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Article *in* Plant Cell Tissue and Organ Culture · May 2008

DOI: 10.1007/s11240-008-9358-0

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ORIGINAL PAPER

Assessment of polyembryony in lemon: rescue and in vitro culture of immature embryos

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Received: 3 December 2007 / Accepted: 28 February 2008

Abstract The 27 lemon cultivars analysed could be considered slightly or moderately polyembryonic, with 25 to 43% of seeds being polyembryonic and from 1.3 to 1.6 embryos per seed. On this basis, it is necessary to rescue zygotic embryos at an immature stage. Rescue and in vitro embryo development have been studied in two *Citrus limon* polyembryonic cultivars. Sucrose (50 and 70 g/l) was combined with Murashige and Skoog and Gamborg's B5 media and tested for optimal growth response. An important effect of genotype was observed: embryos from cultivar 'Eureka' had greater survival, germination percentage, and radical development. While the sucrose concentration in the medium did not have an effect on germination, the medium affected the embryo survival and root development of the seedlings, Gamborg's B5 medium giving the best results. The ability to form plants in vitro was affected by an increase of embryo developmental stage. The germination and seedling height were greater with embryos of seeds collected 135–150 days after anthesis.

Keywords Culture medium · Developmental stage · *Citrus limon* · Genetic improvement · Nucellar embryos

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Abbreviations

DAA	Days after anthesis
GA	Giberellic acid
G-B5	Gamborg's B5 medium
LSD	Least Significant Difference test
MS	Murashige and Skoog medium
MT	Murashige and Tucker medium
NAA	Naphthalene acetic acid

Introduction

Polyembryony is one of the main obstacles in citrus breeding by conventional methods. Most citrus species are polyembryonic, the seeds producing nucellar embryos similar to the mother plant that germinate and develop into plantlets. The zygotic embryos have difficulty in surviving (Frost and Soost 1968) since they must compete for nutrients and space with the embryos that develop from nucellar tissue (Soost and Rose 1996). Soares-Filho et al. (1992) determined that sexual embryo size and survival were inversely related to the number of embryos per seed. The nucellar embryos limit the range of genetic variability that can be observed in the progeny of a cross, and thus the possibility of finding new genotypes. Accordingly, in vitro embryo culture is a useful tool in citrus breeding, since it assures embryo germination and development of hybrid seedlings (Ohta and Furusato 1957).

The success of embryo culture is, to a large extent, determined by the embryo developmental stage and the nutrients and plant growth regulators provided by the culture medium. Generally, young citrus embryos have been excised from polyembryonic seeds approximately 100 days after pollination (Deng et al. 1996; Tusa et al. 1996); in contrast, seeds from monoembryonic seed parents have been collected 7 months after pollination or at maturity (Oiyama and Kobayashi 1990; Sykes and Lewis 1996). The ability to form plants in vitro increases strongly with increasing embryo developmental stage (Carimi et al. 1998)

Rescued citrus embryos are generally cultured on Murashige and Skoog medium (MS) (Murashige and Skoog 1962) or Murashige and Tucker medium (MT) (Murashige and Tucker 1969). These two media have been adjusted to induce embryo germination in numerous citrus species, resulting in variable success. High germination rates of aborted embryos obtained from monoembryonic seed progenitors have been induced on MS (Starrantino 1992) or MS supplemented with malt extract and adenine sulphate (Starrantino and Reforgiato-Recupero 1981). However, mandarin embryos did not germinate well on an identical basal medium supplemented with giberellic acid (GA) (Khan et al. 1996). Increasing the sucrose concentration and adding GA enhanced the germination rates of mandarin embryos (Sykes and Lewis 1996).

A study of embryo developmental stage and medium composition, using polyembryonic diploid citrus, revealed that pro-embryos required GA and a high sucrose concentration in MT medium, while early cotyledonary embryos germinated well on MS with malt extract (Carimi et al. 1998).

Most lemon cultivars produce substantial numbers of embryos per seed (Soost and Roose 1996). Therefore, it is necessary to rescue zygotic embryos at an immature stage to reduce the risk or their abortion and competition with nucellar embryos. Very few studies on the embryo culture of lemon fruit have been carried out. Tusa et al. (1996) used undeveloped lemon embryos that germinated on MT medium with malt extract but no plant growth regulators and Viloria et al. (2005) studied acid citrus fruit embryos derived from interploid hybridisation.

