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MICROPROPAGATION

The effect of gibberellic acid on the *in vitro* germination of coconut zygotic embryos and their conversion into plantlets

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Abstract The effect of gibberellic acid (GA_3) was tested on germination of coconut zygotic embryos, their conversion into plantlets and *ex vitro* survival. There were four treatments consisting of 5 wk of culture in semi-solid medium or liquid medium, with or without GA_3. Embryos were then transferred to GA_3 free-liquid medium for the rest of a 32-wk culture. Germination and conversion percentages were higher in semi-solid medium than in liquid medium, and with both media percentages increased with GA_3 treatment (with the exception of the highest GA_3 concentration). Embryos of two varieties (MGD and MYD) were used. The following are the results with MGD embryos. Optimum GA_3 concentration in liquid medium was 0.46 μM, with 80% germination (62% in the control without GA_3) and 4.6 μM in semi-solid medium with 98% germination (71% in the control). With GA_3 treatment, germination was also faster. Conversion in semi-solid medium with GA_3 was 87% (60% in the control), and 45% in liquid medium with GA_3 (25% in the control). Once the plantlets had at least three bifid leaves and three primary roots at the time of transfer to *ex vitro*, they survived independently of the treatment. When MYD embryos were used, germination and conversion percentages were higher in semi-solid medium than in liquid medium, and they increased when GA_3 was used, although percentages were lower than those obtained with MGD embryos. The results showed that the use of GA_3 benefited coconut embryos in culture because it favored germination and conversion to plants on semi-solid medium, and hence improved previous protocols.

Keywords *Cocos nucifera* · Zygotic embryo culture · Gibberellin treatment · Semi-solid and liquid media · Growth *ex vitro*

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Introduction

The coconut palm (*Cocos nucifera* L.) is an important cash and subsistence crop for many communities in the tropics. Unfortunately, it is severely affected by the devastating phytoplasma-associated disease lethal yellowing (LY) (McCoy, 1972; Plavsic-Banjac et al., 1972) in America (Oropeza and Zizumbo, 1997) and the lethal yellowing-like diseases in Africa (Eden-Green, 1997). In Mexico, LY was introduced in 1977 and has since affected most of the country's coconut growing areas (Oropeza and Zizumbo, 1997). The most effective way to deal with this disease is by replanting with resistant palm genotypes (Been, 1995). Field trials of Mexican coconut germplasm have identified several genotypes with LY resistance (Zizumbo et al., 1999). Nevertheless, testing of more genotypes is needed to have a wider genetic base for replanting and for genetic improvement programs. Thus, it is necessary that coconut genotypes from other parts of the world be introduced into Mexico. To do this safely, the Food and Agriculture Organization/International Board for Plant Genetic Resources has recommended the use of the technique of *in vitro* embryo culture that allows embryo germination and conversion into plantlets in a controlled environment (Frison et al., 1993). However, assays with this technique in different laboratories in the

world showed low efficiency in embryo germination and post-germinative development, resulting in less than 40% conversion compared to about 80% from seed (see Batugal and Engelmann, 1998), thus limiting the application of this technique because the loss of germplasm due to inefficient *in vitro* protocols may lead to unintentional genetic selection. In our laboratory, we have carried out studies to improve the efficiency of coconut embryo culture and have shown that aerobic respiration facilitates embryo germination and conversion into plantlets. This was achieved when embryos were cultured in semi-solid medium and placed with their micropyle end facing upward exposed to the vial atmosphere (Pech y Aké et al., 2004), which is not possible when embryos are cultured in liquid medium. Then, comparing the effect of culturing embryos (var. Malayan Green Dwarf, MGD) in liquid medium *versus* semi-solid medium, germination increased from 65 to 93% and conversion into plantlets (ready for transfer to *ex vitro* conditions) increased from 46 to 89% (Pech y Aké et al., 2004).

To further improve the efficiency of the embryo culture technique for coconut, the effect of gibberellic acid (GA_3) was tested on germination of zygotic embryos and their conversion into plantlets and *ex vitro* survival. It is known that this phythormone promotes seed germination in other species in nature (Evans et al., 1996; Gaspar et al., 1996; Yoshida and Hirawasa, 1996; Kaur et al., 1998) and *in vitro* for embryos (Geerts et al., 1999; White and Rivin, 2000) and for seeds (Arnalte et al., 1991; Jacobsen et al., 1995). There are also reports showing that GA_3 promotes post-germinative development and conversion into plantlets in different species (Evans et al., 1996; Rascio et al., 1998; Lev-Yadun et al., 1999). In the case of coconut embryo culture, the effect of GA_3 was first investigated by Karun et al. (2001). No significant differences in embryo germination were found between media containing the hormone, a control (hormone-free) medium. Neither was there an effect of the hormone on shoot length and root length. This paper reports a positive effect of GA_3 on *in vitro* germination and conversion into plantlets of coconut zygotic embryos.

