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In vitro development and germination of immature olive embryos

By A. TRONCOSO,[1,*] M. CANTOS,[1] J. LIÑÁN,[1] J. TRONCOSO[1] and H. F. RAPOPORT[2]
[1]Instituto de Recursos Naturales y Agrobiología, C.S.I.C., P. O. Box 1052, 41080 Sevilla, Spain
[2]Instituto de Agricultura Sostenible, C.S.I.C., P.O. Box 4084, 14080 Córdoba, Spain
(e-mail: cantos@irnase.csic.es)
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SUMMARY

In vitro culture methods were used to germinate olive embryos prior to maturation. Fruit, seed and embryo development were established with consecutive sampling from 20 to 100 days after bloom. For that same period, embryo development and germination success were determined by *in vitro* culture trials using one-third strength MS medium with or without the addition of zeatin. For early developmental stages, when isolation of the embryo was difficult, a cut portion of the seed containing the embryo was used for culture. The embryos cultured within the cut seed portions germinated and formed normal plantlets. Histological observations indicated a close similarity between the natural and *in vitro* immature embryo differentiation pattern, progressing through preglobular, globular, heart-shaped and torpedo-shaped stages. In some cases, however, the *in vitro* immature embryos developed or germinated abnormally. The presence of zeatin (0.25 mg l^{-1}) in the culture medium and the use of a cut seed-portion containing the immature embryo allowed *in vitro* germination sooner after bloom than previously obtained. On the contrary, zeatin was a handicap for mature olive embryo *in vitro* germination, which reached 100% seedling formation when no plant growth regulators were used.

Seed germination is the most frequently used procedure for plant propagation. Due to the heterozygosity of the generated individuals and the added possibility of genetic recombination after cross pollination, it is also an excellent means for maintaining and even increasing plant biodiversity. Frequently, however, seed germination is limited, giving low percentages of seedlings (Totterdell and Roberts, 1981) and requires extended periods of time to occur (Nickell, 1951). Olive, *Olea europaea* L., seed, covered by a thick stony endocarp, presents both of these problems, as well as a lengthy period required for embryo development (Scaramuzzi and Baldini, 1963; Lagarda *et al.*, 1983; Mitrakos and Diamantoglou, 1984; Crisosto and Sutter, 1985; Troncoso *et al.*, 1998). Such difficulties represent an important handicap for genetic improvement programs in this species (Rugini, 1986; Bellini, 1993).

In vitro mature embryo culture has been used successfully with many species to overcome such problem germination and to accelerate obtaining seedlings (Vázquez and Vieitez, 1966; Cantos *et al.*, 1998). The olive embryo has been shown to have a very good *in vitro* germination (Voyiatzis, 1995; Acebedo *et al.*, 1997; Liñán *et al.*, 1999).

Germination of immature embryos has been successfully utilized in a range of plant species (melon, Adelberg *et al.*, 1994; wheat, Altpeter *et al.*, 1996; maize, Bronsema *et al.*, 1997; Matthys-Rochon *et al.*, 1998; clover, Rybczynski, 1997; *Mimosa*, Burns and Wetzstein, 1998; pine, Li *et al.*, 1998) to regenerate plants through somatic embryogenesis and provide suitable plant material for obtaining transformed plants (Labus-Schneider and Abel, 1991; Kuo and Smith, 1993; Altpeter *et al.*, 1996; Matthys-Rochon *et al.*, 1998). Until now, however, *in vitro* germination of olive embryos has been successful only after the embryo has reached a definitive size, 70–80 d after bloom (Voyiatzis, 1995; Liñán *et al.*, 1999). Consequently, developing methods for the precocious germination of less mature embryos is highly desirable. To achieve this objective, examining embryo development both under natural and *in vitro* culture conditions is an instrumental and critical step.

*Author for correspondence.

MATERIALS AND METHODS

Fruits were obtained from 30 year old, irrigated 'Manzanillo' olive trees growing at the experimental farm "La Hampa" in Coria del Río, Seville, Spain. The fruits were sampled randomly around the canopy, at eye level, on consecutive dates following full bloom. Full bloom was determined each year as the time when more than 50% of the flowers had opened in at least 75% of the inflorescences (Rallo and Fernández-Escobar, 1985), and ranged between 15 and 30 April. At the sample times, the fruits were divided into three groups, for (1) determination of fruit and seed development, (2) *in vitro* germination trials, and (3) morphological and histological observation of embryo differentiation.

