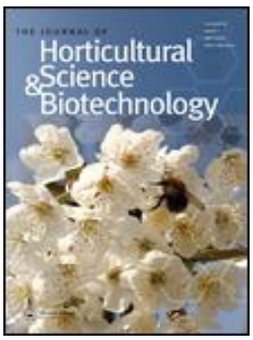

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Effect of 22(S),23(S)-homobrassinolide on somatic embryogenesis in plumule explants of *Cocos nucifera* (L.) cultured *in vitro*

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SUMMARY
The increasing demand for coconut palms resistant to diseases requires massive multiplication of improved or selected palms. This could be achieved through micropropagation. A reproducible regeneration protocol via somatic embryogenesis from plumule explants has already been reported, but its efficiency is still low. The protocol is based on the use of an auxin to induce embryogenic callus. Since brassinosteroids are known to act synergistically with auxins and might be involved in the control of plant embryogenesis, the effect of the brassinosteroid 22(S),23(S)-homobrassinolide on initial callus, embryogenic callus and somatic embryo formation in coconut plumule explants was tested. The explants were exposed (during a 3 or 7 d pre-culture) to different concentrations (0.01, 0.1, 1, 2 and 4 μM) of the brassinosteroid. The explants responded favourably to the brassinosteroid increasing their capacity to form initial callus, embryogenic callus and somatic embryos. The largest amount of somatic embryos formed, 10.8 somatic embryos per explant, was obtained exposing the explants for 3 d to the brassinosteroid at 0.01 or 0.1 μM, whereas 3.8 somatic embryos per explant were obtained from untreated explants. This is a very promising result considering the very slow progress of micropropagation research for this very recalcitrant species, that has taken now three decades since it started. This effect of a brassinosteroid on somatic embryogenesis of coconut (or of any other plant species) is as far as we know the first report.

The coconut palm (*Cocos nucifera* L.) is a very important crop in tropical areas providing cash and subsistence to small holders worldwide. However, most coconut groves require replanting because of loss due either to palm senescence or to diseases such as lethal yellowing in America (Arellano and Oropeza, 1995), the lethal diseases in Africa (Eden-Green, 1995) and cadang-cadang in Asia (Hanold and Randles, 1991). Unfortunately, improved disease-resistant planting materials are scarce and seed propagation does not yield sufficient material to satisfy the rapidly growing demands. Therefore, alternative approaches for the propagation of improved planting materials must be considered and *in vitro* cloning *via* somatic embryogenesis seems to provide a convenient alternative for the future due to its potential for massive propagation. During the 1970s and early 1980s, coconut somatic embryogenesis was reported in various laboratories using different explant sources (see Blake, 1990). Further research utilizing inflorescence (Verdeil *et al.*, 1994) and plumule (Chan *et al.*, 1998) explants has allowed the development of reproducible regeneration protocols attaining measurable efficiencies for the first time, in particular for the case of the plumule protocol with approximately 100 plantlets for every 100 plumules (Sáenz *et al.*, 1999). Unfortunately, this efficiency is still very low for practical application.

Somatic embryogenesis in plants is in general induced using phytohormones (George, 1993). In the particular case of coconut plumule explants, successful induction of embryogenic callus formation has been obtained in the presence of the synthetic auxin dichlorophenoxyacetic acid (2,4-D) (Hornung, 1995; Chan *et al.*, 1998). Somatic embryo formation on these calli is obtained by reducing 100 fold the 2,4-D concentration and adding a cytokinin (Chan *et al.*, 1998). However, the use of a cytokinin during induction of embryogenic capacity has proved detrimental (Sáenz, 2000) and according to the literature no other phytohormones have been tested on plumule explants for this purpose. Therefore, it would be necessary to test other phytohormones with potential to promote morphogenetic responses such as the brassinosteroids (BRs) that (among other physiological effects) are known to promote cell division and cell differentiation (Mandava, 1988). It has also been shown that BRs act synergistically with auxins, including 2,4-D (Katsumi, 1985), and this has been proposed to occur by enhancement of tissue sensitivity to auxins (Mandava, 1988).

