 'D97407', observando su germinación y posterior crecimiento. Estas plántulas han sido caracterizadas molecularmente mediante marcadores isoenzimáticos y microsatélites. Además, hemos identificado los cariotipos mitóticos en los ápices radiculares. El porcentaje de semillas con embriones múltiples donde una de las plantas procedente de uno de estos embriones mostró un crecimiento aberrante fue del 25%, con un índice de mortalidad de las plántulas aberrantes procedentes de estos embriones del 90%. Se ha observado un origen sexual de estos embriones múltiples. En algunos casos estos dos embriones son genéticamente iguales y dan lugar a dos plantas idénticas, mientras que en el caso de semillas con embriones aberrantes el embrión secundario pierde parte de la dotación genómica, dando lugar a plantas aneuploides junto a las plantas diploides procedentes del embrión primario. El uso y utilización de este material en los programas de mejora genética del almendro se discute también en este trabajo.

**Palabras clave:** *Prunus dulcis*, poliembriónia, aneuploidía, marcadores moleculares, isoenzimas, SSRs, cariotipo, mejora genética, germoplasma.

### Introduction

Polyembryony or presence of multiple embryos in the same tegument, is a phenomenon that occurs

spontaneously in seeds from certain plants. According to Maheswari and Sachar (1963), this phenomenon is more frequent in gymnosperms than in angiosperms. In fruit tree crops this phenomenon occurs in kiwi (Crete, 1944), mango (Sobrinho and Gurgel, 1953), peach (Toyama, 1974), almond (Gulcan, 1975) and especially in different citrus crops

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(Leroy, 1947; Sobrinho and Gurgel, 1953; Koltunow *et al.*, 1996).

The existence of multiple embryos has been described for different almond cultivars including 'Nonpareil', 'Price', 'Sonora', 'Jiml', «Carmel», 'Johlyn' and 'Mission' (Kester and Gradziel, 1996; Micke *et al.*, 2000). The percentage of seeds with multiple embryos varies greatly, and is strongly affected by environmental conditions (Micke *et al.*, 2000). In the mature state, these seeds are distinguished by the presence of more than one embryo in the same tegument, orientated from the basal to the distal region and commonly called «twin seeds». The plants growing from the same polyembryonic seed are viable, although in some cases one of the plants reveals a weak development with deformed leaves. Gulcan (1975) characterised these aberrant plants as haploid, although the results were not very conclusive.

The causes of polyembryony are not clear. Although Leroy (1947) attributed it to hormonal disorders, most hypotheses tend to associate it with genetic causes (Maheswari and Sachar, 1963; Castle and Meinke, 1993). In different lupine cultivars, Atabekova (1957) observed different tendencies in polyembryonic seed production. Most research carried out supports an apomictic origin (early embryos' somatic mother tissue) of the polyembryos. This occurs in the case of mango (Sobrinho and Gurgel, 1953) and most *Citrus* species (Cameron and Soost, 1979; Koltunow *et al.*, 1995; 1996). On the other hand, a possible sexual origin has been described with the fertilisation of one or more ovules in some *Citrus* species (Cameron and Garber, 1968; Cameron and Soost, 1979), in soybean (Kenworthy *et al.*, 1973) and in some strawberry species (*Fragaria* spp.) (Lebegue, 1952).

The aim of this work was to study the origin and nature of multiple embryos in the Californian almond cultivar 'Nonpareil' and the two Spanish almond selections 'D97396' and 'D97407', by phenotypic, molecular and cytological analyses.

## Material and methods

### Plant material

Polyembryonic seeds were collected in open pollination conditions from trees of the Californian almond cultivar 'Nonpareil' (grown in Davis, USA), and the Spanish selections 'D97396' and 'D97407', obtai-

ned from the cross between the new French cultivar 'R1000' and the Spanish cultivar 'Desmayo Largueta' (grown in Murcia, Spain). Polyembryonic seeds were stratified on moist filter papers and placed in Petri dishes at 5°C for 60 days. Later, the tegument was removed and embryos from the same polyembryonic seed were individually planted on the Oasis® growth medium (Oasis® Grower products, Kent, Ohio, USA), in a growth chamber. After three weeks, seedlings of approximately 8-10 cm in height and a diameter of about 2-3 mm were grown in a greenhouse in one litre pots containing 30% perlite and 70% peat. Percentage of germination of the planted embryos and seedling growth were recorded in the three studied genotypes.

