Occurrence and variability of sexual polyembryony in olive cultivars

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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.

Keywords: *Olea europaea*, polyembryonic, twin embryos, monozygotic cleavage, seedlings.

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.

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 (5,287 seeds in total).

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. (2002). 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 20µl, containing 2ng of genomic DNA, 1X supplied PCR buffer (Biotools, Spain), 200µM 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 94ºC for 5 min, 35 cycles of 94ºC for 20 s, 50 ºC for 30 s and 72ºC for 30 s, and a final extension at 72ºC 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 most of the cultivars (Table 1). In contrast, the frequency of double seeding ranged between 2.4% and 23.7% for the set of cultivars studied by Farinelli et al. (2012).

Despite we dissected a high number of seeds per cultivar we cannot discard the occurrence of polyembryony in the cultivars in which it was not found, given the general low frequency of polyembryonic events. Cultivars 'Meski' and 'Cornicabra' yielded significantly more polyembryonic seeds than the other cultivars according to the Chi-square test at $P = 0.05$ (Table 1). Thus, we highlight these cultivars as the most interesting for future studies about polyembryony in olive.

The majority of the polyembryonic seeds consisted of two embryos (duplet), with one of them typically surrounding the other (Figure 1). The seeds had a regular endosperm covered by a common seedcoat. Only one polyembryonic seed had three embryos (triplet). Although it was not quantified, the biomass partitioning appears to be similar among the embryos for most of the olive polyembryonic seeds. In contrast, in *Citrus* species, an unequal biomass distribution of the embryos is the most usual situation (Kishore et al., 2012).

When both embryos showed equivalent biomass their germination and development were generally similar to those of a regular monoembryonic seedling (Figure 2a). Conversely, when the embryos showed an unequal size, one of them exhibited abnormal development and a

137 low growth rate (Figure 2b). Despite their abnormal size, some of these seedlings were viable,
138 achieving a regular size after the first weeks after germination.
139 We applied SSR markers to determine the sexual or asexual origin of the polyembryonic
140 seedlings in olive. To do so, we genotyped 35 polyembryonic seedlings (16 duplets and 1
141 triplet) using six SSR markers, achieving a consistent result: seedlings from polyembryonic
142 seeds were genetically identical and distinguishable from their mother parent. The six SSR
143 markers amplified correctly, showing most of them heterozygous profiles (Table 2). This
144 pervasive heterozygosity discards the possibility of having haploid genotypes among our
145 polyembryonic seedlings; however, the possibility of aneuploidy cannot be completely ruled
146 out. Aneuploidy was reported in almond, being particularly frequent in polyembryonic seedlings
147 with unequal biomass (Martínez-Gómez and Gradziel, 2003). Flow cytometry or karyotype
148 analyses would be required to assess this possibility in olive because our six SSR markers are
149 way far from covering the 46 olive chromosomes. Aneuploid genotypes might be especially
150 interesting to determine the location, effects and interactions of the genes present on the absent
151 chromosome. However, aneuploidy usually has harmful consequences that make the survival of
152 the seedlings impossible.
153 According to our findings, the polyembryony observed in olive is sexual, resulting from
154 monozygotic cleavage after a normal fertilization. This type of polyembryony, which leads to
155 what often is called “twin embryos”, occurs when the original zygote splits into multiple
156 genetically identical embryos (Batygina and Vinogradova, 2007). This phenomenon has been
157 reported in other tree species, such as almond (Martínez-Gómez and Gradziel, 2003), *Citrus*
158 (Aleza et al., 2010) and *Araucaria* species (Agapito-Tenfen et al., 2012).
159 The occurrence of “twin embryos” and the possibility of finding aneuploidy in olive can be
160 useful for genetic and breeding studies. In addition, studies of twin plants can improve our
161 understanding of how genetic, environmental and stochastic factors impact upon epigenetics,
162 affecting for instance developmental changes such as the transition from juvenile to adult.
163 However, the low frequency of polyembryony events imposes the screening of a large number

of progenies even in cultivars particularly prone to exhibit this phenomenon, such as 'Meski' or 'Cornicabra'.

