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In vitro rescue of immature avocado (*Persea americana* Mill.) embryos

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Abstract

An in vitro culture protocol was developed as a means of avocado embryo rescue. Different factors including presence of cotyledons, medium texture and cold or gibberellic acid pretreatments, were studied. To better understand the germination process in this recalcitrant species, immature zygotic embryos at different stages were used in these experiments. Optimum results were dependant on the embryo developmental stage. Whereas smaller embryos (5 mm long) germinated better in M1 liquid medium, 15 mm long embryos responded better when precultured in B5m medium supplemented with 1 mg l^{-1} GA_3, and fully mature embryos were capable of germinating directly in solid M1 medium. Our results suggest the existence of two types of dormancy in avocado embryos: an embryo-dormancy caused by cotyledons, and another type of dormancy, mainly occurring in 35 mm long embryos and revealed by the formation of dwarfing rosette seedlings, that can be released by a GA_3 pretreatment.

Keywords: Avocado; Embryo rescue; Germination; Gibberellic acid; *Persea americana* Mill.

1. Introduction

Avocado is an important subtropical woody crop cultivated by its nutritious fruits. For this reason, majority of cultivars traditionally utilized have been selected on the basis of superior yield and fruit quality (Ray, 2002). However, average productivity of avocado orchards is low as consequence of a series of problems that harass this crop including *Phytophthora* root rot, excessive tree vigour at expense of fruit set, soil stress, or fruit diseases (anthracnose, cercospora spot or avocado scab) (Litz et al., 2005).

In avocado, a vast gene pool exists and is available for further genetic gain within conventional breeding programs (Ray, 2002). However, despite the number of breeding programs carried out in different countries, leading cultivars are still mostly derived from open pollinations (Litz et al., 2005).

Avocado, as other subtropical woody crops, has a particularly high rate of fruit abscission (Gómez-Lim and Litz, 2004). Premature abortion of the developing embryos results in a dramatic reduction of the viable hybrid progeny, which significantly reduces the efficiency of avocado breeding programs.

In vitro embryo culture may allow immature embryos to germinate and convert into plants (Burgos and Ledbetter, 1993). Embryo rescue techniques have been developed for numerous species and are routinely used in breeding programs. This technology enables the recovery of an increased progeny, including progeny from interesting crosses that otherwise would be lost (Scemama and Raquin, 1990; Sharma et al., 1996).

The aim of the present investigation was to develop an in vitro culture protocol for recovery of immature avocado embryos by manipulating culture media and conditions. Along this line, the effects of different factors influencing the germination process were studied utilizing immature embryos at different developmental stages.

2. Material and methods

2.1. Plant material and culture conditions

Avocado fruits (*Persea americana* Mill.), cv. ''Hass'', were harvested from open-pollinated trees growing in a monovarietal orchard located at La Mayora Experimental Station

Abbreviations: BA, benzyladenine; B5m, Gamborg major salts and Murashige and Skoog minor salts and vitamins; B5m + GA_3, B5m medium supplemented with 1 mg l^{-1} GA_3; GA_3, gibberellic acid; M1, germination medium of Skene and Barlass; MS, Murashige and Skoog

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(Algarrobo-Costa, Málaga, Spain). After harvesting, fruits were surface sterilized by immersion in a 0.5% (v/v) sodium hypochlorite solution supplemented with Tween 20 (1 drop/100 ml) for 10 min, and rinsed three times with sterile distilled water. Fruits were carefully cut lengthways under sterile conditions and zygotic embryos were excised from seeds.

Zygotic embryos corresponding to different developmental stages were used in the experiments. Developmental stages were defined by their average length and included embryos within a 2–3 mm interval.

The pH of all media was adjusted to 5.74 before autoclaving. Solid media were warmed 7 min to melt the gelling agent and subsequently, 25 ml were dispensed into 25 mm × 150 mm test tubes (Bellco Glass Inc., NJ, USA) or 50 ml into 85 mm × 80 mm cylindrical glass jars. For liquid medium, 5 ml were dispensed into 25 mm × 150 mm test tubes. Finally, media were autoclaved at 121 °C and 0.1 MPa for 15–20 min.

Germination was carried out under a 16 h light photoperiod, provided by Grolux lamps (Sylvania, Germany) (40 μmol m^{-2} s^{-1}), while pre-germination treatments were applied under dark conditions. Unless otherwise indicated, cultures were maintained at 25 ± 1 °C.