The present research is aimed at acquiring knowledge that could support a genetic improvement programme for lemon based on hybridisation. To this end, the purpose of this study was to analyze polyembryony in lemon and to establish an efficient in vitro culture system and an optimum developmental stage for the germination and normal seedling development of two lemon cultivars.

Materials and methods

Plant material

To study polyembryony in lemon, mature fruits from 27 cultivars and selections were analysed. To gain a better understanding, the cultivars and selections were grouped by origin or similarity in the following groups:

*Fino I: Spanish selections of 'Fino' cultivar ('Fino 46', 47, 48, 49, 77 and 95 and 'Chaparro').
*Fino II: 'Fino' type lemon cultivars ('Lisbon', 'Laphitos' and 'Mezsara').
*Verna: 'Verna' type lemon cultivars and Spanish selections of 'Verna' cultivar ('Vakalov', 'Verna 50', 51, 62, 70 and 96).
*Italian lemon trees ('Adamo', 'Flor de Arancio' and 'Rubitaro').
*Femminello: selections of 'Femminello' ('Santa Teresa', 'Campisi', 'Greco' and 'Messina').
*Eureka: lemon cultivars 'Eureka' type ('Eureka', 'Villafranca' and 'Betera').

To study the embryo rescue of 'Eureka' and 'Verna 51', lemon fruits from natural pollination were used.

Material was obtained from the collection of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), at La Alberca (Murcia) and at the Torreblanca farm (Campo de Cartagena-Murcia).

Polyembryony analysis

Fruits were collected and harvested between December and January. They were cut at the equatorial zone, avoiding the core where seeds are embedded, and opened. Seeds were removed, washed in running water and detergent (Mistol®, Henkel Ibérica, S.A.) and dried. By means of a longitudinal cut, the inner integument was removed. Embryo extraction and counting were performed under a stereoscopic microscope, using a scalpel and forceps.

Experimental design and statistical analysis

Twenty-five mature fruits and 11–50 seeds were analysed per cultivar, depending on fruit availability and seed number per fruit. The number of embryos per seed and the monoembryonic seeds per cultivar were recorded.

The cultivar effect on the number of embryos per seed was analysed by analysis of variance and differences between cultivars were tested by a Least Significant Difference (LSD) test.

The effect of cultivar on the monoembryonic seeds percentage was tested by means of maximum likelihood ANOVA. Differences among cultivars were analysed by maximum likelihood contrasts.

Immature embryo rescue

Embryo excision

Embryos to be grown in vitro were excised from fruits of 3.5-cm equatorial diameter. The fruits were surface-sterilised by washing with water and detergent (Mistol®, Henkel Ibérica, S.A.) and then shaken for 30 min in 20% commercial bleach (5.25% NaClO) with 2–3 drops/l of detergent. Finally, the fruits were rinsed three times with sterile water, under aseptic conditions.

The fruits were cut, in sterile conditions, at the equatorial zone, avoiding the core where seeds are embedded, and opened. The seeds were dissected and placed into sterile water, to avoid drying. The seed size was 4–5 mm.

Immature embryos were excised carefully, under a stereomicroscope, from the micropilar end of the seed, after removing the seed coat. Excised embryos were plated immediately on nutrient media, to induce germination. All embryos, independent of their growth stage, were used.

Induction of immature embryo germination

The germination treatments were combinations of two culture media, MS and Gamborg's B5 (G-B5) (Gamborg et al. 1968), and two sucrose concentrations (50 and 70 g/l). All media contained 0.5 g/l malt extract, 1.5 mg/l GA and 6 g/l agar (Hispanlab). After addition of plant growth regulators and adjustment of pH to 5.7 with 1 N NaOH, the media were sterilised in an autoclave, at 121°C for 21 min, and were dispensed in 55-mm Petri plates. Embryos were grown at 25 ± 1°C with white light (5,000 lux) and a 16-h photoperiod.

To induce further seedling growth, 15–20 days after germination, embryos were transferred onto MS supplemented with 20 g/l sucrose and 0.02 mg/l naphthalene acetic acid (NAA). The medium was dispensed in 20 × 150-mm culture tubes covered with permeable membrane caps, before autoclave sterilisation. Medium sterilisation and culture conditions were as described above.