Determination of fruit and seed development (average of eight years' sampling)

For 25 fruits per sampling date, length of the longitudinal and transversal axes (fruit size) were measured with a calliper. Then, the fleshy pulp and stony endocarp surrounding the seed were removed, and the two main seed axes measured as above (seed size). Subsequently, the seed was opened longitudinally for observation and photography of the embryo under a binocular microscope. For the early dates, i.e. prior to 60–65 d after bloom, additional fruits per sampling date were fixed for histological observation of embryo development stage as described below.

In vitro *germination trials (average of five years' sampling)*

Until 65–70 d after bloom, the fruits were first disinfected by immersion in commercial bleach with 2% active chlorine and a few drops of Tween 20, for 15 min at 26°C with stirring, and then washed three times with sterile distilled water. Then the seed was extracted and cut in half transversely, to obtain a cut seed portion containing the undisturbed embryo surrounded by a small volume of gelatinacious endosperm, all of which was placed in *in vitro* culture. For later dates, the stony pit was isolated and eliminated without first disinfecting the fruit (Sotomayor-León and Caballero, 1990). The extracted seed was sterilized by immersion in a solution of commercial bleach (0.33% active chlorine) for 20 min at 26°C with stirring, followed by three washings with sterile distilled water, and then the embryo taken out of the seed coat and cultured *in vitro* (Sarmiento *et al.*, 1994).

For *in vitro* culture, half the number of either embryo-containing seed portions or complete embryos of each sampling date were placed individually in sterile test tubes (21 × 150 mm) containing 10 ml of one third strength MS (Murashige and Skoog, 1962) medium. The other half of the embryo-containing seed portions or complete embryos were identically cultured *in vitro* but with the addition to the medium of 0.25 mg l^{-1} zeatin. The culture tubes were covered with plastic caps, sealed with parafilm and placed in a growth chamber at 23°C and a 16 h photoperiod (light intensity, 30 $\mu mol\ m^{-2}s^{-1}$). The *in vitro* embryo development was monitored by continuous visual observation and photos, and selected *in vitro* plant material was fixed for histological preparation and microscope observation as described below.

Morphological and histological evaluation

Morphological studies were made by photographing the samples under a binocular microscope. Histological observations were made to determine the stages at which the still microscopic embryos were placed in culture and to observe the degree and form of within-seed *in vitro* embryo development. The embryo-containing seed portions were fixed in FAE (formalin: acetic acid: 60% ethanol = 2:1:17 v/v), dehydrated in a tertiary butyl alcohol series, imbedded in paraffin and sectioned at 10–12 μm. Staining was with tannic acid, iron chloride, safranin and fast green modified from Jensen (1956), adapting the procedures of Ma *et al.* (1993) and Kiernan (1996) for staining prior to paraffin removal.

RESULTS AND DISCUSSION

Table I presents fruit, seed and embryo size, and embryo developmental stage in relation to time from bloom, and the percentage of *in vitro* embryo germination obtained with and without zeatin in the medium. For each sampling time, a range of embryo stages was present, due to the variability in the fruit and seed maturation process. Such developmental differences are especially likely to be encountered in periods of rapid morphogenetic change, such as those occurring during the sampling and culture-initiation period. Nevertheless, it was possible to define representative stages of development, which are shown in Table I according to the indices defined by Rapoport (1994), which can also be used as developmental indicators for olive trees growing in other environmental conditions.

The olive fruit was spherical in the first 20–30 d after bloom, after which it developed a more ovoid structure as a consequence of the greater growth of its longitudinal axis (Table I). The seed, which increased in size in parallel with fruit development, was covered by a tough but flexible seedcoat and contained a dense liquid endosperm. The presence of a very young embryo, corresponding with stages 1–2 described by Rapoport (1994), was observed by 30–35 d after bloom (Figure 1). In a second period, 35–80 d after bloom, the fruit and seed grew rapidly, reaching maximum size by the end of that time. During that period, the embryo, surrounded by a dense, pasty endosperm, differentiated quickly through globular, heart and torpedo stages (Figures 2, 3, 4) to form a developed but not yet full-size embryo. As the embryo reached torpedo form, the fruit endocarp became totally lignified. The embryo continued to grow and reached its maximum size in the final period utilized for embryo culture, 80–100 d after bloom (Table I).