Materials and Methods

Plant material. Embryos were obtained from nuts of Malayan Green Dwarf (MGD) and Malayan Yellow Dwarf (MYD) coconut palms, collected in Dzidzantun, Yucatan, Mexico. The nuts were 12–14 mo. post-anthesis when harvested. They were cut transversely to excise the endosperm enclosing the embryo from the open nuts using a cork borer (1.6 cm diameter).

Sterilization. Immediately after excision, endosperm cylinders were placed for 20 min in a 0.6% (v/v) NaOCl solution (diluted commercial bleach), subsequently surface-sterilized for 3 min with 70% ethanol, rinsed three times with sterile distilled water, washed with agitation for 20 min with a 3% NaOCl solution and finally rinsed three times with sterile distilled water. Once in the laboratory, under aseptic conditions, the endosperm cylinders were washed for 3 min with 70% ethanol, rinsed three times with distilled sterile water, washed for 20 min with a 3% NaOCl solution and rinsed three times with distilled sterile water. The embryos were then excised from the endosperm cylinders and washed for 10 min with a 0.6% NaOCl solution and rinsed three times with sterile distilled water.

Culture media and culture conditions. All chemicals were purchased from Sigma (St. Louis, MO). Each embryo was cultured in a 50-ml culture vessel containing 10 ml of Y3 medium (Rillo and Paloma, 1992), with the addition of activated charcoal (2.5 g l^{-1}). Media were prepared with or without 3 g l^{-1} gelrite resulting in semi-solid medium or liquid medium correspondingly. The pH was adjusted to 5.75 with a 1 M NaOH solution before autoclaving for 20 min at 120°C and 105 kPa.

Germination. To test the effect of GA_3 on germination of MGD embryos, they were cultured for 5 wk in a vessel containing 10 ml of liquid (without shaking) or semi-solid media with different phytohormone concentrations (0.046, 0.46, 4.6, and 46 μM). The GA_3 solution was filtered through a 2-μm Millipore membrane and then added to the sterilized media. When embryos were cultured in semi-solid medium, they were placed with their micropylar end facing upwards (to expose it to the vial atmosphere) according to Pech y Aké et al., (2004). Tests were carried out in the dark at 27±2°C.

Post-germinative development and conversion. To test the effect of the germination conditions and transfer to solid or liquid medium on post-germinative development and conversion, MGD and Malayan Yellow Dwarf (MYD) embryos were germinated at the optimal concentrations of GA_3 already determined for semi-solid or liquid media. After germination, they were transferred to 145-ml vessels with 25 ml of liquid or semi-solid GA_3-free medium. Five wk later, when shoots started growing, all of them, regardless of previous treatment, were transferred to 500-ml vessels with 50 ml of GA_3-free fresh liquid medium. During further development of shoots into plantlets, they were subcultured in 50 ml of fresh Y3 liquid medium every 6 wk. Finally, isotactic isopropylene bags were placed on top of the containers to increase the space for plantlet growth (according to Rillo, 1998). Plantlets were kept *in vitro* until they developed at least three bifid leaves and three primary roots. All post germinative tests were carried

out under 16 h photoperiod (45–60 μmol m^{-2} s^{-1} photosynthetic photon flux density, PPFD) at 27±2°C.

Plantlet development. This was evaluated only for MGD shoots and plantlets for the following parameters: height, fresh weight, number of bifid leaves, and primary roots formed. For the evaluation, at the end of 32 wk of *in vitro* culture, shoots or plantlets were separated into four groups. The first group included shoots 10 cm or less of height, whereas those that had converted into plantlets were separated into three groups depending on the height: 10–20, >20–30, and >30–40 cm.

Plantlet acclimation. After 32 wk plantlets were transferred to a greenhouse and planted in black polyethylene bags containing a mixture of peatmoss and soil (1:1, w/w) and covered with transparent polyethylene bags with 15 1.5-cm slits on each side to increase the atmospheric exchange. After 1 wk, the transparent covers were removed and plantlets were left uncovered for a further 3-wk period in the greenhouse conditions (16 h photoperiod; 218 μmol m^{-2} s^{-1} photosynthetic photon flux density, PPFD; at 33±2°C) before being transferred to shaded nursery (16 h photoperiod; 506 μmol m^{-2} s^{-1} PPFD; at 36±2°C).