### Isoenzymatic analysis

The isoenzymes aminotransferase aspartate (ATA) and phosphoglucomutase (PGM) were studied by horizontal starch gel electrophoresis. These isoenzymatic systems had previously been found to show a polymorphic behaviour in the 'Nonpareil' almond cultivar (Hauagge *et al.*, 1987). The young leaves used for this study came from seedlings 9-11 cm in height, which had already started the growth and development process.

Protein extraction, electrophoretic parameters and staining, were all carried out according to the method described by Arulsekar and Parfitt (1986).

### Application of microsatellites (SSRs)

Genomic DNA extraction from young leaves of plants was done following the method described by Gepts and Clegg (1989), quantifying the DNA by a DyNAQuant200® fluometer (Amersham-Pharmacia, Piscataway, New Jersey, US).

In order to analyse the DNA polymorphism using SSRs (Simple Sequence Repeats) type markers, ten markers developed in peach were chosen (UDP-96001, UDP-96003, UDP-96005, UDP-96008, UDP96-013, UDP96-018, UDP96-019, UDP97-401, UDP97-403 and UDP98-407) (Cipriani *et al.*, 1999). The oligonucleotides were synthesized by Gibco-BRL (Gibco BRL, Carlsbad, California). DNA amplification by the polymerase chain reaction (PCR) was carried out in a final volume of 25 µl and the reaction mixture contained: 10 mM Tris-HCl (pH 8.2), 50 mM KCl, 100 µM of each dNTP, 1.9 µM MgCl<sub>2</sub>, 0.125 µM of each

oligonucleotide (direct and reverse), one unit of Taq DNA Polymerase and 50 ng of genomic DNA as a template.

The amplification conditions were: 1 cycle at 95°C 3 min; 35 cycles at 94°C 1 min, 57°C 1 min, 72°C 2 min; and a final cycle of 72°C for 10 min. Finally, the samples were stored at 4°C. The reactions were done in a Robocycler (Stratagene Inc, La Jolla, California, US). The reaction products were analysed in Metaphor® agarose gels (Biowittaker, Rockland, Maine, US) at 3% in TBE 1X buffer. Finally, these gels were stained with ethidium bromide, and the 123-bp molecular weight marker (Gibco BRL).

### Mitotic karyotype

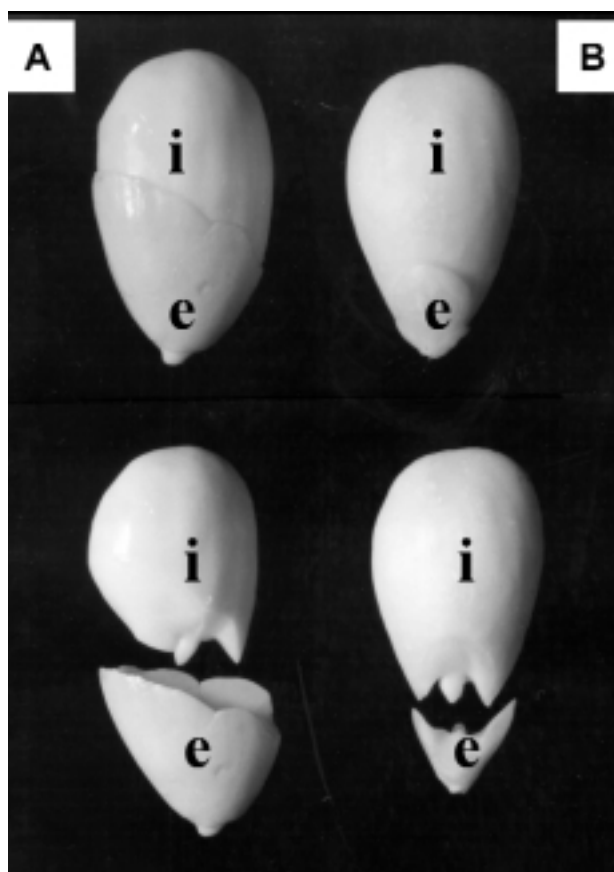
To characterise the karyotype root tips of the study plants were used as plant material. These were treated with 2% colchicine for 3 hours at 5°C. They were then fixed with a mixture of methanol, propionic acid and chloroform in a ratio of 6:3:2 for 24 hours at 5°C, leaving them finally in ethanol (70%). After, the plant material was hydrolysed with 1M HCl at 60°C for 20 min and stained with acetic acid orcein (45%) for 2 hours, according to the method described by Darlington and Lacour (1979). Finally, the root tips were placed on a slide and examined with a Nikon Eclipse EG00 optical microscope (Nikon Inc. Tokio, Japan) (500X).