In vitro applications of BRs have also been reported. Ponsamuel *et al.* (1996) reported the stimulation of somatic embryo conversion in tea explants. Ponsamuel *et al.* (1998) reported a stimulation of the organogenesis process in peanut. Although there are no reports in the literature on the effect of these compounds on the formation of somatic embryos, there is evidence that sterols and BRs play a role in the regulation and signalling in cell division and cell expansion in embryogenic and post-embryogenic development in plants (Jang *et al.*, 2000). The present paper reports the utilization of a brassinosteroid compound to promote the formation of initial callus, embryogenic callus and somatic embryos in coconut plumular tissue.

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MATERIALS AND METHODS

Plant material

The fruit were harvested 12–14 months after pollination (except where otherwise indicated) from 15 year old Malayan Dwarf coconut palms at San Crisanto, Yucatán, México. The fruit were cut transversely with a machete revealing the embryos surrounded by solid endosperm. Embryos were excised from the open fruit using a cork borer (1.6 cm diameter) and placed in distilled water. Under aseptic conditions, the endosperm enclosing the embryo was washed in 70% ethanol in water (v/v) for 3 min and rinsed three times with sterile distilled water, washed in a 6% NaClO solution (w/v) for 20 min and rinsed three times with sterile distilled water. The embryos were excised from the endosperm and washed in a 0.6% NaClO solution (w/v) for 10 min and rinsed with sterile distilled water three times. Embryos were 5–7 mm long and each weighed approximately 100 mg. The plumules were excised from these embryos using a stereoscopic microscope and placed directly in culture medium.

Culture media and conditions

All chemicals were obtained from Sigma (USA). Each explant was cultured in 35 ml culture vessels containing 10 ml of Y3 medium (Eeuwens, 1976), added with gelrite (3 g l^{-1}) and charcoal (acid washed – PCCT, 2.5 g l^{-1}). Growth regulator concentrations were: 0.55 mM 2,4-D for medium I (this concentration was reduced when explants were pre-cultured in modified medium I, see below); and 6 μM 2,4-D and 300 μM 6-benzylaminopurine (6-BAP) for medium II. The pH of the medium was adjusted to 5.75 before autoclaving for 20 min at 120°C. The explants in medium I were incubated in conditions 1: darkness for 3 months (or as indicated in the text) at 27 ± 2°C without subculturing. When transferred to medium II they were incubated in conditions II: 16 h illumination (45–60 μmol m^{-2} s^{-1} PPFD)/8 h darkness photoperiod at 27 ± 2°C, and subculturing every two months.

Experiments with 22(S),23(S)-homobrassinolide (HBr)

The brassinosteroid HBr (Sigma) was dissolved in ethanol, sterilized by filtration (Millipore 0.22 μm) and added to the sterile medium, prior to gelling, to final concentrations of 0, 0.01, 0.1, 2 and 4 μM. Explants were exposed to HBr during a 3 or 7 d pre-culture following previous reports (Katsumi, 1985; Zurek *et al*., 1994) and the medium I formulation was modified: 2.5 g l^{-1} polyvinylpolypyrrolidone (PVPP) was added instead of charcoal (according to Sáenz, 2000), to avoid accumulation of phenols and other compounds toxic to the explants, and a reduced 2,4-D concentration of 1 μM instead of 0.55 mM. During pre-culture explants were kept in conditions I. At the end of pre-culture, the cultures were transferred to unmodified brassinosteroid-free medium I and kept there for three months in conditions I. Then they were transferred to medium II and conditions II, and kept there to allow embryo formation.

Histology

The histological procedures were carried out according to Buffard-Morel *et al*. (1992), with slight modifications. Tissue samples were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2) for 24 h under negative pressure. Samples were dehydrated in a stepwise manner with 30%, 50%, 70%, 80%, 90%, 95% and 100% aqueous ethanol solutions, for 60 min each. This was followed by impregnation with JB-4 resin (Polyscience, USA). Three micrometer sections were prepared from the resin-impregnated tissues and stained with PAS-Naphthol blue black.