Experimental design and statistical analysis. Unless otherwise indicated, each treatment included 20 embryos or plantlets for assays in liquid medium and 30 for assays with semi-solid medium, and each of the experiments was carried out three times. ANOVA and Newman and Newman–Keuls test (significance level, P=0.05) were applied for multiple comparisons of means, using Sigma Stat for Windows version 1.0. For data in Fig. 2, sigmoid curves were adjusted for the observed data using the program Jandel Table curve 2D version 3 for Windows 3.2 and plotted using Jandel Scientific Sigma.

Results and Discussion

Embryo germination. An initial assay was carried culturing MGD coconut embryos for 5 wk in liquid or semi-solid medium with different concentrations of GA_3 (0, 0.046, 0.46, 4.6, or 46.0 μM) to evaluate if it had any effect on the frequency of germination. After 5 wk the embryos were transferred to GA_3-free-liquid medium. When they were transferred to semi-solid medium, the development was arrested with shoots developing very slowly and no conversion occurred in any of the four treatments, whereas when they were transferred to liquid medium there was development leading to conversion, as previously reported by Pech y Aké et al. (2004). Therefore, for the results presented below, embryos were subjected during the initial 5-wk culture to different treatments (using either liquid or semi-solid medium) before being transferred only to liquid medium, independently of the treatment.

At the end of the 5-wk culture, the germination frequency was higher in semi-solid medium than in liquid medium, and also either with semi-solid or liquid medium the addition of GA_3 was beneficial (Fig. 1*a*). The best GA_3 treatment in liquid medium was with 0.46 μM, with 30% germination compared to 6% in the control (without GA_3), whereas the best treatment in semi-solid medium was with 4.6 μM GA_3, with 80% germination compared to 55% in the control. The need for a higher GA_3 concentration in semi-solid medium compared with liquid medium, probably resulted from a binding of the gibberellin molecules to gelrite.

Similar trends with respect to the type of medium and GA_3 concentration were observed at the end of 30 wk of culture, but germination frequencies were higher (Fig. 1*b*). The best GA_3 treatment in liquid medium was 0.46 μM,

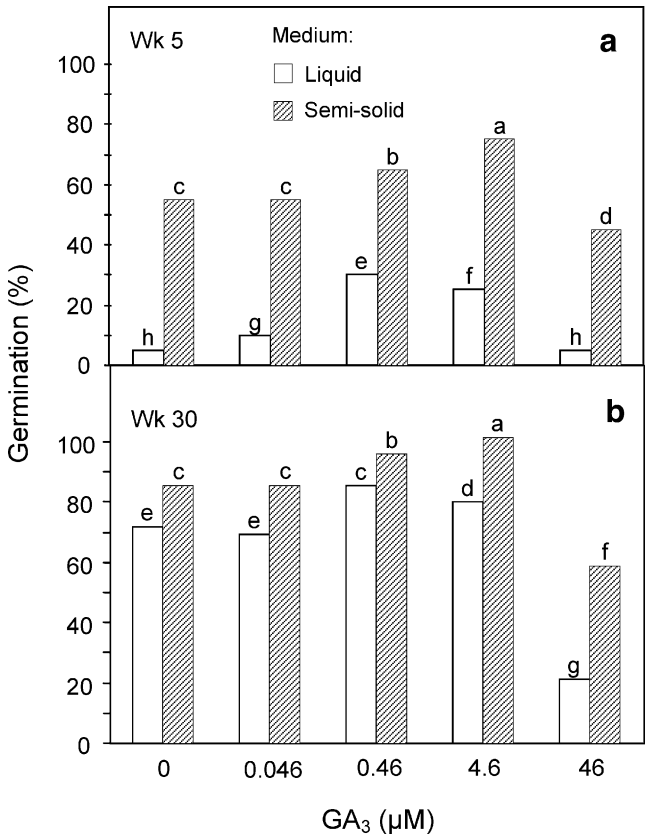

Figure 1. Effect of different concentrations of gibberellic acid (GA_3) on the germination of coconut (Green Malayan Dwarf variety) zygotic embryos after: (*a*) 5 wk or (*b*) 30 wk of culture. Each treatment included three batches of 20 or 30 embryos for liquid or semi-solid medium, respectively. Different *letters* above the *bars* denote significant differences among them.