## Results

### Phenotypic characterization

Most polyembryonic seeds had two embryos (95%), only 5% had three embryos and none had more than three in the three studied genotypes. Therefore, multiple embryos in the almond species usually corresponded to twin embryos (two embryos in each seed).

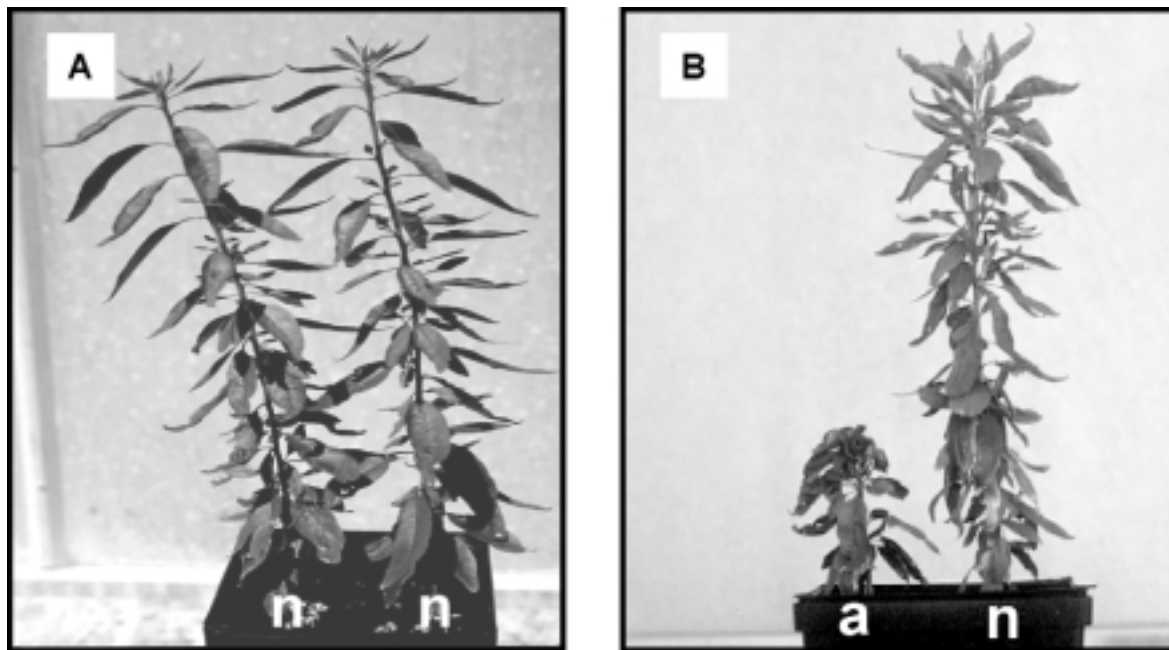
In around 75% of the studied polyembryonic seeds the cotyledon mass had an irregular distribution, and 65-80% corresponded to the distal or internal embryo and the rest to the basal or external embryo. However, in the remaining 25% of polyembryonic seeds a similar size distribution was observed for the cotyledon mass (around 50% for each embryo) (Fig. 1). In the case of seed with three embryos, the mass distribution of the cotyledon was approximately 70% for the in-



**Figure 1.** Polyembryonic seeds of the almond cultivar ‘Nonpareil’ in its original state (above) and after having separated the embryos (below): (A) revealing a similar distribution of the cotyledon mass between the internal embryo (i) distal and the external embryo (e) basal and (B) showing an unequal distribution.

ternal (distal) zone and 15% for each of the other two embryos.

On the other hand, 450 seedlings from polyembryonic seeds of ‘Nonpareil’, and 100 seedlings from polyembryonic seeds of ‘D97396’ and ‘D97407’ were studied. Seedlings from the same seed revealed a similar growth in 75% of the multiple seeds analysed. In the other population, one of the plants from each polyembryonic seed revealed less growth and less development. These aberrant (dwarf) plants are distinguished by their reduced vigour, shorter internodes and deformed leaves (Fig. 2). No correlation was observed between the cotyledon mass proportion of the embryo and its development, although in 90% of cases the seedlings with aberrant growth grew from the basal or external embryo. Moreover, these seedlings presented a death rate of 90% in the three studied genotypes.