Optimal developmental stage for immature embryo rescue

To study the optimum developmental stage for immature embryo rescue, fruits of 'Eureka' with equatorial diameter of 2, 2.5, 3, 3.5, 4, 4.5 or 5 cm were used, corresponding to 50, 65, 80, 100, 135, 150 or 165 days after anthesis (DAA), respectively (García Lidón 2003).

The germination culture medium was composed of G-B5 medium and 50 g/l sucrose. Fruit sterilisation, embryo excision, media and culture conditions were as described above.

Experimental design and statistical analyses

Four embryos per Petri dish were cultured, eight dishes were used per germination treatment and a single seedling was grown per test tube to induce plant growth. Treatments applied to induce immature embryo germination were arranged in a 2 × 2 factorial experiment. We studied the effect of culture medium on survival and germination percentage 15–20 days after culture initiation, and on shoot height and root length after culturing for 4 weeks in growth medium.

To study the optimum developmental stage, we used 10 dishes per treatment, with four embryos per dish; the effects of embryo developmental stage on the survival, germination and later embryo growth were recorded.

Statistical analysis was performed using the percentages within each Petri dish as replicates. The treatment effects on survival and germination were analysed by means of maximum likelihood ANOVA; differences between treatments were tested by

maximum likelihood contrasts. Embryo growth, shoot height and root length were analysed by analysis of variance. Differences among treatment means were tested by a Least Significant Difference (LSD) test. If necessary, the data were transformed to achieve homogeneity of variances, prior to analysis.

Results and discussion

Polyembryony analysis

Most citrus cultivars are polyembryonic (Soost and Cameron 1975) and polyembryony constitutes one of the major problems in citrus improvement because it makes the recuperation and identification of hybrids difficult, particularly in crosses involving taxonomically closely related parents.

In lemon, significant differences ($P < 0.001$) were observed between the lemon tree groups for the monoembryonic seeds percentage (Table 1). The highest percentage of monoembryonic seeds was in the Italian group (average of 75.0%) and the lowest was in the Fino I group (average of 56.9%). Significant differences ($P < 0.05$) within groups were observed for the Femminello and Fino II groups. When cultivars in these groups were analysed separately, we observed significant differences between 'Messina' and 'Campisi' or 'Sta. Teresa' in the Femminello group (Table 1). Differences from 'Greco' were not significant and the highest percentage of monoembryonic seeds was observed in 'Messina'. In the Fino II group, significant differences were observed between 'Lisbon' and 'Laphitos' or 'Mezsara' and the highest percentage of monoembryonic seeds was observed in 'Lisbon' (Table 1).

Between 1 and 6 embryos per seed were found (Table 1) and, in seeds with more than two embryos, several malformations in the cotyledons were observed, with one embryo being more developed and some deformed embryos being smaller. Significant differences ($P < 0.001$) were observed between groups and within the Femminello group ($P < 0.05$). The highest number of embryos per seed was in the Fino I group (1.6 on average) and the lowest was in the Fino II group (1.3 on average). When cultivars in the Femminello group were analysed, we observed significant differences between 'Messina' and 'Campisi' or 'Sta. Teresa' (Table 1). Differences from 'Greco' were not significant and the lowest number of embryos per seed was observed in 'Messina'. These values are lower than those obtained in sweet orange, with up to 15 embryos per seed (Moreira et al. 1947), or 'Pera' sweet orange and 'Cleopatra' mandarin, with 6.3 and 10.1 embryos per seed, respectively (Soares-Filho et al. 1992). Polyembryony differs as much within as between species, having both genetic (Cameron and Soost 1979; Deidda and Chessa 1982) and environmental causes (Ikeda 1982; Tusa et al. 1983).

Cameron and Soost (1979) defined as monoembryonic those cultivars whose number of polyembryonic seeds does not reach 7%. All the lemon cultivars analysed could be considered slightly or moderately polyembryonic: the lowest percentage of polyembryonic seeds was observed in the Italian group, with 25.0% on average, and the highest percentage was in the Fino I group, with 43.0% on average. The cultivars in the Italian group could be good candidates for use as mother plants for sexual hybridisations in lemon.