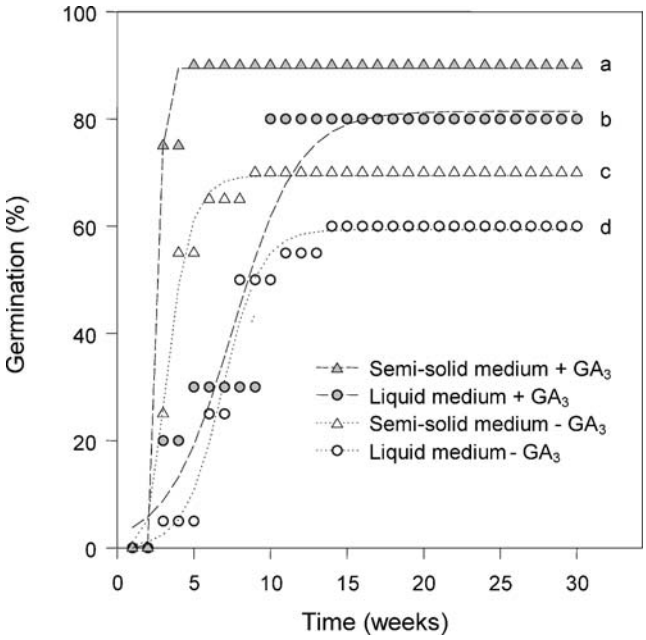

Figure 2. Time course of the effect of gibberellic acid (GA_3) on the germination of coconut (Green Malayan Dwarf variety) zygotic embryos. Each treatment included three batches of 20 or 30 embryos for liquid or semi-solid medium, respectively. Different *letters* associated with the treatments denote significant differences among them ($p<0.05$).

with 85% germination compared to 71% in the control, and the best treatment in semi-solid medium was 4.6 μM GA_3, with 98% germination compared to 86% in the control. These results demonstrated that the germination was favored by semi-solid medium as previously reported by Pech y Aké et al., (2004), but also that GA_3 improved this response using either of the two media, although the best response was achieved using the combination of semi-solid medium and GA_3.

To define more accurately the responses to GA_3, a time course assay was carried out using only the best gibberellin concentrations for solid and liquid medium (Fig. 2). The results were similar to those of the first experiment. Germination was more frequent in semi-solid medium than in liquid medium, and GA_3 not only promoted the frequency but also accelerated the process compared with the control. In only 5 wk in semi-solid medium with GA_3, germination reached 98%, whereas without GA_3 9 wk were required to reach 71%. Germination was also faster with gibberellin in liquid medium, but the extent of the increase was lower than in semi-solid medium, as it required 10 wk to reach 80%. The lowest and slowest germination occurred in liquid medium without GA_3. After 14 wk germination was only 62% (Fig. 2).

The effect of GA_3 (0.01 to 1.0 μM) on the germination of coconut zygotic embryos was tested by Karun et al., (2001) and, although they found increased germination in relation to the untreated embryos, the differences were not significant. This result might differ from ours because they used a lower range of gibberellin concentration.

The promoting effects of GA_3 on both the frequency and the rapidity of germination of coconut embryos might suggest that there is a hormonal imbalance in some of the embryos, probably related to embryo immaturity, a phenomenon known to be frequent in palms (Orozco-Segovia et al., 2003). Furthermore, increased germination frequency and rapidity as a result of GA_3 treatment have also been reported with seeds of other palm species such as *Aiphanes erosa*, *Arenga microcarpa*, *Hyphaene schatan*, *Sabal palmetto*, *Phoenicoforium borsiqianum* (29–72 μM, Odetola 1987) and *Howea forsteriana* (290 μM, Chin et al., 1988). GA_3 also promoted germination of non-palm species such as Zea mays (seeds, 10–100 μM, White and Rivin, 2000) and *Panax ginseng* (somatic embryos, 2.9 μM, Kwang-Tae et al., 2000).

The embryos germination was asynchronous, both in liquid and in semi-solid medium, which has also been reported in the nut (Zizumbo-Villarreal and Arellano-Morin, 1988). However, since the treatment with GA_3 promoted the germination rapidity, it concomitantly improved the synchrony of embryo germination, particularly in the semi-solid medium.

Conversion and development of plants. Conversion into plantlets was studied with embryos of two coconut varieties, MGD (used in the previous experiments) and Malayan Yellow Dwarf (MYD). For both at the end of wk 36, conversion was affected by the type of medium (semi-solid or liquid) and the GA_3 treatment during the first 5 wk of culture (Fig. 3). As mentioned before, it was also found that after this initial culture, if embryos were transferred to semi-solid medium, shoots developed very slowly and no conversion occurred with any of the treatments. In contrast, conversion occurred if the embryos were transferred to liquid medium and values varied according to the treatment.