Statistical analysis

ANOVA and Newman and Keuls test (significance level $P<0.05$) were used for multiple comparisons of means from the treatments. For studies on the formation of initial calli and embryogenic calli, for each treatment every experiment consisted of 15 individual explants and was replicated four times. For studies on the formation of somatic embryos, for each treatment every experiment consisted of 10 calli and was replicated four times.

RESULTS

The formation of initial callus, embryogenic calli and somatic embryos was evaluated in plumule explants exposed during a 3 d or a 7 d pre-culture to different concentrations of HBr (0, 0.01, 0.1, 1, 2, 4 μM).

Histological and morphological evaluation

Initial callus formation: In explants cultured in medium I (and conditions I) with HBr (independently of the brassinosteroid concentration or time of pre-culture) initial callus formed after 30 d (Figure 1A) that was identical to initial callus formed in explants cultured in HBr-free medium (not shown). In histological sections at this stage meristematic areas were observed at the periphery of the calli (Figure 1B) as observed in calli formed in explants cultured in HBr-free medium (not shown).

Embryogenic callus formation: After 90 d of culture, a major proportion (see below) of initial calli, formed with or without HBr, developed embryogenic structures (Figure 1C and 1F) characteristic of embryogenic callus. These structures were more abundant in embryogenic calli formed in the presence of 0.01 or 0.1 μM HBr and 3 d preculture. In histological sections, embryogenic calli formed without HBr showed the occurrence of meristematic nodules that were present only at their periphery (Figure 1D) but not in inner tissues of the calli (Figure 1E). In the case of calli formed in the presence of 0.01 or 0.1 μM HBr, meristematic nodules were present in their periphery (Figure 1G) but also in inner tissues of the calli (Figure 1H). In these calli, proembryos were also occasionally observed (Figure 1I) whereas this was not the case in calli formed in medium without HBr. In calli formed in media containing 1, 2 or 4 μM HBr, meristematic nodules or proembryos were not observed.

Somatic embryo formation: When embryogenic calli were transferred to medium II (and conditions II), they formed somatic embryos (SE) identical morphologically (Figure 1J) or histologically (Figure 1K) to those formed in calli grown in medium without HBr (not shown). They show the presence of characteristic structures such as the coleoptile, the leaf primordia, the shoot apex and root apex (Figure 1K). Initial calli that did not form embryogenic structures did not develop somatic embryos eventually.

