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Short communication

Occurrence and variability of sexual polyembryony in olive cultivars

Carlos Trapero[a,*], Diego Barranco[a], Antonio Martín[b], Concepción M. Díez[a,c]

[a] Departamento de Agronomía, Universidad de Córdoba-Campus de Excelencia Internacional Agroalimentario ceiA3, Edificio C4, Campus de Rabanales, 14014 Córdoba, Spain
[b] Instituto de Agricultura Sostenible, IAS-CSIC, 14080 Córdoba, Spain
[c] Department of Ecology and Evolutionary Biology, U.C. Irvine, CA, USA

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ABSTRACT

The occurrence of spontaneous sexual polyembryony is described and characterized for cultivated olive (*Olea europaea* L.). We screened seeds from 24 olive cultivars and found significant differences in the frequency of polyembryonic seeds between them. Cultivar 'Cornicabra' and, especially, 'Meski' yielded the highest ratio of polyembryonic seeds (1.6% and 3.0%, respectively), indicating that polyembryony is a low-frequency (0.95% of 5287 observed seeds) but cultivar-dependent feature in olive. Polyembryonic seeds consisted of two and eventually three embryos with a normal endosperm. Simple sequence repeat (SSR) markers were used to characterize the nature of the polyembryonic seedlings. DNA profiles indicated that polyembryonic seedlings in olive have a sexual origin because their profiles were identical and distinguishable from the mother parent. Therefore, polyembryony in olive is of a sexual origin and is due to monozygotic cleavage after normal fertilization. To the best of our knowledge, this is the first evidence of polyembryony in olive and its occurrence in a representative number of cultivars.

1. Introduction

Polyembryony is defined as the development of multiple embryos within the same seedcoat (Webber, 1940). This phenomenon was discovered by Leeuwenhoek in 1719 and can be divided into two main types based on the cellular origin of the embryogenesis: gametophytic and sporophytic. Gametophytic polyembryony includes apogamety and apospory. Sporophytic polyembryony includes monozygotic cleavage and nucellar, integumental and endospermal polyembryony. All these forms of polyembryony except monozygotic cleavage and endospermal polyembryony are asexual reproduction mechanisms through seeds (apomixis) (Batygina and Vinogradova, 2007; Webber, 1940). It is important to distinguish between polyembryony and the presence of several seeds within the same endocarp. While polyembryony has not been described for olive yet, the presence of double-seeded fruits, originated from different fertilization events in two of the four ovules of the flower (Rapoport and Rallo, 1990), is known in this species (Cuevas et al., 1994). Doubled-seeded fruit is not a common phenomenon in olive and its frequency depends on the mother cultivar (Cuevas and Oller, 2002; Farinelli et al., 2012.

Polyembryony is a relevant phenomenon in the breeding of some species, where its occurrence has been reported as relatively frequent. This is the case of citrus species where most apomictic embryos arise from nucellar tissue and therefore bear the same genotype as their female genitor. Polyembryony facilitates the rootstock breeding process (García et al., 1999; Koltunow et al., 1996) and the generation of disease-free citrus plants (Bruno, 1962; Koltunow et al., 1996). This phenomenon is advantageous for breeding other fruit crop species, such as mango (Aron et al., 1998; Knight, 1970; Sauco et al., 2001). Conversely, polyembryony might also be a serious drawback. For instance, apomictic embryos in citrus seriously hinder the identification of true hybrids, which are the product of crossing between different cultivars in a breeding program (Oliveira et al., 2002).

Olive breeding has been developed over the last several decades (Bellini et al., 2002; Lavee, 1990; Ozdemir et al., 2013; Rallo et al., 2007). The germination of vast numbers of seedlings within the olive-breeding program carried out by the University of Cordoba, Spain, allowed us to observe for first time cases of polyembryony in olive. This phenomenon might be a possible source of new lines such as haploids or aneuploids that could be useful for breeding and understanding the genetic mechanisms ruling important agronomical characters (Kimber and Riley, 1963).

The goal of this study was to describe and characterize the nature of polyembryony in olive as well as its variability in a representative group of olive cultivars.

* Corresponding .author. Tel.: +34957218570.
E-mail address: carlostrapero@uco.es (C. Trapero).

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Table 1
Maternal (cultivar) effects on the frequency of polyembrionic seeds (%).[a]

Cultivar	Dissected seeds	Polyembryonic seeds (%)	Chi-Square value[b]
'Meski'	644	3.0	27.4*
'Cornicabra'	1343	1.6	5.4*
'Zaity'	200	1.0	0.0
'Changlot Real'	629	0.6	0.6
'Gordal Sevillana'	172	0.6	0.2
'Manzanilla de Almería'	181	0.6	0.3
'Empeltre'	206	0.5	0.5
'Lechín de Sevilla'	200	0.5	0.4
'Arbequina'	184	0.0	1.7
'Blanqueta'	100	0.0	0.9
'Carolea'	100	0.0	0.9
'Cornezuelo de Jaén'	128	0.0	1.2
'Frantoio'	100	0.0	0.9
'Gemlik'	100	0.0	0.9
'Hojiblanca'	100	0.0	0.9
'Jabaluna'	100	0.0	0.9
'Koroneiki'	100	0.0	0.9
'Manzanilla de Sevilla'	100	0.0	0.9
'Memecik'	100	0.0	0.9
'Morisca'	100	0.0	0.9
'Picual'	100	0.0	0.9
'Racimal'	100	0.0	0.9
'Sevillenca'	100	0.0	0.9
'Villalonga'	100	0.0	0.9
Total	5287	1.0	–

[a] Overall Chi-Square value = 51.04 ($P = 0.001$).
[b] Values followed by an asterisk correspond to polyembryony frequencies significantly higher than the mean of all cultivars according to Chi-Square test at $P = 0.05$.