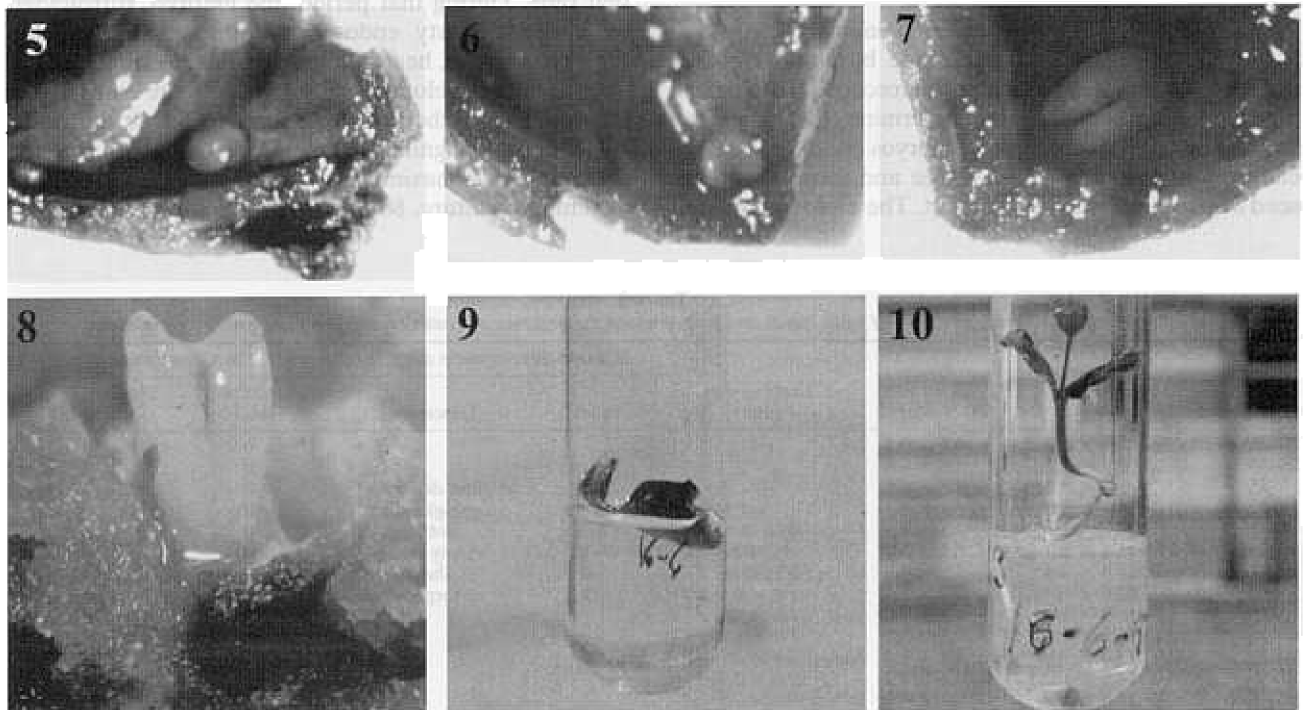
TABLE I

The influence of developmental stages of fruits, seeds and embryos and the presence of zeatin on in vitro *olive embryo germination*