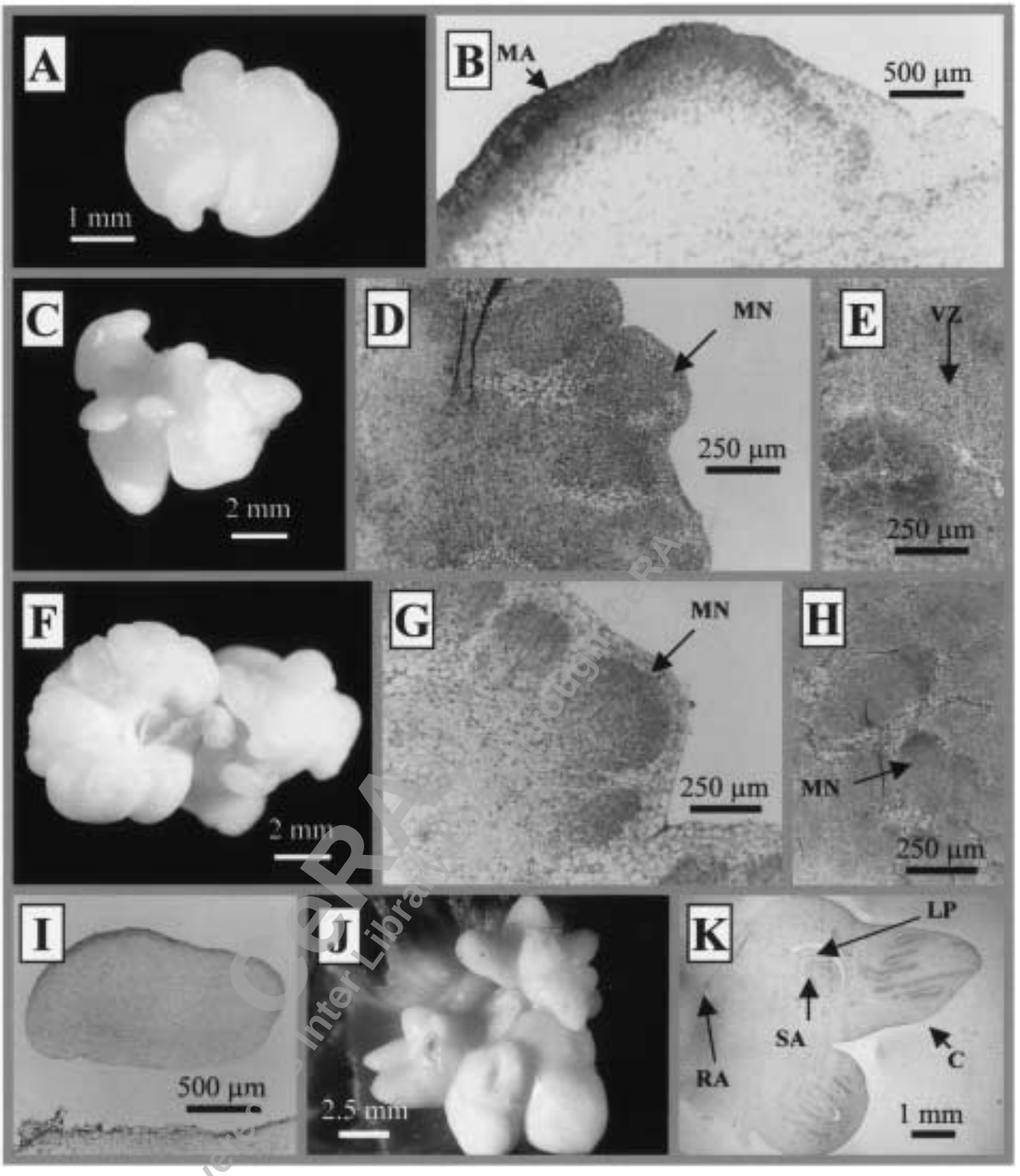

Fig. 1
(A) Callus tissue developed in coconut plumule explants treated with 22(S),23(S)-homobrassinolide (month 1, medium I) and (B) histological section showing the meristematic area [MA]. (C) Embryogenic callus from untreated explants of culture (month 3, medium I) and histological sections showing (D) the meristematic nodules [MN] and (E) the vascularized zone from the inner tissue [VZ]. (F) Embryogenic callus from brassinolide-treated explants and histological sections showing the MN (G) in peripheral and (H) inner tissues. (I) Proembryo formed in initial calli derived from brassinolide-treated explants (month 3, medium I). (J) Clump of germinating somatic embryos formed in calli derived from brassinolide-treated explants (month 2, medium II). (K) Histological section showing the coleoptile [C], leaf primordial [LP], shoot apex [SA] and root apex [RA] of a somatic embryo.

Quantitative evaluation

Initial callus formation: Brassinosteroid treatment was found to affect the percentage of explants forming initial callus. When explants were cultured for 3 d in medium I, the best response was observed with 0.1 µM HBr, 96% of the treated explants formed initial callus whereas only 76% of the untreated (no HBr and no pre-culture) explants did. Although lower, increases were also obtained with the other HBr concentrations used 0.01 (93%), 1 (86%), 2 (93%) and 4 µM (90%) (Figure 2). The pre-culture itself was found to have a positive effect (86%) on initial callus formation. In the case of a 7 d pre-culture there were no significant treatment effects at any of the concentration of HBr used (Figure 2).