2.2. Effect of embryo developmental stage on germination

Avocado fruits were periodically collected in order to cover the avocado zygotic embryo developmental period. Embryos corresponding to seven specific developmental stages based on embryo length (4, 10.5, 16.5, 20.5, 25.5, 30.5 and 40 mm) were induced to germinate by culturing on M1 medium (Skene and Barlass, 1983). M1 medium consisted of MS (Murashige and Skoog, 1962) formulation at half strength supplemented with 0.5 mg l^{-1} benzyladenine (BA) and solidified with 1.7 g l^{-1} gelrite (Kelco, St. Diego, CA, USA).

Germination was carried out during 15 weeks with recultures onto fresh medium at 5-week intervals.

2.3. Effect of medium texture (liquid or solid) and cotyledon removal on germination of embryos at early developmental stages

Intact embryos or embryos with their cotyledons partially excised were cultured on liquid or solid M1 medium. Average sizes of embryos tested were 5, 10 and 15 mm. In embryos with their cotyledons partially excised, one of the cotyledons was snapped apart carefully to avoid damaging the embryonic axis and the remaining cotyledon was partially eliminated. Cotyledons were partially removed only in embryos 10 mm in length or bigger, since it was very difficult to do so in 5 mm long embryos without damaging the embryonic axis. Embryos cultured in liquid medium (6 weeks) were placed in a roller-drum at 5 rpm and recultured twice at 2-week intervals. Subsequently, they were placed onto solid M1 medium for an additional 9-week period. Embryos in solid medium were recultured twice onto fresh medium at 5-week intervals.

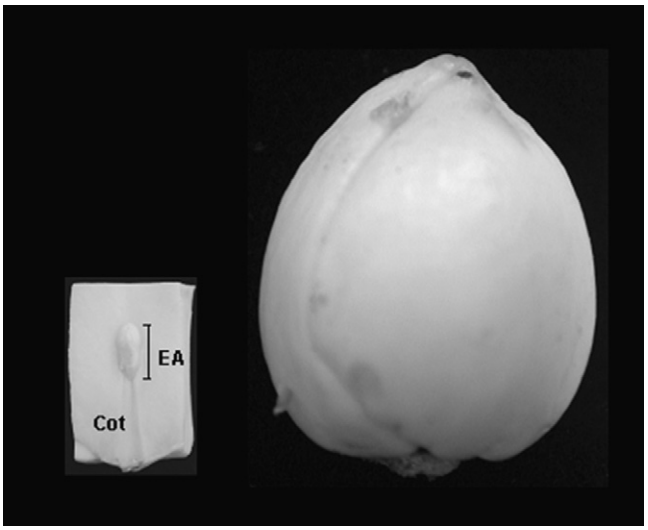

Fig. 1. Zygotic embryo 23 mm in length, intact and with its cotyledons partially excised. In the embryo with partially excised cotyledons (Cot), the segment indicates the embryonic axis (EA).

2.4. Effect of cotyledon removal on germination of embryos at advanced developmental stages

Two embryo developmental stages, 23 and 29 mm in length, were tested in this experiment. In this case, intact embryos or embryos with their cotyledons partially excised were tested for germination on solid M1 medium. In Fig. 1, a 23 mm intact embryo and the same embryo with its cotyledons partially excised can be observed.

Germination was carried out during 15 weeks with recultures onto fresh medium at 5-week intervals.

2.5. Effect of low temperature and gibberellic acid pretreatments on germination of embryos at different developmental stages

The effects of cold or gibberellic acid (GA_3) pretreatments were tested in embryos corresponding to five developmental stages (5, 15, 25, 35 and 40 mm in length).

For the low temperature pretreatment, intact zygotic embryos were cultured in 85 mm × 80 mm cylindrical glass jars containing solid B5m medium. B5m medium consisted of MS medium (Murashige and Skoog, 1962) with the major salts of the B5 formulation (Gamborg et al., 1968) solidified with 1.7 g l^{-1} gelrite. Incubation was carried out at 9 °C and dark conditions during 5 weeks.