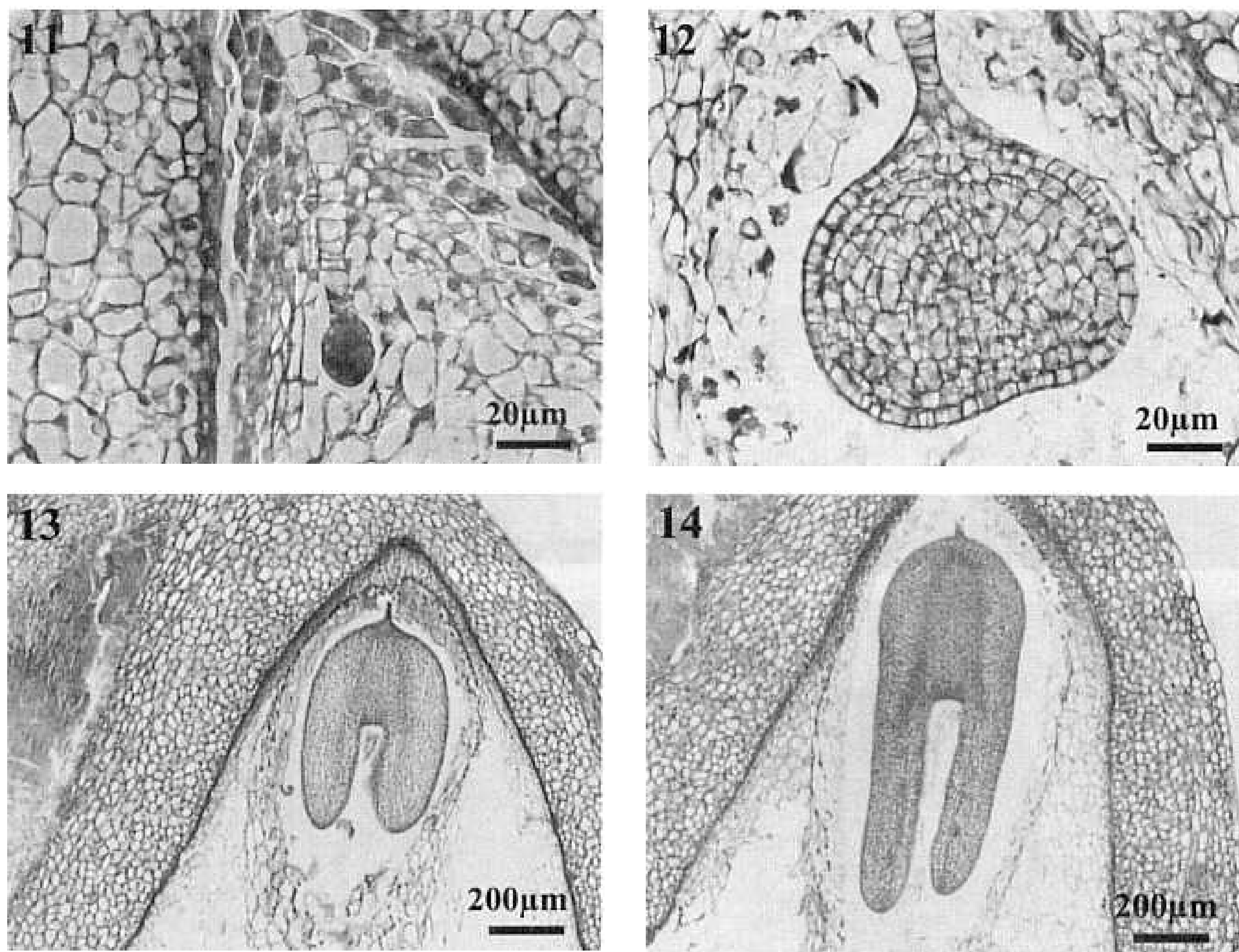
Days after bloom	Fruit size (mm)	Seed size (mm)	Embryo size (mm)	Embryo development stage: Index[1]	Embryo development stage: Description	% Germination: Medium OM/3	% Germination: Medium OM/3 and zeatin
20–25	3.0 × 3.0	0.7 × 0.5	–			0	0
25–30	3.7 × 3.7	1.8 × 0.8	–			0	0
30–35	5.5 × 5.0	2.8 × 1.1	–	1–2	first divisions	0	0
35–40	8.5 × 6.0	3.4 × 1.3	–	3–5	pre-globular	0	0
40–45	10.0 × 6.5	4.3 × 1.8	0.05–0.15	6–8	globular	0	12
45–50	13.0 × 9.0	5.3 × 2.0	0.2–0.5	9–11	early heart	0	14
50–55	14.0 × 9.5	7.5 × 3.0	0.7.5–1.0		–heart	0	54
55–60	15.0 × 10.0	7.7 × 3.4	1.0 × 0.5	12	torpedo	0	64
60–65	16.0 × 11.0	8.0 × 3.0	2.0 × 1.0			0	75
65–70	16.5 × 12.0	8.5 × 3.0	2.0 × 1.0			0	70
70–75	17.0 × 12.0	8.8 × 4.0	3.5 × 2.1			0	70
75–80	18.0 × 12.0	9.2 × 4.0	5.0 × 2.2			60	73
80–85	18.0 × 12.0	9.2 × 4.0	6.0 × 2.5			80	35
85–90	18.0 × 12.0	9.2 × 4.0	7.0 × 3.0			100	30
80–95	18.0 × 12.0	9.2 × 4.0	8.5 × 3.5			100	29
95–100	180 × 12.0	9.2 × 4.0	90.0 × 4.0			100	25