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239

240 **Figure captions**

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

243

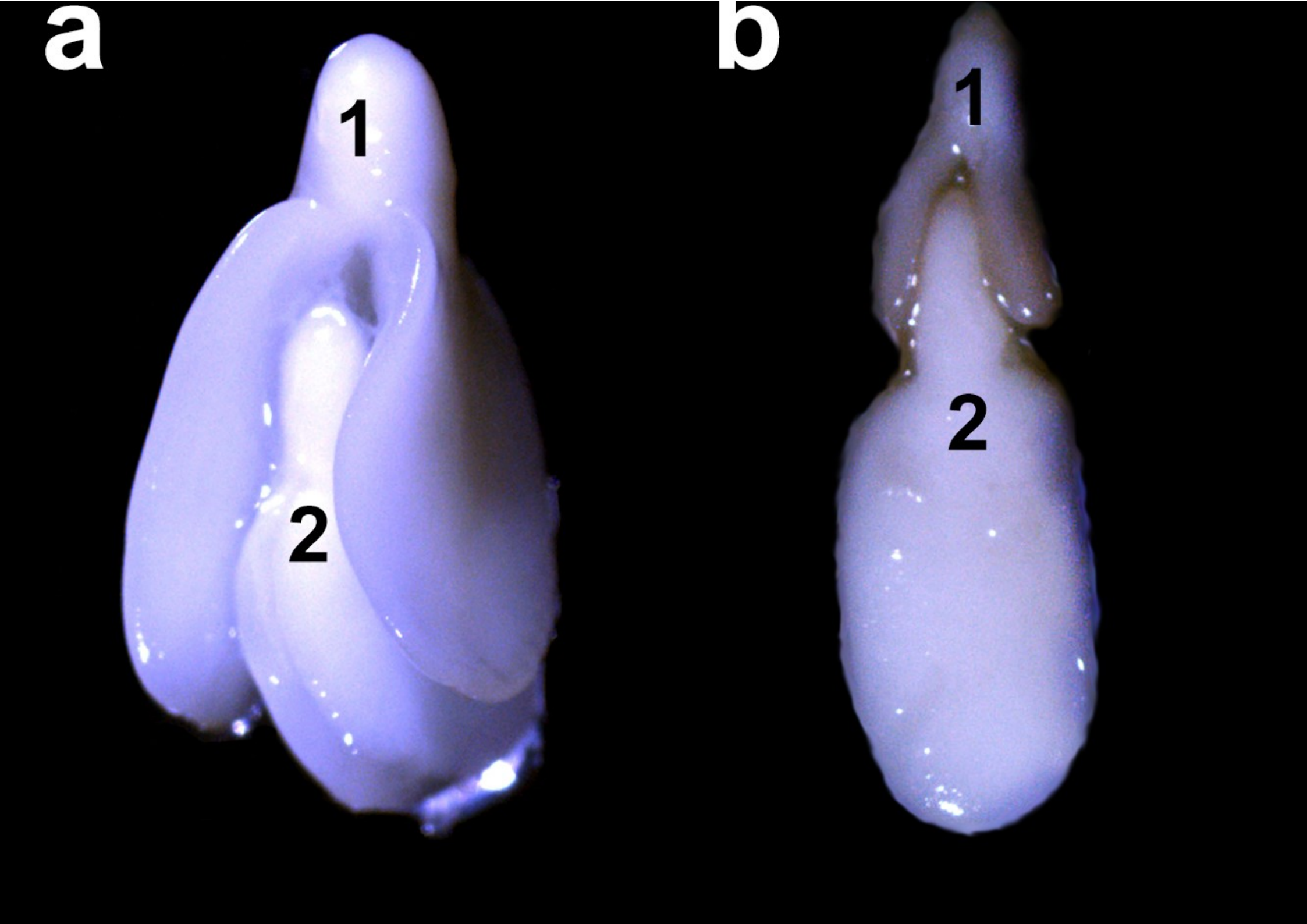
244 **Fig. 2** Polyembryonic olive seedlings emerging from the same seed. Seedlings germinated from
245 similar (a) and different (b) embryo sizes