**Figure 2.** Seedlings from polyembryonic seeds: (A) with a normal development (n) in both cases and (B) with a normal development (n) and an aberrant development (a).

### Isoenzymatic polymorphism

When isoenzymatic patterns of ATA and PGM were compared a similar polymorphism was observed for seedlings (embryos) from the same polyembryonic seed in the case of the cultivar ‘Nonpareil’ (Table 1). These results reveal a similar origin for multiple embryos in spite of the fact that these have a normal or aberrant phenotype. In the different seedlings studied different alleles were observed for isoenzymes ATA-1/ATA-2 and PGM-1/PGM-2.

### Polymorphism of the microsatellites

Most microsatellite markers have a similar polymorphism in seedlings from the same polyembryonic seed in spite of them having a normal or aberrant phenotype in the three studied genotypes (Figure 3). However, some specific markers of some alleles were not found in dwarf seedlings from polyembryonic seeds in the case of ‘Nonpareil’ (marker UDP96-013; pair 1) (Table 1; Figure 3).

### Mitotic karyotype

In the karyotypic study of ‘Nonpareil’ seedlings growing from polyembryonic seeds it was found that

those with a normal phenotype had a diploid chromosome complement ( $2n=16$ ), while the chromosome contents observed in the aberrant phenotype with a loss of SSR alleles was aneuploid ( $2n-1=15$ ) (Fig. 4).

## Discussion

### Genetic nature of almond seedlings with aberrant growth from polyembryonic seeds

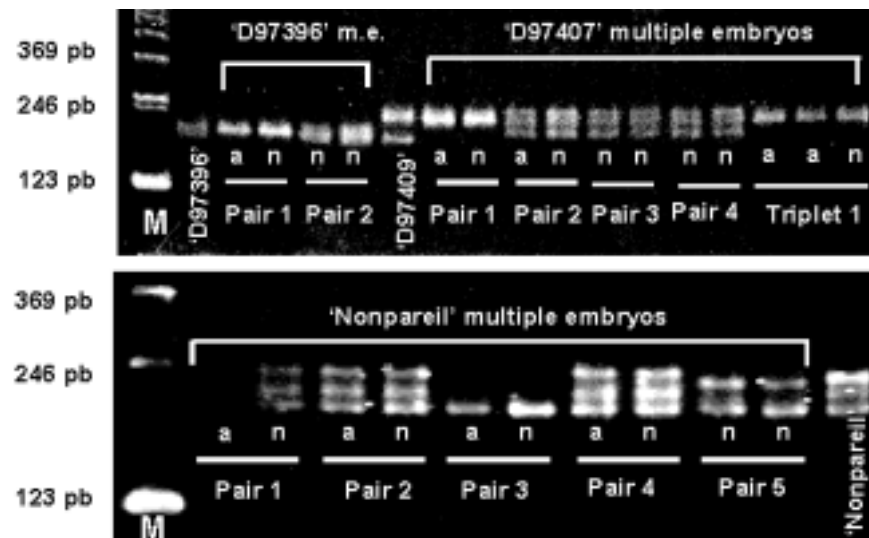
Aberrant development (dwarfism) with short internodes, deformed leaves, and the high mortality observed in ‘Nonpareil’, ‘D97396’, and ‘D97407’ almond seedlings from polyembryonic seeds, agree with other authors descriptions for haploid peach (Hesse, 1971; Toyama, 1974) and almond seedlings (Gulcan, 1975).

The results of the isoenzymatic study showed a similar allelic segregation for seedlings from ‘Nonpareil’ polyembryonic seeds in spite of these having a normal or aberrant phenotype. These results suggest the existence of a diploid genotype in all the plants studied rather than a haploid genotype as Gulcan (1975) described for dwarf almond seedlings. From studies of different haploid peach plants, Arulsekar *et al.* (1986) described the absence of an allelic system for the isoenzyme malate dehydrogenase, as would be expected for true haploids, results that have not been observed in our assays.