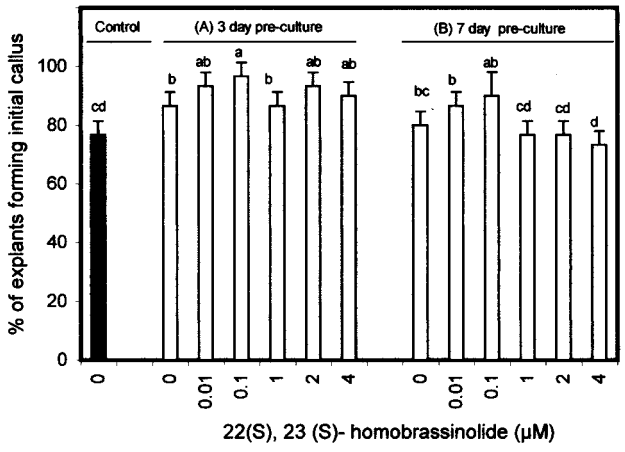

FIG. 2
Effect of 3 d (A) and 7 d (B) pre-culture in modified medium I, containing different concentrations of 22(S),23(S)-homobrassinolide, on the formation of initial callus in coconut plumule explants. After the pre-culture period the explants were transferred to unmodified medium I. The response was evaluated one month later. The control was cultured in unmodified medium I from the beginning. Bars denote SD of four replicates (n = 15). Different letters denote significant differences ($P<0.05$).

Embryogenic callus formation: After 90 d of culture, brassinosteroid treatment was also found to affect the percentage of explants forming embryogenic callus. For cultures originally pre-cultured during 3 d, the best response was observed with 0.01 and 0.1 μM HBr, 90% of the treated explants formed embryogenic callus, whereas only 63% of the untreated (no HBr and no pre-culture) explants did (Figure 3). In all cases embryogenic callus developed only from initial callus and not from explants unable to form initial callus. Although lower increases were also obtained with the other HBr concentrations used 1 (83%), 2 (80%) and 4 μM (80%) (Figure 3). The pre-culture itself was found to have a positive effect (70%) on embryogenic callus formation. In the case of a 7 d pre-culture, the best results were obtained with 0.01 (70%) and 0.1 μM HBr (76%). With other concentrations there was only a slight

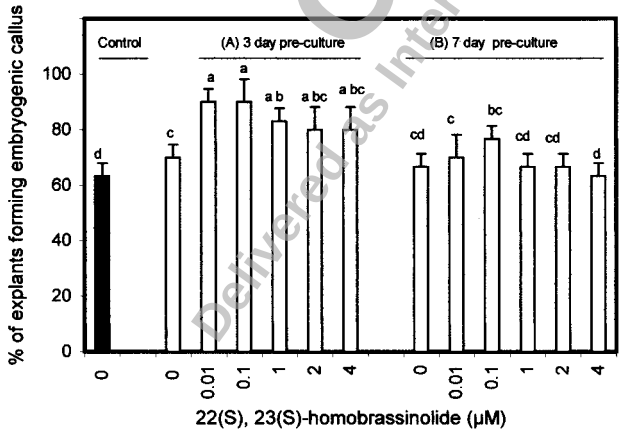

FIG. 3
Effect of 3 d (A) and 7 d (B) pre-culture in modified medium I, containing different concentrations of 22(S),23(S)-homobrassinolide, on the formation of embryogenic callus in coconut plumule explants. After the pre-culture period the explants were transferred to unmodified medium I. The response was evaluated three months later. The control was cultured in unmodified medium I from the beginning. Bars denote SD of four replicates (n = 15). Different letters denote significant differences ($P<0.05$).

TABLE I
Effect of a 3 d pre-culture with 22(S),23(S)-homobrassinolide (HBr) on the formation of somatic embryos (SE) in plumule cultures. Different letters denote significant differences (P<0.05), four replicates (n = 10)

Concentration HBr (μM)	Number of SE/explant
Control	3.8 e
0	6.3 c
0.01	10.88 a
0.1	10.89 a
1	9.54 b
2	8.96 b
4	5.53 cd

effect (1 and 2 μM, 66%) or no change (4 μM). Again, the pre-culture itself was found to have a positive effect, although slight in this case (66%).