Embryo rescue

Induction of immature embryo germination

The analysis of maximum likelihood ANOVA showed that the embryo survival of 'Eureka' and 'Verna 51' was affected significantly by the medium salt ($P < 0.01$) and the cultivar ($P < 0.05$). The effect of sucrose concentration was not significant. Survival in 'Eureka' was greater than in 'Verna 51' (90.0% and 78.3%, respectively, averaged over all treatments) and G-B5 medium (90.3%, average of the two cultivars) produced greater survival than MS medium (77.9%, average of the two cultivars) (Table 2).

Embryo germination was only affected significantly by the cultivar ($P < 0.01$) and by the cultivar x medium interaction ($P < 0.05$). Germination of 'Eureka' (53.6%, averaged over all treatments) was greater than for 'Verna 51' (36.0%, averaged over all treatments) (Table 2). Embryos which failed to develop into plants showed incomplete germination or remained embryonic. Some embryos produced embryogenic callus.

MS and G-B5 media, with certain degrees of modification, are the most widely used basal media in

Table 1 Mean and range for the number of embryos per seed and percentage of monoembryonic seeds in 27 cultivars and selections of lemon tree

Cultivars	Total seeds	Embryos per seed	Range		Monoembryony (%)
			Min.	Max.	
EUREKA					
'Eureka'	46	1.6 ± 0.8	1	4	52.2 ± 7.4
'Betera'	50	1.5 ± 0.8	1	4	60.0 ± 6.9
'Villafranca'	50	1.4 ± 0.7	1	4	68.0 ± 6.6
FINO I					
'Fino 46'	38	1.3 ± 0.5	1	2	65.8 ± 7.7
'Fino 47'	50	1.8 ± 0.9	1	4	46.0 ± 7.1
'Fino 48'	50	1.6 ± 0.9	1	6	57.1 ± 7.1
'Fino 49'	50	1.7 ± 0.9	1	4	52.0 ± 7.1
'Fino 77'	50	1.5 ± 0.8	1	5	63.3 ± 6.9
'Fino 95'	48	1.5 ± 0.8	1	4	66.7 ± 6.8
'Chaparro'	50	1.6 ± 0.7	1	4	50.0 ± 7.1
FINO II					
'Lisbon'	50	1.3 ± 0.6	1	3	50.0 ± 9.1b
'Laphitos'	50	1.3 ± 0.5	1	3	74.0 ± 6.2a
'Mezsara'	50	1.2 ± 0.4	1	2	82.0 ± 5.4a
FEMMINELLO					
'Sta. Teresa'	50	1.5 ± 0.6b	1	3	60.0 ± 6.9b
'Campisi'	14	1.6 ± 0.8b	1	3	57.1 ± 13.2b
'Messina'	50	1.2 ± 0.4a	1	3	86.0 ± 4.9a
'Greco'	50	1.3 ± 0.5ab	1	3	70.0 ± 6.5ab
ITALIAN					
'Adamo'	11	1.3 ± 0.7	1	3	81.8 ± 11.6
'Flor de Arancio'	50	1.4 ± 0.6	1	3	68.0 ± 6.6
'Rubitaro'	31	1.2 ± 0.6a	1	4	83.9 ± 6.6
VERNA					
'Verna 50'	32	1.5 ± 0.7	1	4	56.3 ± 8.8
'Verna 51'	50	1.4 ± 0.6	1	3	68.0 ± 6.6
'Verna 62'	50	1.4 ± 0.6	1	3	70.0 ± 6.5
'Verna 70'	32	1.4 ± 0.8	1	4	71.9 ± 7.9
'Verna 96'	35	1.4 ± 0.6	1	3	68.6 ± 7.8
'Vakalov'	50	1.3 ± 0.5	1	3	74.0 ± 6.2

Data are means ± standard error. Different letters indicate significant differences ($P < 0.05$) according to contrasts of maximum likelihood, for monoembryony, or according to the LSD test, for the mean number of embryos per seed

embryo culture (Bridgen 1994). High germination rates of aborted embryos obtained from monoembryonic seed progenitors have been induced on MS (Starrantino 1992). Furthermore, triploid lemon hybrids have been rescued on MS supplemented with malt extract and adenine sulphate (Starrantino and Reforgiato-Recupero 1981) or MT (Tusa et al. 1996), although the germination rates were not given in these studies. However, mandarin embryos did not germinate well on an identical basal medium supplemented with giberellic acid (Khan et al. 1996) and Gamborg's medium induced the highest germination in acid citrus fruit (Viloria et al. 2005). In our work, when cultivars were studied separately, the germination of 'Verna 51' was significantly higher in MS, contrasting with a lack of effect of the media on the germination of 'Eureka'.