**Table 1.** Diagram representing the allelic segregation of two isoenzymatic systems and ten microsatellite (SSR) markers in pairs of seedlings [with a normal development (n) with an aberrant development (a)] from polyembryonic seeds of the almond cultivar ‘Nonpareil’

	‘Nonpareil’	Pair 1		Pair 2		Pair 3		Pair 4	
		a	n	a	n	a	n	a	n
ATA-1 <sup>(1)</sup>	— —	—	—	—	—	—	—	—	—
ATA-2	—	—	—	—	—	—	—	—	—
PGM-1 <sup>(1)</sup>	— —	—	—	—	—	—	—	—	—
PGM-2	— —	—	—	—	—	—	—	—	—
UDP96-001 <sup>(2)</sup>	—	—	—	—	—	—	—	—	—
UDP96-003 <sup>(2)</sup>	— —	—	—	—	—	—	—	—	—
UDP96-005 <sup>(2)</sup>	— —	—	—	—	—	—	—	—	—
UDP96-008 <sup>(2)</sup>	— —	—	—	—	—	—	—	—	—
UDP96-013 <sup>(2)</sup>	— —		—	—	—			—	—
UDP96-018 <sup>(2)</sup>	—	—	—	—	—	—	—	—	—
UDP96-019 <sup>(2)</sup>	— —			—	—	—	—	—	—
UDP97-401 <sup>(2)</sup>	—	—	—	—	—	—	—	—	—
UDP97-403 <sup>(2)</sup>	—			—	—	—	—	—	—
UDP97-407 <sup>(2)</sup>	— —	—	—	—	—	—	—	—	—

<sup>(1)</sup> Isoenzymatic systems. <sup>(2)</sup> Microsatellites.



**Figure 3.** Allelic segregation of the microsatellite UDP96-013 in pairs and triplets of seedlings from polyembryonic seeds of 'D97396', 'D97407', and 'Nonpareil', with a normal (n) and aberrant development (a). M: molecular weight marker 123-bp (Gibco BRL).

Most SSRs studied also showed a similar polymorphism in seedlings from the same polyembryonic seed with both the normal and dwarf phenotype in the three genotypes studied. These results agree with those obtained by Lebegue (1952), who revealed the existence of genetically identical sexual polyembryos in wild strawberries (*Fragaria vesca*). In addition, Cameron and Garber (1968) described the existence of genetically identical polyembryos resulting from interspecific crosses in *Citrus* species.

On the other hand, the absence of certain microsatellite alleles in some dwarf seedlings from 'Nonpareil' polyembryonic seeds could indicate that a chromosome is lost as the result of monosomic aneuploidy rather than from haploidy. Allard (1960) described a phenotype of dwarfed growth for several monosomic aneuploid species. However, to date, diploid-aneuploid seeds have not yet been described. Maheswari and Sachar (1963) reveal that the most common reason for the presence of multiple embryos in plants is a combination of diploid-diploid and haploid-diploid individuals.

Swanson *et al.* (1981) concluded that the loss of a chromosome in a diploid organism has a more damaging effect than haploidy or the addition of an extra chromosome, since this alters the chromosome balance and any recessive gene can be expressed. This could explain the high mortality rate observed in seedlings with an aberrant development (dwarfs). On the other hand, Kimber and Riley (1963) proposed the hypo-