[1]Rapoport (1994).

FIGS. 1–4
Histological observations of early embryo development stages at successive times after bloom: Fig. 1. Early division in the embryo proper (Stage 2) at 30–35 d. Fig. 2. Full globular embryo (Stage 7) at 40–45 d. Fig. 3. Early heart-shaped embryo (Stage 9) at 45–50 d. Fig. 4. Advanced heart-shaped embryo (Stage 11) at 50–55 d.



FIGS. 5–10
Development and germination of olive embryos cultured *in vitro* within cut-seed portions. Fig. 5. Globular embryo (Stage 7). Fig. 6 Early heart-shaped embryo (Stage 9). Fig. 7. Advanced-heart shaped embryo (Stage 11). Fig. 8. Cotyledons emerging from the cut surface. Fig. 9. Young germinated seedling with the root tip still within the cut seed. Fig. 10. Normal, fully developed seedling.

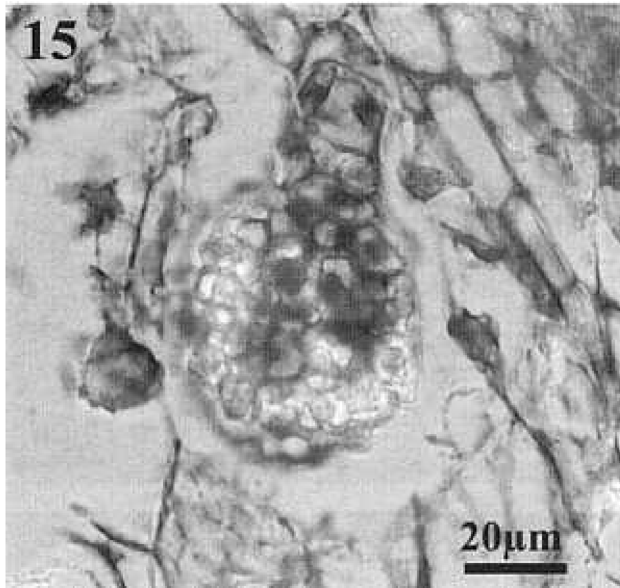


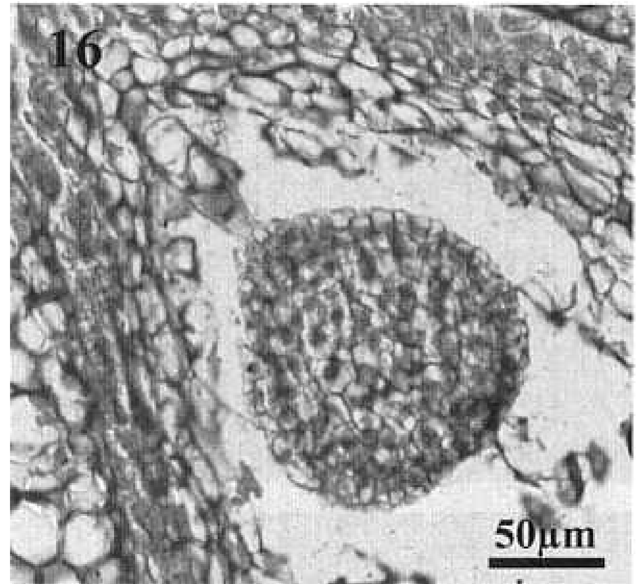
FIGS. 11–14
Histological preparations of normal *in vitro* olive embryo development within cut seed portions. Fig. 11. Pre-globular embryo (Stage 5) after 10 d *in vitro*, placed in culture at 30–35 d after bloom. Fig. 12. Globular embryo with apical end flattened prior to cotyledon formation (Stage 8) after 10 d *in vitro*, placed in culture at 40–45 d after bloom. Fig. 13. Advanced heart-shaped embryo (Stage 11) after 20 d *in vitro*, placed in culture at 45–50 d after bloom. Fig. 14. Torpedo-shaped embryo (Stage 12) after 10 d *in vitro*, placed in culture at 55–60 d after bloom.