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	5,287	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.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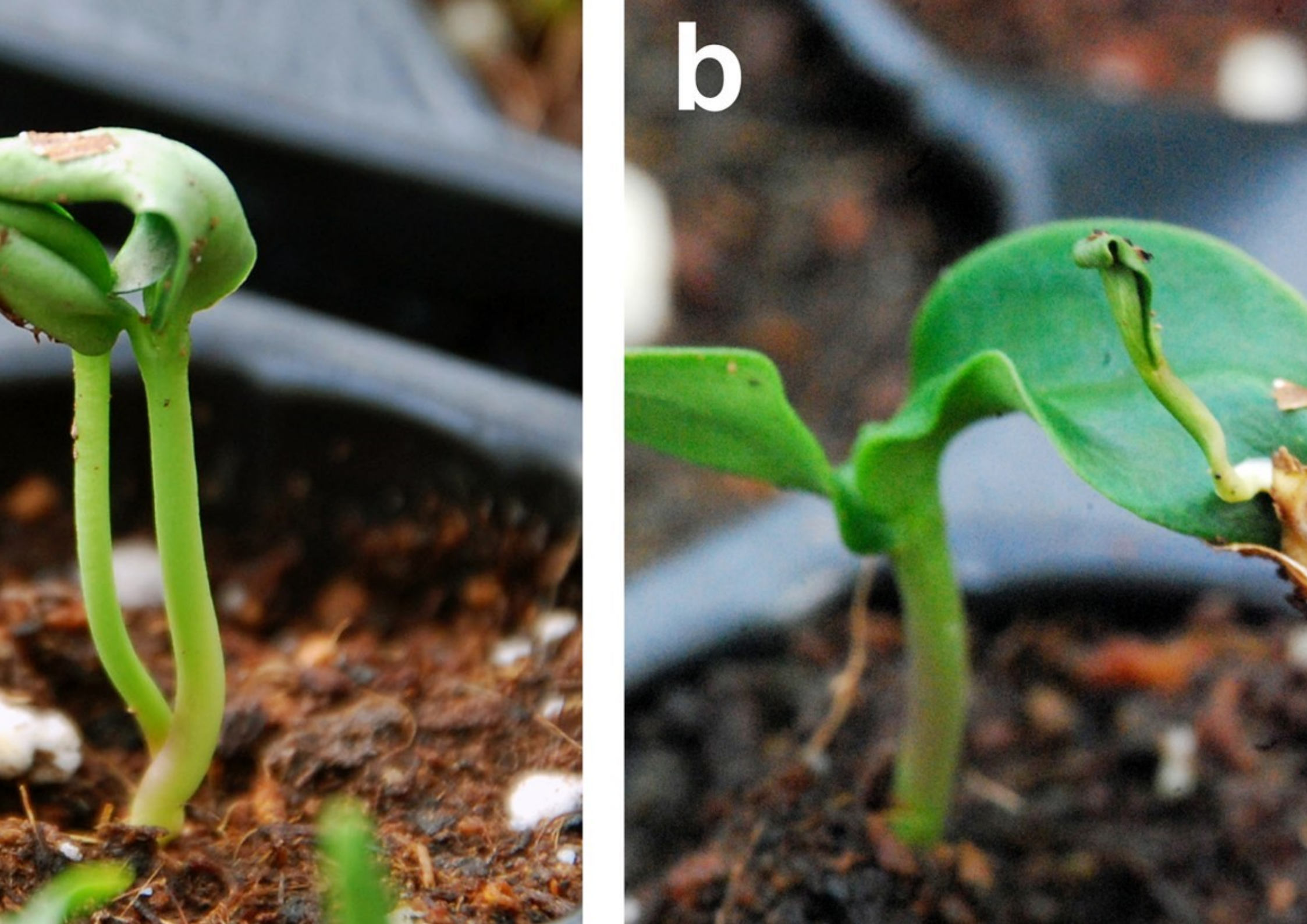
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