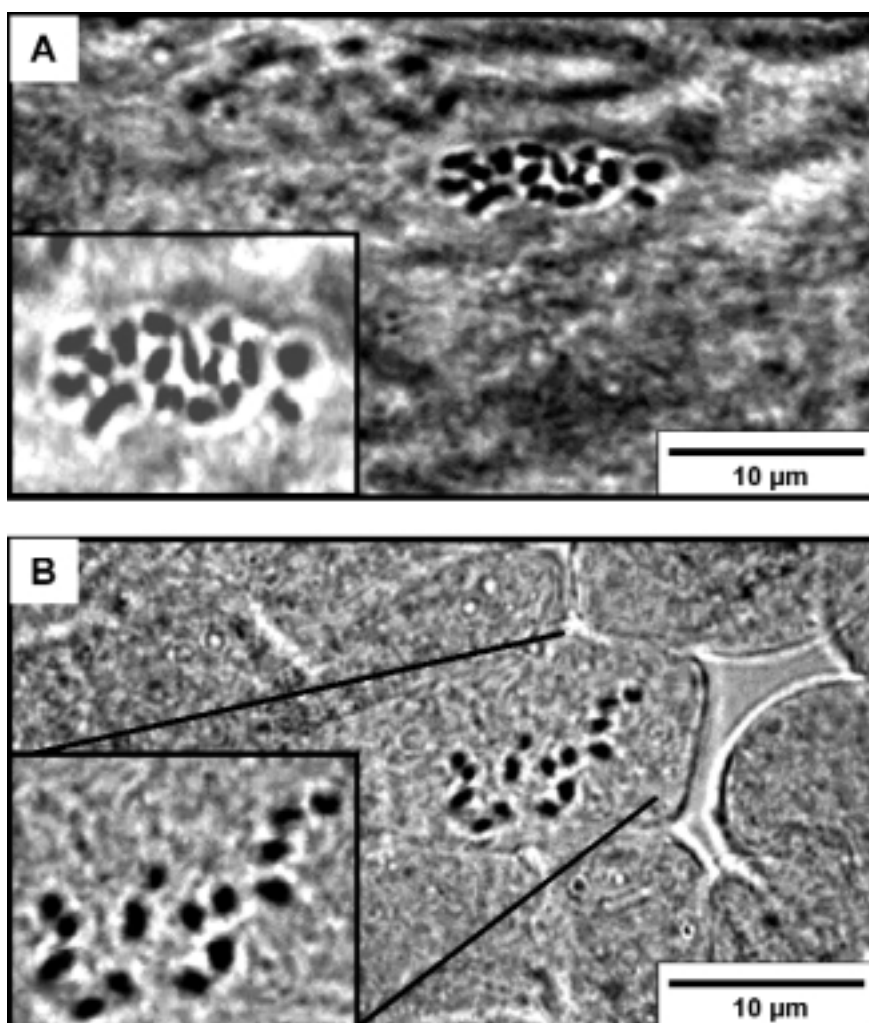
thesis that the existence of multiple embryos is an important source of haploid organisms in angiosperms. However, Specht *et al.* (2001) described an unequal distribution of chromosomes in some plants from polyembryonic seeds in species of the *Allium* genus.

The results of the karyotypic study also suggest a monosomic aneuploidy for the two 'Nonpareil' aberrant seedlings studied although molecular and cytological studies are required in a greater number of dwarf plants from polyembryonic seeds to confirm this hypothesis.

### Origin of multiple embryos in almond

The observation of a similar allelic segregation in seedlings from the same polyembryonic seed different to the parental, in the three genotypes studied suggests a sexual origin for both embryos that is partially corroborated by the contributions of other authors in some *Citrus* species (Cameron and Garber, 1968), wild strawberry (Lebegue, 1952) and in soybean (Kenworthy *et al.*, 1973).

One mechanism that could be responsible for polyembryonic development is the separation of a secondary embryo (external or basal) from a primary embryo (internal or distal) (Fig. 1), as Kenworthy *et al.* (1973) proposed in soybean. Alternatively, development and fertilisation of these two or three embryonic sacs could also occur within the same ovule. This latter



**Figure 4.** Root tips of 'Nonpareil' seedlings from a polyembryonic seed: (A) with a diploid chromosome complement [ $2n=16$ ] and (B) with aneuploidy [ $2n-1=15$ ].

hypothesis was suggested by Lebegue (1952) in *Fragaria* species.

Loss of part or all of a chromosome could occur as a consequence of separation of the new embryo. This phenomenon could be explained by a lack of binding or by the incomplete division of chromosomes in the meiotic or mitotic anaphase, as Swanson *et al.* (1981) suggested for some animal cells.

#### **The use of multiple embryos of sexual origin in the genetic improvement of almond**

Apomictic or adventitious polyembryos (genetically identical to the mother) are used for the vegetative propagation of genetically uniform material of mango and

citrus plants (Sobrinho and Gurgel, 1953; Koltunow *et al.*, 1996). The use of haploid plants from multiple embryos has also been described to obtain homozygotic diploid genotypes by processes of chromosome duplication (Kimber and Riley, 1963). However, since polyembryony in the almond is not of apomictic origin and none of the aberrant multiple embryos studied are haploid, the material available is not genetically uniform nor can it be duplicated to obtain genetically homozygotic individuals.

Nevertheless, the chromosomal aneuploidy found in plants with an aberrant development could be very useful for genetic studies, including the chromosomal localisation of specific genes and specific chromosome transfer (Allard, 1960; Riley and Law, 1984). It would also be interesting to conduct studies of mole-

cular isolation and the sequencing of altered genes with this plant material.

Future studies will aim to confirm the results found here (aneuploidy of aberrant seedlings) with a larger sample size. Also, the phenotypes of trees will be studied (time of flowering, fruit production, growth habit) and the kernel (size, shape, flavour) of plants from these multiple embryos permitting the different horticultural characteristics of the almond to be characterised in greater depth.

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