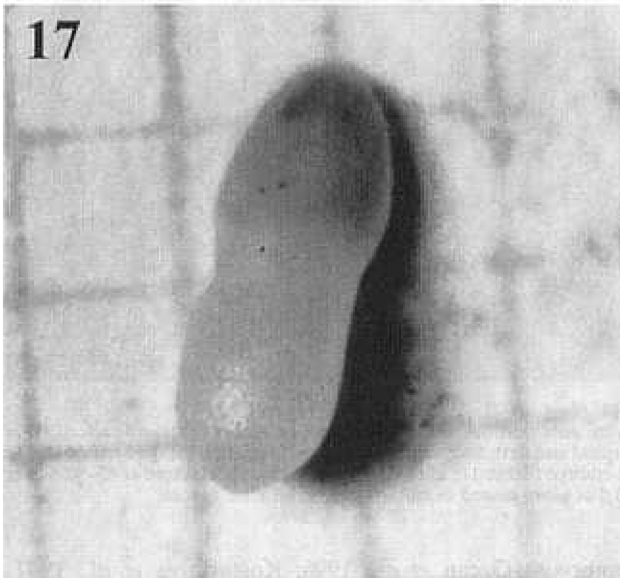
Although different plant growth regulators have been used for *in vitro* propagation of mature olive embryos (Rugini, 1986), Voyiatzis (1995) and Liñán *et al.* (1999) showed that *in vitro* olive embryo germination and seedling formation depend mainly upon embryo development and size. Both groups (Voyiatzis, 1995; Liñán *et al.*, 1999), using a culture medium without growth regulators, obtained the earliest germination 12 weeks after bloom with 6 mm long torpedo-shaped embryos. After that, germination and seedling formation increased successively with increased embryo development. Similar results were obtained in the current studies when the medium lacking plant growth regulators was used. No germination occurred until approximately 75 d after bloom, when the embryo was quite mature, torpedo-shaped and measured 5 × 2.2 mm. Germination increased rapidly with further embryo development, reaching 100% 85 d after bloom when the differentiated embryo had obtained practically the final size (Table I).

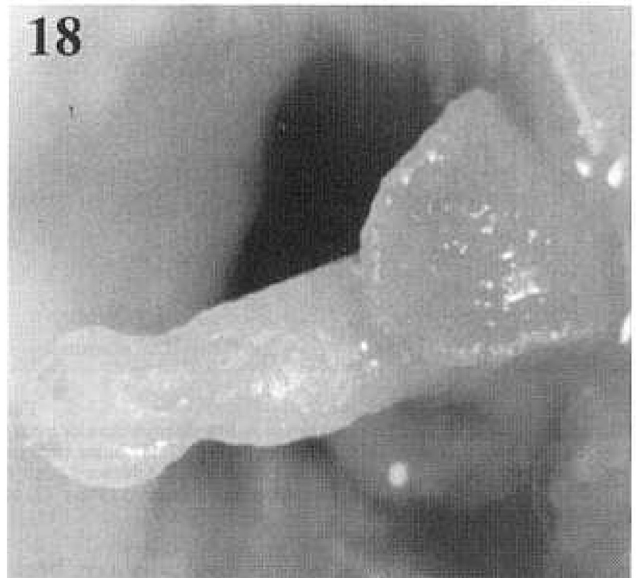
In vitro germination of immature olive embryos improved when the cytokinin zeatin was added to the culture medium. Different plant growth regulators are normally used for *in vitro* germination of immature embryos (Ozcan *et al.*, 1996; Kosturkova *et al.*, 1997) and the use of zeatin with *in vitro* olive plant material was recommended by Rugini (1986) and Clavero and Pliego (1988). With the addition of zeatin, germination began at very early stages of development, 40–50 d after bloom (Table I), when the embryos were globular or early heart-shaped. In this period, however, it was not possible to culture isolated embryos due to their extremely small size. Additional problems were found during the attempted isolation of these very young embryos from the seeds, in that rapid oxidation of the tissues occurred. Thus, as described above, a cut portion of the seed containing the embryo was used for the *in vitro* germination.