The effect of a GA_3 pretreatment was tested by culturing intact embryos in 85 mm × 80 mm cylindrical glass jars containing solid B5m medium supplemented with 1 mg l^{-1} GA_3. Cultures were maintained at 25 °C and darkness for 5 weeks.

After the cold or GA_3 pretreatments, embryos were induced to germinate by partial removal of the cotyledons and subsequent culture on solid M1 medium. Embryos of the control treatment were directly cultured on M1 medium. Germination was carried out over 15 weeks with recultures onto fresh medium at 5-week intervals.

2.6. Data taken and statistical analysis

Twenty to 30 zygotic embryos were utilized per treatment. Embryos were considered germinated when shoot and/or root elongation was ≥2 mm. Data on percentage of germinated embryos, type of germination (shoot, root or shoot and root) as well as length of the shoots and/or roots obtained were evaluated at the end of each reculture.

Germination speed was determined, according to Hartmann et al. (1997), by calculating the average number of days required for shoot or root development:

$$\text{Mean days} = \frac{N_1T_1 + N_2T_2 + N_3T_3}{\text{total number of embryos germinating}}$$

where N_i are the numbers of embryos germinating within each reculture and T_i values indicate the times between the beginning of the germination phase and the end of each reculture.

Percentage data were subjected to frequency analysis with an R×C test of independence or with a three-way log-linear analysis to evaluate interactions between variables. Length data were analysed by ANOVA and differences among means were tested by the LSD. The significance level used was 0.05 in all cases.

3. Results

3.1. Effect of embryo developmental stage on germination

Germination on solid M1 medium was not significantly affected by embryo developmental stage (Table 1). Interestingly, low germination rates, ranging between 0 and 11.76%, were observed in small or medium-sized embryos (4–20.5 mm in length) while no germination was obtained in embryos larger than 20.5 mm.

Germinated embryos of smaller sizes (4–10.5 mm) developed only shoots while complete germination (development of shoot and root) was only observed in 20.5 mm long embryos (data not shown).

3.2. Effect of medium texture (liquid or solid) and cotyledon removal on germination of embryos at early developmental stages

Effects of medium texture and cotyledon removal were evaluated in 5–15 mm long embryos. A 40% germination rate was obtained in 5 mm long embryos cultured with intact cotyledons in liquid medium (Fig. 2). However, embryos of larger sizes (10 and 15 mm in length) cultured in the same conditions failed to germinate. Culturing on solid M1 medium induced a decrease in the germination percentage of smaller embryos (5 mm long) but favoured that of the larger embryos (15 mm long). However, the obtention of high germination rates (50%) in 15 mm long embryos cultured on solid medium was dependant upon the partial removal of the cotyledons. These results revealed a significant influence of medium texture ($P = 0.0084$), embryo size ($P = 0.0008$) and medium texture - × embryo size ($P = 0.0065$) on avocado zygotic embryo germination.

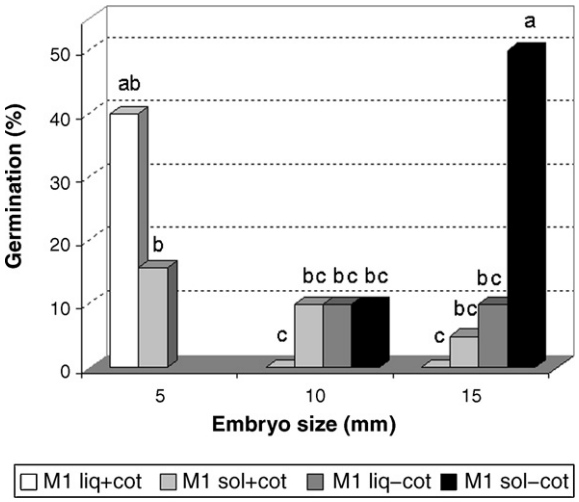

Fig. 2. Effect of medium texture and cotyledon removal on germination rate of avocado zygotic embryos, cv. Hass, at early developmental stages. Different letters indicate significant differences obtained by frequency analysis with a significance level of 0.05.

In most cases, germination consisted only of shoot development, although some of the 15 mm long embryos occasionally formed only roots or complete plantlets (data not shown). No significant differences were found regarding size of the shoots or roots developed (data not shown). A considerable number of hyperhydric shoots were obtained in this experiment, mainly in material derived from 5 mm long embryos.