Somatic embryo formation: After three months of culture in medium I, the embryogenic calli were subcultured to medium II. Brassinosteroid treatment was found to affect the somatic embryo (SE) yield. The yield in callus derived from originally untreated (no HBr and no pre-culture) explants was 6 SE per embryogenic callus (Figure 4). In comparison, for cultures originally pre-cultured for 3 d, the best responses were observed with 0.01 and 0.1 μM HBr with 12.1 SE per embryogenic callus. Responses with 1 and 2 μM HBr were 11.5 and 11.2 SE per embryogenic callus correspondingly, although they were not statistically different from those obtained with 0.01 and 0.1 μM HBr. The pre-culture itself was found to have a positive effect (9 SE per embryogenic callus). In the case of a 7 d pre-culture, the formation of SE was inhibited by all HBr treatments. Again, the pre-culture itself was found to have a positive effect (7.2 SE per explant) (Figure 4).

Results on SE formation were also expressed on explant basis (Table I), to show that the overall effect combining the increase in initial and embryogenic calli yields and SE per callus yield. The patterns are similar to those observed for SE per embryogenic calli (Figure 4), but the increases are greater in proportion. For instance, for cultures originally pre-cultured during 3 d, the highest yields observed (with 0.01 and 0.1 μM HBr) were 10.8 SE per explant whereas it was 3.8 SE per explant in originally untreated (no HBr and no pre-culture) explants – an increment of nearly three-fold compared with to two-fold when expressed on a embryogenic callus basis.

DISCUSSION

The increasing demand for palms resistant to diseases such as lethal yellowing, which has devastated coconut plantations worldwide, requires massive multiplication of improved or selected palms. This could be achieved through micropropagation. The use of coconut plumule explants has allowed important progress (Hornung, 1995; Chan *et al.*, 1998) to develop a reliable protocol, however the regeneration efficiency is still low. Since brassinosteroids are known to act synergistically with auxins (Katsumi, 1985), phytohormones required for the induction of embryogenic callus formation in coconut explants (Hornung, 1995; Chan *et al.*, 1998), and might be related to embryo development in plants (Jang *et al.*, 2000), we tested the effect of the brassinosteroid HBr on

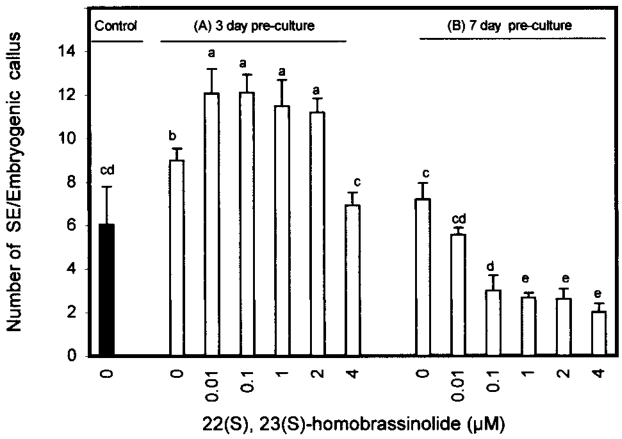

FIG. 4
Effect of 3 d (A) and 7 d (B) pre-culture in medium I, containing different concentrations of 22(S),23(S)-homobrassinolide, on the formation of somatic embryos in embryonic calli obtained from coconut plumule explants. After the pre-culture period the explants were transferred to unmodified medium I and three months later the derived embryogenic calli were transferred to medium II. The response was evaluated after two months in medium II. The control was cultured in unmodified medium I from the beginning. Bars denote SD of four replicates (n = 10). Different letters denote significant differences ($P<0.05$).

morphogenesis in plumule explants, to increase the regeneration efficiency of coconut.

Different concentrations of HBr were tested (0.01, 0.1, 1, 2 and 4 μM) exposing the plumule explants to the brassinosteroid during a 3 d or 7 d pre-culture. The results showed that the explants responded favourably to HBr increasing their capacity to form initial callus, embryogenic callus and doubled the amount of somatic embryos formed per embryogenic callus when the brassinosteroid was applied during a 3 d pre-culture, but no effects or very small effects were observed when it was applied during a 7 d pre-culture. These results agree with previous reports that brassinosteroid action depends on the length of time to which tissues are exposed, and that a short exposure is more convenient than a longer one (Katsumi, 1985; Zurek *et al.*, 1994). This differential effect of exposure time might be related to a brassinosteroid-induced ethylene formation during the longer pre-culture time and this phytohormone in turn affecting the embryogenic response in plumule explants, since brassinosteroids have been reported to be able (even synergistically with auxins) to promote ethylene formation (Arteca *et al.*, 1983; Arteca, 1984; Arteca *et al.*, 1984; Arteca *et al.*, 1988) and ethylene has been reported to inhibit somatic embryogenesis in coconut zygotic embryo explants (Adkins *et al.*, 1999).