2. Materials and methods

2.1. Plant material and frequency of polyembryony events

Olive seeds were collected from trees grown under homogeneous conditions in the World Olive Germplasm Bank of Cordoba (WOGBC), located in the IFAPA research center in Cordoba, Spain.

We first collected open-pollinated seeds from 24 different olive cultivars and screened 100–200 of them to assess the occurrence of polyembryony and whether this phenomenon might have a variable frequency among cultivars (Table 1). Seeds were dissected and individually observed under a stereoscopic microscope (Nikon SMZ-2T, Nikon Corporation, Tokyo, Japan) to determine the existence of polyembryonic seeds. We increased the number of the assessed seeds (depending on their availability) in those cultivars showing polyembryony. Subsequently, we sowed seeds of these cultivars and others such as 'Picual' that although did not show polyembryony in the first screening, are massively used as genitors in the olive breeding program giving rise to large progenies (Trapero et al., 2011). Seeds were stratified in a mixture of peat, coir and perlite (55:30:15) at 14 °C for 30 days and then grown in a greenhouse at 22 ± 5 °C and continuous light. Polyembryonic seedlings were identified just after germination, transplanted to 1.5-l pots and grown under the conditions described above.

The differences in the frequency of polyembryony events between the 24 evaluated cultivars were analyzed by a Pearson's Chi-squared nonparametric test at $P = 0.05$ (Table 1), considering the observed and expected frequencies of polyembryonic seeds in each cultivar. Statistical analyses were performed using the program Statistix 10.0 (Analytical Software, Tallahassee, USA) and taking into account the total number of seeds evaluated and sown (5287 seeds in total).

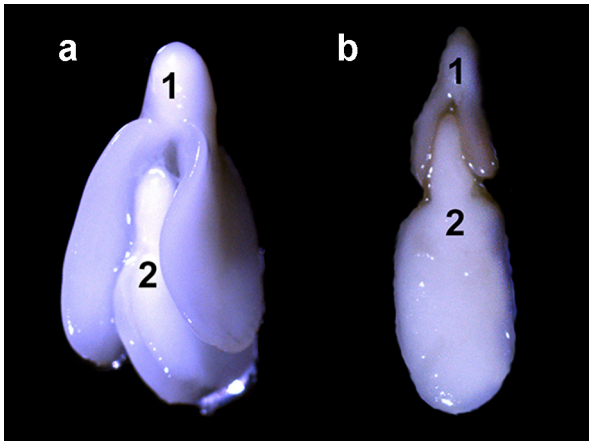

Fig. 1. Olive embryos from polyembryonic seeds. The distribution of size between the outer (1) and the inner (2) embryo was found to be similar (a) and different (b).

2.2. Genotyping polyembryonic seedlings

With the main goal of determining the sexual or asexual nature of the polyembryony in olive, we analyzed 35 seedlings from 17 polyembryonic seeds with outstanding SSR markers previously used in the characterization of olive germplasm (Díez et al., 2012; Haouane et al., 2011; Trujillo et al., 2013). SSR markers have been successfully used to distinguish apomictic and sexual embryos in other fruit species, such as citrus (Aleza et al., 2010; Oliveira et al., 2002) and almond (Martínez-Gómez and Gradziel, 2003).

Total genomic DNA was extracted from completely developed leaves using the CTAB method proposed by Murray and Thompson (1980) with the modifications described by de la Rosa et al. (2004). DNA quality and quantification were assessed by electrophoresis on 0.8% (w/v) agarose gels. Subsequently, the samples were genotyped using six SSR markers: ssrOeUA-DCA03, ssrOeUA-DCA09, ssrOeUA-DCA11, ssrOeUA-DCA16 and ssrOeUA-DCA18 (Sefc et al., 2000), and UDO99-043 (Cipriani et al., 2002). The SSR amplification was performed in a total volume of 20 μl, containing 2 ng of genomic DNA, 1× supplied PCR buffer (Biotools, Spain), 200 μM of each dNTP (Roche), 0.25 units of Taq DNA polymerase (Biotools, Spain) and 0.2 μM of forward (fluorescently labeled) and reverse primers. The PCR reactions were carried out on a thermal cycler (Perkin-Elmer-9600) using the following program: denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 20 s, 50 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. Detection of amplification products was carried out with an automated sequencer ABI 3130 Genetic Analyzer (Applied 181 Biosystems/HITACHI) using the internal standard GeneScan 400 HD-Rox. Two cultivars, 'Arbequina' and 'Frantoio', were used as controls in all runs.

3. Results and discussion

In this study we describe for first time the occurrence of polyembryony in olive. This phenomenon was observed when germinating a large number of progenies within the framework of an olive-breeding program. Poliembriony was detected only in eight out of the 24 screened olive cultivars. This cultivar specificity is in agreement with other fruit species in which polyembryony is also a genetically regulated character (Aron et al., 1998; Batygina and Vinogradova, 2007; Kishore et al., 2012).

The frequency of polyembryonic seeds was lower than the phenomenon of double-seeding reported by Farinelli et al. (2012). However, both cases were highly cultivar-dependent. Polyembryony ranged between 3.0% for the cultivar 'Meski' and 0% shown by