On the other hand, increasing the sucrose concentration enhanced the germination rates of mandarin embryos (Sykes and Lewis 1996) and Carimi et al. (1998) observed that high sucrose concentrations in MT medium were necessary for the germination of

Table 2 Survival and germination percentages of 'Eureka' and 'Verna 51' embryos obtained with four different germination media

Cultivar	Medium	Sucrose	Survival (%)	Germination (%)
'EUREKA'	MS	50	87.5 ± 5.9	46.4 ± 9.4
	MS	70	84.4 ± 6.4	51.9 ± 9.6
	G-B5	50	91.2 ± 4.9	51.6 ± 9.0
	G-B5	70	96.9 ± 3.1	64.5 ± 8.6
'VERNA 51'	MS	50	62.5 ± 8.6	40.0 ± 10.0
	MS	70	77.4 ± 7.5	50.0 ± 10.2
	G-B5	50	93.8 ± 4.3	23.3 ± 7.7
	G-B5	70	79.3 ± 7.5	30.8 ± 9.1

Data are means ± standard error

pro-embryos from *Citrus aurantium*. For lemon embryos, the sucrose concentration in the medium did not affect germination, in agreement with the results obtained by Viloria et al. (2005) for acid citrus fruit.

Induction of seedling development

Optimal seedling development was achieved after four weeks in the growth medium (Fig. 1).

The sucrose concentration and MS medium strength influenced the growth and development of the aerial parts as well as the plantlet root system in the culture of immature embryos of mandarin fruits (Pasqual et al. 2003). In lemon, although plant height, after four weeks in the growth medium, was not affected by any variable (cultivar, sucrose or germination medium), root length was affected significantly by the cultivar ($P < 0.05$) and the nutrient medium on which embryos were germinated ($P < 0.001$). Roots of 'Eureka' were longer than those of 'Verna 51' and seedlings growing on germination medium with G-B5 produced longer roots (Table 3). These results are the opposite of those obtained by Viloria et al. (2005) since, although root length was the only variable affected by the medium for acid citrus fruit, MS medium significantly enhanced root growth compared with G-B5.

Fig. 1 Seedlings of 'Eureka' and 'Verna 51' after 4 weeks in the growth medium

Optimum developmental stage for immature embryo rescue

The success of embryo rescue is determined, to a great extent, by the embryo development stage. Different authors have reported that younger embryos are the most difficult to rescue in vitro (Bridgen 1994; Hu and Wang 1986; Pierik 1987).

In 'Eureka' lemon fruits, none of the immature seeds taken from fruits harvested at 50, 65 or 80 DAA (fruits of equatorial diameter 2, 2.5 and 3 cm, respectively) contained visible embryos. In contrast, at 165 DAP (5-cm equatorial diameter), the seeds had developed too much and it was impossible to separate the different embryos. These fruits were not used in the study. These findings are in agreement with those of Carimi et al. (1998) for *Citrus aurantium*. These authors observed, in immature seeds dissected from fruits harvested 65 and 85 days after pollination, no visible embryos, and in seeds collected 125–220 days after anthesis, the zygotic embryo (if not aborted) was mixed with many nucellar embryos and these could not be distinguished from each other on the bases of size and position in the seed.

Rangan et al. (1969) observed that nucellar embryos had not yet been found in the developing seeds of sour orange 120 days after anthesis, while the zygotic embryo had already reached the heart-shaped stage. Hence, embryo culture could make screening of nucellar and zygotic seedlings in polyembryonic cultivars unnecessary. In our study, most of the immature seeds harvested at 100–110 DAA (3.5-cm equatorial diameter) contained one embryo though some seeds with two or three embryos could be observed, and fruits at 135–150 DAA (equatorial diameter of 4–4.5 cm) contained several embryos per seed (Fig. 2).