Concerning morphological and histological development, initial callus formed in explants cultured in HBr-containing medium showed no differences in relation to initial callus formed in medium without HBr. On the other hand, embryogenic calli formed in explants cultured in HBr-containing medium showed developmental differences in relation to embryogenic callus formed in medium without HBr. Morphologically, they showed a greater abundance of nodular structures. Histologically, they showed the occurrence of meristematic nodules in inner tissues besides those formed in tissues in their periphery. Embryogenic capacity has been associated with meristematic nodules in different species, (Schwendiman *et al.*, 1990; Alemanno *et al.*, 1996) including coconut (Verdeil *et al.*, 1994; Chan *et al.*, 1998), and with nodular structures in coconut (Verdeil *et al.*, 1994; Sáenz *et al.*, 1999). Therefore a greater abundance of these two types of structures in embryogenic callus derived from HBr treated explants may explain the increased number of somatic embryos formed in these calli.

This promotion of morphogenesis by HBr in coconut plumule explants might be related, as reported for other plant species, to the synergistic action of brassinoesteroids with auxins (Katsumi, 1985) or their possible role on the regulation of signalling in cell division and cell expansion in embryogenic development (Jang *et al.*, 2000). On the other hand, brassinoesteroids are also known to act on the endogenous levels of cytokinins. Gaudinová *et al.* (1995) reported that in tobacco callus tissue the effect of two brassinosteroids could be related to their effect of endogenous levels of cytokinins. The effect was inhibitory or stimulatory depending on the brassinosteroid used. Although the brassinosteroids used by Gaudinová *et al.*, were different from the one used here, analysis of endogenous levels of cytokinins in coconut plumule calli showed an inverse relationship between cytokinin content and their capacity to form somatic embryos (Sáenz, 2000). Hence HBr could be reducing the endogenus levels of cytokinins of the plumule explants, an hypothesis that should be tested.

Efficiency-wise the overall effect of HBr increases the total amount of somatic embryos formed per explant 2.8 times. This is a very important increase considering the very slow progress of micropropagation research for this very recalcitrant species, that has been worked on for three decades. This effect of HBr (or any brassinosteroid) on somatic embryogenesis of coconut (or of any other plant species) is, as far as we know, now reported for the first time. These results are encouraging but yet not enough to have a large scale micropropagation protocol considering the efficiencies achieved for other species. For instance in date palm, Omar *et al.* (1992) claim that the number of somatic embryos that can be obtained from each gram of embryogenic calli is 230 after two months of culture and can be as large as 235,000 after 12 months. In the present case, 1 gram of embryogenic calli (about eight) yield approximately 100 somatic embryos. Therefore, to further increase yield efficiency, it will be interesting to test other brassinosteroids, but in addition it is important to consider several strategies. It has been shown that polyamines (Adkins *et al.*, 1999), or abscisic acid (ABA) in combination with osmotic agents (Samosir *et al.*, 1999) in slices of coconut zygotic embryos; and ABA alone (Fernando and Gamage, 2000) in zygotic embryos cultured *in vitro* have important effects on the formation of somatic embryos. Both strategies have been tested in our laboratories on plumule cultures, but unfortunately without any positive effect (A. Azpeitia, unpublished). Large somatic embryo yields have been achieved for oil palm through cell suspension culture (Touchet *et al.*, 1991; Texeira *et al.*, 1995). In the case of coconut, this approach has been attempted, but unfortunately without success so far (Magnaval, 1995). On the other hand other strategies currently being tested in our laboratory, secondary embryogenesis and the use of substances such

as anticytokinins, have shown promising results (unpublished). We believe that the combination of different strategies, including those above and the use of brassinosteroids, will increase the chances of improving significantly the efficiency of coconut regeneration provided their effects are cumulative.

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