The MGD embryos cultured in semi-solid medium with 4.6 μM GA_3 conversion was 87% compared to 60% in the control without gibberellin; and in liquid medium with 0.46 μM GA_3, it was 45% compared to 25% in the control (Fig. 3*a*). Conversion of MYD embryos in semi-solid medium with 4.6 μM GA_3 was 60% compared with 43% in the control without gibberellin; and in liquid medium with 0.46 μM GA_3, it was 32% compared to 20% in the control (Fig. 3*b*). Therefore, for both varieties, conversion percentage of embryos initially cultured in semi-solid medium was higher than when cultured in liquid medium, and it increased further when the medium contained GA_3. According to these data, the trends in conversion apparently were influenced by the semi-solid medium and GA_3

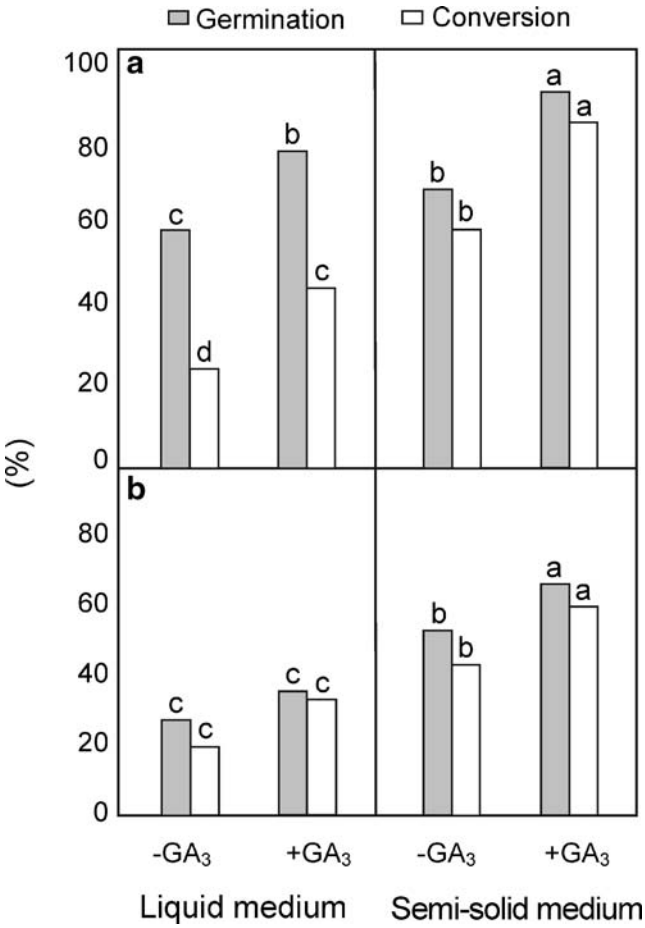

Figure 3. (*a*) The effect of gibberellic acid (GA_3) on the germination and conversion to plantlets of coconut (Green Malayan Dwarf variety) zygotic embryos in 36 wk of culture. Each treatment included three batches of 20 or 30 embryos for liquid or semi-solid medium, respectively. (*b*) Same as above but using embryos of the Yellow Malayan Dwarf variety. Different *letters* associated with the treatments denote significant differences among them ($p<0.05$) of two varieties.

treatment on germination, since the conversion frequency increased proportionally with the germination (Fig. 3*a* and *b*). These results also showed that a protocol for coconut embryo germination based on the use of semi-solid medium and the addition of GA_3 could be useful with different genotypes, although in the case of MYD embryos, optimization might be necessary, because the GA_3 concentrations tested were those defined for MGD embryos, and they might not be optimal for MYD embryos. Previous results reported by Pech y Aké et al., 2004) on the effect of semi-solid and liquid medium on conversion of MGD embryos, also showed that with semi-solid medium, higher germination (93%) and conversion (89%) were obtained compared with liquid medium (66% and 46%, respectively), although the values were higher than those obtained in our work, probably as a result of differences between batches or seasonal conditions.