3.3. Effect of cotyledon removal on germination of embryos at advanced developmental stages

Partial removal of the cotyledons significantly improved germination in embryos 23–29 mm in length ($P = 0.0000$). In this type of embryos, following excision of the cotyledons an increase in germination percentage was observed as embryo development progressed (57.14% in 23 mm long embryos versus 76.19% in 29 mm long embryos) while intact embryos did not germinate.

Concerning the type of germination obtained, for most embryos only sprouting of the shoot was observed, although occasionally, complete germination could be obtained (data not shown). Length of the structures that developed also increased with embryo size. While shoots and roots derived from 23 mm

Table 1
Germination percentage of avocado zygotic embryos, cv. Hass, at different developmental stages

Embryo size (mm)	Germination (%)	
4.0	5.48	a
10.5	10.17	a
16.5	11.76	a
20.5	5.26	a
25.5	0	a
30.5	0	a
40.0	0	a

Different letters indicate significant differences obtained by frequency analysis with a significance level of 0.05.

long embryos were 3.08 and 3.90 cm in length, respectively, those arising from 29 mm long embryos averaged 4.29 and 6.65 cm in length, respectively.

3.4. Effect of low temperature and gibberellic acid pretreatments on germination of embryos at different developmental stages

In this experiment, in which germination was induced in embryos with partially excised cotyledons, embryo age had a significant effect on germination rate ($P = 0.0000$) and a clear correlation could be established between both factors (Fig. 3). As expected, embryo maturation significantly affected germination capacity, e.g., 5 mm long embryos seldom germinated on solid medium (3.33%), an important increase in germination rate was observed in 25 mm long embryos and, at full maturity (40 mm in length), virtually all embryos (95.45%) were capable of giving rise to shoots and/or roots.

Whereas cold pretreatment did not show noticeable effects on germination, with the exception of a slight promotion in 15 mm long embryos, incubation in B5m medium supplemented with GA_3 significantly affected shoot and/or root emergence ($P = 0.0012$) (Fig. 3). The influence of GA_3 pretreatment was significantly dependant on embryo size ($P = 0.0047$), with maximum positive effects at the 15 mm stage.

In general, germination speed increased as embryo development progressed (Table 2). A significant effect of GA_3 pretreatment on this parameter could also be observed, since pretreated embryos always required a lower number of days for germinating than control embryos. The effect of GA_3 was more noticeable with embryos at the earlier developmental stages. Cold pretreatment did not show any effect, except for 15 mm long embryos where a clear increase of germination speed was evident.

Both, the type of germination and quality of the seedlings obtained, varied significantly with embryo size. As embryo development progressed, a higher proportion of complete

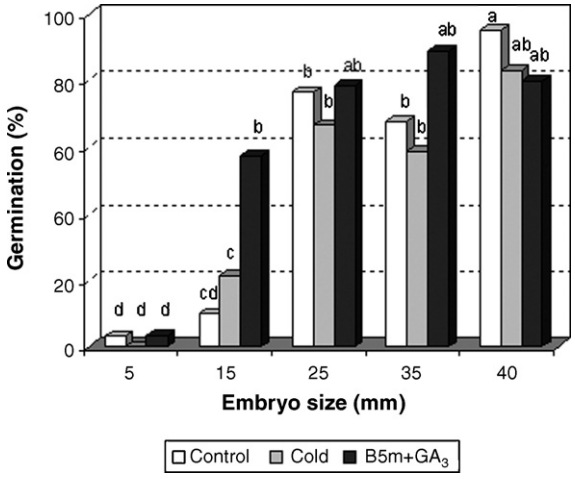

Fig. 3. Effect of low temperature and gibberellic acid pretreatments on germination of avocado zygotic embryos, cv. Hass, at different developmental stages. Different letters indicate significant differences obtained by frequency analysis with a significance level of 0.05.

Table 2
Effect of low temperature and gibberellic acid pretreatments on germination speed of avocado zygotic embryos, cv. Hass, at different developmental stages

Embryo size (mm)	Germination speed		
	Control	Cold	B5m + GA_3
5	94.00	–	37.00
15	85.67	51.17	67.00
25	54.57	65.13	47.36
35	60.79	60.20	52.00
40	39.29	38.60	37.50

Germination speed was calculated according to Hartmann et al. (1997) as the average number of days required for shoot or root elongation.

plantlets was formed and the shoots and roots developed were progressively larger (data not shown).