The analysis of variance shows that, in seeds harvested 100–150 DAA, survival was not affected by the developmental stage of the immature embryo, and was high in all cases (Table 4). However,

Table 3 Plant height and root length of 'Eureka' and 'Verna 51' seedlings, obtained from embryos germinated in vitro on four different media, after 4 weeks in the growth media

Cultivar	Medium	Sucrose (g/l)	No. of seedlings	Plant height (mm)	Root length (mm)
'EUREKA'	MS	50	13	7.9 ± 4.6	20.6 ± 17.5
	MS	70	14	6.6 ± 2.5	20.0 ± 15.2
	G-B5	50	16	6.9 ± 2.7	34.2 ± 21.1
	G-B5	70	20	8.1 ± 4.0	35.0 ± 23.4
'VERNA 51'	MS	50	9	11.8 ± 7.9	17.9 ± 14.0
	MS	70	12	6.1 ± 4.0	15.5 ± 12.3
	G-B5	50	6	7.8 ± 3.0	27.2 ± 25.7
	G-B5	70	8	6.9 ± 3.0	20.6 ± 10.8

Data are means ± standard error

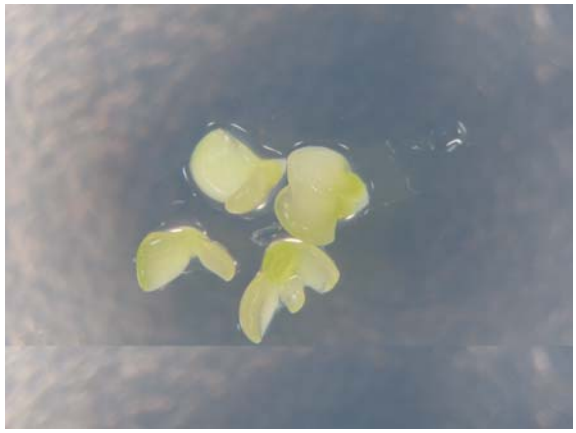

Fig. 2 Immature embryos dissected from only one seed, in a fruit harvested 150 days after anthesis (DAA)

germination was affected by the developmental stage ($P < 0.01$). When contrasts of maximum likelihood were applied, we observed that the germination percentages were significantly higher in embryos of immature seeds harvested at 135 or 150 DAA. Significant differences were not observed between 135 and 150 DAA (Table 4). On the other hand, seedling height, after 4 weeks in the growth medium, was affected significantly by the fruit age ($P < 0.001$), but differences were not observed for root length (Table 5). The LSD test showed that embryos from immature seeds harvested 135 or 150 DAA generated significantly longer shoots (Table 4). These results are in agreement with those of Carimi et al. (1998) who found, in sour orange, that the frequency of germination and plant formation increased with increasing age; while for germination of globular embryos the plant formation was reduced.

Table 4 Survival and germination percentages of 'Eureka' embryos obtained at three different developmental stages

DAA	Survival (%)	Germination (%)
100	85.7 ± 5.0	55.1 ± 7.1b
135	93.9 ± 3.4	75.5 ± 6.1a
150	98.0 ± 2.0	87.7 ± 4.7a

Data are means ± standard error. DAA: days after anthesis. Different letters indicate significant differences according to contrasts of maximum likelihood

Table 5 Plant height and root length of 'Eureka' seedlings, obtained from embryos at three different developmental stages, after 4 weeks in the growth medium

DAA	N° Seedlings	Plant height (mm)	Root length (mm)
100	27	7.5 ± 5.2b	46.8 ± 27.3
135	36	12.5 ± 4.3a	56.7 ± 24.0
150	43	11.6 ± 5.7a	48.8 ± 28.4

Data are means ± standard error. DAA: days after anthesis. Different letters indicate significant differences according to the LSD test

Conclusions

The lemon cultivars analysed could be classified as having limited or moderate polyembryony. In the study of the rescue and in vitro culture of immature embryos, an important effect of genotype was observed, and, while the sucrose concentration in the medium did not have an effect on germination, the medium salt composition affected the embryo survival and root development of the seedlings; Gamborg's B5 medium giving the best results. As expected, the ability to form plants in vitro was affected by an increase of embryo developmental stage. The germination and seedling height were

greater with embryos of seeds collected 135–150 days after anthesis.

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