At the end of 36 wk, significant differences were observed in the height of the plantlets among treatments. The greatest percentage of taller plants were obtained when embryos were cultured in semi-solid medium with 4.6 μM GA_3 (Fig. 4), with most of them >20–30 cm tall. A slower development was obtained when embryos were cultured in liquid medium without GA_3, with most of them developing only into shoots. The other two treatments resulted in plants of height scattered within the three ranges (Fig. 4). The evaluation of the other parameters (fresh weight, number of bifid leaves and of primary roots) was carried out only on plantlets; shoots were not considered. The results did not show significant differences in any of the parameters (data not shown). There were two or three bifid leaves and two to three primary roots on all plantlets regardless of treatment. The data variation was proportionally smaller with semi-solid medium and GA_3 treatment than with the other treatments; therefore, this treatment increased developmental uniformity, probably owing to the synchronous germination.

GA_3 has been reported to promote postgerminative development and conversion into plantlets in several species (Evans et al., 1996; Rascio et al., 1998; Lev-Yadun et al., 1999). For coconut, no significant differences were found on root and shoot length between untreated embryos

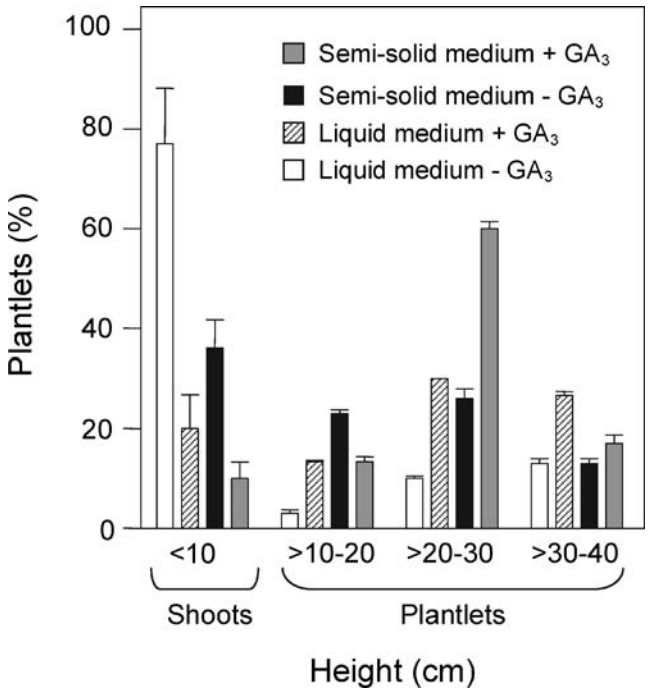

Figure 4. The effect of gibberellic acid (GA_3) on the height of shoots and plantlets obtained from coconut (var. Green Malayan Dwarf) zygotic embryos after 36 wk of culture. Analyses were carried out in four different groups defined according to height range. Each treatment included three batches of 20 or 30 embryos for liquid or semi-solid medium, respectively. *Bars* denote standard deviation and significant differences between treatments ($p<0.05$).

and embryos treated with different GA_3 concentrations (Karun et al., 2001). In similar studies with other species, GA_3 also affected plant size. In the case of *Lesquerella fendleri*, embryos treated with this gibberellin produced plantlets that were taller with fewer but longer leaves (Evans et al., 1996). Embryos of *Cercis siliquastrum* treated with GA_3 during germination, also converted into taller plantlets, but in this case with a greater number of leaves (Rascio et al., 1998). In the present work, the number of leaves of coconut did not change with GA_3.

Plantlet survival. The effect of each of the four treatments on plantlet survival *ex vitro* was evaluated at 48 wk, after they had already been in the greenhouse for 4 and 12 wk in the nursery. Plantlets were >20 cm height with at least three bifid leaves and three primary roots at the time of transfer to *ex vitro*. All plants survived independently of the treatment: liquid medium without GA_3 (10 out of 10), liquid medium with 0.46 μM GA_3 added (15 out of 15), semi-solid medium without GA_3 (33 out of 33) and solid medium with 4.6 μM GA_3 added (42 out of 42). The difference in the number of plants tested reflected availability as a result of treatment. These results showed that the survival was not dependent on the treatment but rather on the stage of development that had to be attained *in vitro* before the transfer to *ex vitro*. Plantlets >10–20 cm of height eventually grew and continue their development, but this took longer, whereas in the case of shoots, most did not develop further.

The results presented above showed that the use of GA_3 benefited coconut embryo culture as it improved the percentages of germination, conversion, and developmental uniformity, particularly when semi-solid medium was used. Although these improvements were observed in two different varieties, it will be important to evaluate this improved embryo culture protocol with a greater number of coconut varieties to determine its full potential.

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