During avocado embryo development, germination sometimes occurred in an abnormal way, giving rise to rosette seedlings (Fig. 4). Rosette seedlings were mainly characterized by disposition of leaves in a rosette pattern; moreover, leaf appearance was very different from that of normal seedlings and apical axis appeared most times arrested, giving rise to dwarf plantlets with no visible stem or stems smaller than 2 mm. This type of abnormal shoot growth occurred specifically in 35 mm long embryos and to a lesser extent in 25 mm long embryos, revealing a significant influence of embryo age ($P = 0.0000$) (Table 3).

Interestingly, GA_3 pretreatment significantly decreased the formation of rosette shoots in 35 mm long embryos, e.g., at this

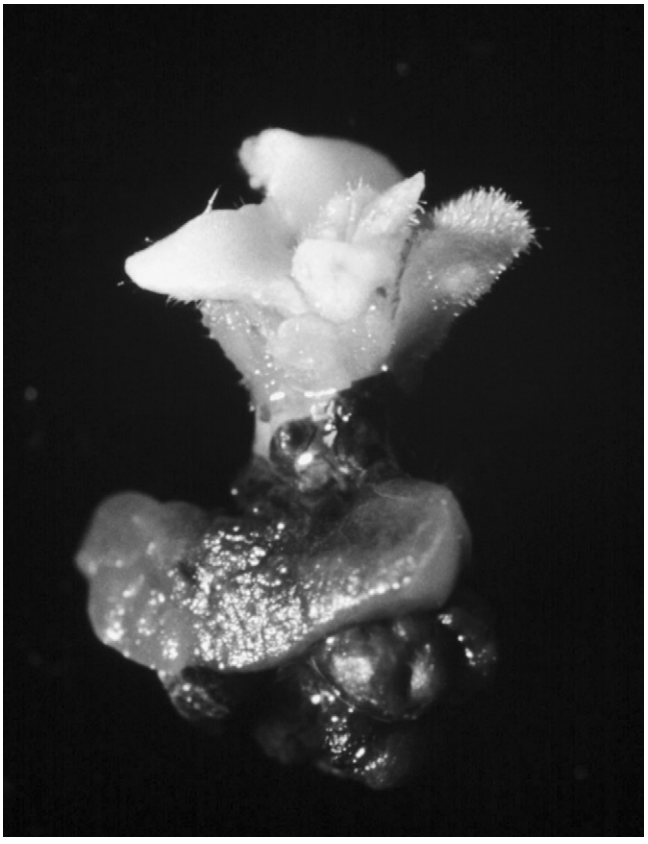

Fig. 4. Rosette seedling developed from an avocado embryo 35 mm in length.

Table 3
Effect of low temperature and gibberellic acid pretreatments on formation of rosette seedlings following germination of avocado zygotic embryos, cv. Hass, at different developmental stages

Embryo size (mm)	Rosette formation (%)					
	Control		Cold		B5m + GA_3	
5	0	c	0	c	0	c
15	0	c	0	c	0	c
25	16.67	b	29.17	ab	21.43	ab
35	46.43	a	41.18	ab	11.11	bc
40	0	c	0	c	0	c

Different letters indicate significant differences obtained by frequency analysis with a significance level of 0.05.

developmental stage, rosette shoot formation was reduced from 46.43% in control embryos to 11.11% in pretreated embryos (Table 3).

4. Discussion

Germination percentages generally increase with embryo development (Bridgen, 1994; Carimi et al., 1998). However, in avocado, small-sized embryos cultured on solid M1 medium germinated at low rates while mid to large embryos failed to germinate.

Generally, solidified media are more suitable to induce germination under in vitro conditions (Hu and Wang, 1986); however, the utilization and renewal of liquid media is one strategy used in cases where low germination percentages are obtained (Wang and Janick, 1984). The investigation carried out revealed that, in avocado, medium texture significantly affects the germination process. Although Skene and Barlass (1983) used liquid medium for germinating avocado embryos, our results showed that only small size embryos (5 mm in length) germinated better under these conditions. The rate of imbibition during the initial stages of germination is an important aspect of the germination process. The embryonic axis needs to achieve a turgor threshold for germination to begin (Finch-Savage et al., 1992) but a rapid imbibition may lead to abnormal germination and appearance of hyperhydricity (Thorpe, 1995). The increase in germination rate observed when intact small size embryos were cultured in liquid medium could be due to the attainment of adequate conditions for embryonic axis water uptake. Moreover, at this developmental stage, cotyledons are only partially developed and may not interfere to a large extent with the germination process. In larger embryos, however, appropriate hydration conditions do not appear to be met and blackening of cotyledons takes place, limiting the occurrence of germination.