The embryos cultured *in vitro* within the cut seed portions normally passed through naturally occurring zygotic stages of development described by Rapoport (1994) prior to germination (Figures 5, 6, 7), germinated successfully (Figures 8, 9) and formed normal plantlets (Figure 10). The histological preparations confirmed a normal zygotic embryo sequence of development within the cultured cut-seed portions, such as, for example, a pre-globular embryo 10 d after placement *in vitro* at 30–35 d (Figure 11), a flattening post-globular embryo 10 d after placement *in vitro* at 40–45 d (Figure 12), an









FIGS. 15–18
Anomalous development of olive embryos cultured *in vitro* within cut-seed portions. Fig. 15. Histological preparation of early globular embryo after 20 d *in vitro*, with tissue discoloration and irregular dermal layer. Fig. 16. Histological preparation of globular embryo after 10 d *in vitro*, with irregular dermal layer. Fig. 17. Young germinating embryo with stunted cotyledons (darker tissue) and irregular swelling of the embryo axis. Fig. 18. Irregular development of the embryo radical during *in vitro* germination.

advanced heart-shaped embryo 20 d after placement *in vitro* at 45–50 d (Figure 13) and a torpedo-shaped embryo 10 d after placement *in vitro* at 55–60 d (Figure 14). Even for the early dates of *in vitro* culture initiation, when germination was unsuccessful or low (Table I), the normal embryo differentiation patterns were observed (Figures 5, 11, 12).

In some cases, structural anomalies were found in the *in vitro* cultured embryos (Figures 15–18). For young embryos within the cut seeds, for example, even though the embryo stage appeared relatively normal, the embryo surface was sometimes irregular and a clearly defined dermal layer was lacking (Figures 15, 16). In some of these embryos, there appeared to be some discoloration as well (Figure 15), but as no histochemical tests were performed, staining anomalies cannot be ruled out. There was no evidence, though, of the possible generation of somatic embryos, either from the zygotic embryos or from other tissues within the cut seed portions. All observed embryos were solitary, a single suspensor was always present (Figure 2) and no residual tissues were observed which might represent a previous or former zygotic embryo.

No embryos were found in 12% of the *in vitro* cultured seed portions examined histologically, as well as in many of the seeds obtained directly from fruits It is quite likely that those cases represent embryo abortion, a not infrequent phenomenon in the olive (Rapoport, 1994), rather than *in vitro* culture failure. The lack of an embryo in the seed due to prior in-fruit abortion could partially account for the low percentages of *in vitro* germination found with the cultured cut seed-portions.

Maintaining the very small, immature olive embryos within cut seed-portions and using zeatin in the culture

medium permitted *in vitro* culture closer to bloom time than had previously been obtained (Voyiatzis, 1995; Liñán *et al.*, 1999). It appears that for any germination to occur in the experimental conditions, however, the embryo required a basic size or form, with differentiation having reached at least the globular stage, which occurred approximately 40–45 d after bloom (Table I). Then, increased germination appears to have been obtained after 50–55 d, as further embryo development occurred with the initiation of the cotyledons in the transition to the heart shape. After 60–65 d, when the embryo reached the initial torpedo shape and could easily be extracted from the seed, still higher germination levels were obtained (Table I).

When the embryo reached full developmental form, the presence of zeatin in the medium was self-defeating. Germination decreased markedly and numerous abnormal growth patterns such as embryo axis swelling (Figure 17) or irregular development (Figure 18) were observed. Even for the 60–65 d embryos cultured within the cut seed, in many cases a seemingly anomalous large size was observed. In consequence, it was concluded that zeatin addition was required for immature olive embryos which had not yet obtained the capacity to germinate. On the contrary, when the embryos reached a certain degree of maturity, and consequently a more appropriate hormonal equilibrium, the presence of zeatin was a handicap.

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