Avocado embryos, as with other recalcitrant species, are characterized by the presence of large cotyledons that at the end of embryo development represent the major part of the embryo weight and constitute a compact tissue surrounding the embryonic axis.

The positive effect of cotyledon removal on avocado embryo germination could indicate the existence of an embryo-dormancy associated with the cotyledons. In other species with recalcitrant seeds such as *Aesculus hippocastanum* (Radojevic, 1988), *Theobroma cacao* (Novak et al., 1986), *Corylus avellana* and *Fraxinus excelsior* (Taiz and Zeiger, 2002), a significant increase in germination capacity has also been observed as result of partial elimination of cotyledons. These large structures typical of recalcitrant seeds could act as physical constraints and, as proposed for seed coat-caused dormancy, probably prevent germination by restricting water uptake, mechanically restricting the growth of the embryonic axis or by the presence of chemical inhibitors (Bradbeer, 1988). Nevertheless, limited uptake of nutrients and plant growth regulators present in the germination medium or limited gaseous exchange should also be considered (Bewley and Black, 1982).

Besides the dormancy caused by the cotyledons, the study carried out with embryos at different developmental stages suggests the existence of another type of dormancy in avocado because, at specific developmental stages (35 mm and to a lesser extent 25 mm), a high proportion of embryos produced rosette plantlets despite cotyledon removal. Occurrence of germination in a rosette pattern has been associated to dormancy so that, although embryos can sometimes bypass it and germinate, resulting seedlings grow abnormally (Tukey, 1938).

Preculturing for 5 weeks in a 1 mg l^{-1} GA_3 supplemented medium, prior to germination, significantly decreased the number of rosette plants. These results agree with previous investigations that pointed out a key role of gibberellins in the regulation of dormancy release (Kucera et al., 2005). Moreover, coinciding with previous observations carried out in other species (Kucera et al., 2005), GA_3 pretreatment also affected the germination process in avocado by accelerating germination onset and enhancing germination rate and speed.

Cold pretreatment did not show any significant effect on dormancy release or germination. The absence of an effect of cold pretreatment could be due to the fact that avocado is a subtropical crop and effectiveness of low temperatures has usually been associated to nontropical species (Bewley and Black, 1994). However, it could also be possible that the very specific low temperature requirements had not been met with the treatment applied.

5. Conclusions

Although only a few cases of dormancy in recalcitrant seeds have been previously described (Bewley and Black, 1994), a period of developmental arrest appears to exist in the avocado zygotic embryo and probably more than one regulatory mechanism could be involved.

The results obtained show that embryos at different developmental stages differ in germination capacity and culture requirements, e.g., embryos at the earlier developmental stage tested (5 mm in length) germinated at an acceptable rate in liquid M1 medium, embryos at a more advanced developmental stage (15 mm in length) responded better when pretreated in B5m medium supplemented with 1 mg l^{-1} GA_3, and virtually

100% of fully mature embryos were capable of germinating directly in solid M1 medium.

Seedlings obtained under optimum conditions for each developmental stage generally exhibited good quality, not requiring further recovery treatments. The unique exception was plantlets derived from 5 mm long embryos germinated in liquid medium, in which symptoms of hyperhydricity were frequent. In cases where roots were not formed, plantlets could be obtained following the protocol of Barceló-Muñoz et al. (1990) for micropropagation of juvenile avocado, e.g., shoot proliferation in the presence of 1 mg l^{-1} BA and rooting following a 3-day pretreatment in the presence of 1 mg l^{-1} indole-3-butyric acid and subsequent culture in basal MS medium with macroelements at 0.3×.

The embryo rescue technique developed in this research could be used in avocado breeding programs to increase plant recovery from valuable crosses. In these crosses many embryos are generally lost due to fruit abscission at very early developmental stages.

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