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Coconut (*Cocos nucifera*) In Vitro Ecology: Modifications of Headspace and Medium Additives Can Optimize Somatic Embryogenesis

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Abstract

Of those explants tested, immature zygotic embryo tissues proved to be the best for initiating callus with potential for somatic embryogenesis. Slicing of this tissue and use of the central sections (near to and including the meristematic tissue) gave the best embryogenic response. Slices that were placed under illumination necrosed more rapidly and to a greater degree than those incubated in the dark. Explant slice necrosis could be prevented or severely retarded by the addition of activated charcoal into the medium. Washing the explants for short periods of time prior to culture was also found to improve callus production. Prolonged washing resulted in low rates of callus production. In an attempt to prevent ethylene accumulation in the culture vessel headspace, AVG, an ethylene biosynthesis inhibitor and STS, a chemical which reduces the physiological action of ethylene, were successfully used to promote somatic embryogenesis. Spermidine, putrescine and spermine, polyamines that are known to delay plant senescence and promote somatic embryogenesis in some plant species, enhanced the rate of somatic embryogenesis when they were introduced into the callus induction medium. The use of polyethylene glycol in combination with abscisic acid helped promote somatic embryo formation and maturation as well as the subsequent formation of plantlets. The use of all of these improvements together has created a new and improved protocol for coconut somatic embryogenesis. This new protocol puts significant emphasis on improving the in vitro ecology of the explant, callus and somatic embryogenic tissues.

INTRODUCTION

Coconut (*Cocos nucifera* L.) is one of the most important oil crops in the tropics. It is called "the tree of life" as it has attained an important socio-economic role in the local communities where it is produced (Persley, 1992). Oil extracted from the endosperm is the main reason for its production and is a major source for short-chain fatty acids. Coconut is produced on 12 m ha in 86 different countries with this area increasing by 2 % per annum. Most of the world's coconuts are grown in the Asia-Pacific region with the Philippines, Indonesia and India being the largest producers. Over 50 million people are directly involved with its production and manufacturing industries. There is a need to produce new disease resistant, high yielding coconut cultivars to meet the production needs of the present day. Once such elite hybrids have been produced through conventional breeding programs they will then need to be made readily available to the farming community. Seed production per tree is low and asexual propagation is not possible. However, one method of clonal propagation is through somatic embryogenesis. Despite several decades of attempting this (Verdeil et al., 1989; Buffard-Morel et al., 1992; Jesty and Francis, 1992) a reliable protocol has not been established yet. The major problems with coconut somatic embryogenesis remain the heterogeneous response of the explanted tissues, slow and low responses of the explant to the culture medium and premature necrosis of explanted tissue. Our earlier work (Adkins, 1992) on cereals has shown that reductions in the rates of somatic embryogenesis and somatic embryo proliferation induced by endogenously produced ethylene can be overcome by either reducing its production (with 1-aminoethoxyvinyl glycine – AVG) or by protecting the

explanted tissue from its action (with silver thiosulphate – STS). The production of well formed, high quality somatic embryos can then be achieved by incorporating certain polyamines (Smith, 1985) into the culture medium. The development and maturation of the somatic embryos can be further improved by using abscisic acid (AbA), alone or in combination with one of several osmotically active agents. Thus, this paper reports a series of experiments aimed at improving the basic protocol of regenerating coconut plants via somatic embryogenesis placing emphasis on headspace and medium additives to optimise somatic embryogenesis.

MATERIALS AND METHODS

Tissue Culture

Unless otherwise stated, Malayan Yellow Dwarf zygotic embryos (ca. 8 to 12 month-old) were isolated from mature fruits in the field at South Johnstone Research Station, and brought to Brisbane using a cold isolation, storage and transportation system (Samosir, 1999). The coconut dwarf type is predominantly selfing and the trees are grown in isolated areas at the station far from other coconut tress. In fact, the station has only few coconut trees] Once in the laboratory, the embryos were surface sterilised with sodium hypochlorite (1.5 % w/v) for 15 minutes and rinsed twice with sterile deionised water. The basic protocol consisted of a series of four media (Table 1) each containing Y3 nutrients (Eeuwens, 1976), supplemented with Morel and Wetmore vitamins (Morel and Wetmore, 1951), agar (7 gL^{-1}) and neutralised activated charcoal (2.5 gL^{-1}). Unless otherwise described, the callus induction (M1), proliferation (M2) and maturation (M3) steps were undertaken in the dark while plantlet regeneration (M4) and root development steps were undertaken in the light (ca. 120 µmol $m^{-2}s^{-1}$) with a 12 h photoperiod. In all steps, the temperature used was a constant (28 ± 1 ^{o}C).

Culture Performance Indicators and Phenolic Analysis

Unless otherwise stated, the amount of callus (nodular, white) produced on the explants was determined 12 weeks after the start of culture on M1. The amount of callus produced in any one treatment was expressed as the percentage of the explants cultured that produced callus. The intensity of necrosis or browning of the explanted tissues was estimated on the area of the surface of the explant that turned brown in relation to the area that remained white (0 was no necrosis, 4 was 75-100 % tissue necrosis). Gas samples (1 mL) taken from the headspace of the culture vessels were analysed by gas chromatography (model 5830A, Hewlett Packard Ltd, USA) containing a Porapak N (Waters Association Inc, USA) column (1.8 m x 3 mm). The oven and injectors of the apparatus were maintained at 90 and 95 ^{o}C respectively and a flame ionisation detector (95 ^{o}C) was used to analyse ethylene in each sample (Adkins, 1992). The number of somatic embryos produced on any explanted tissue of callus mass was confirmed by viewing under a binocular microscope and expressed on a per explant basis. The total number of shoots (at least 1 cm long) produced following 12 weeks on M4 was expressed on a per culture vessel basis.

For determination of the amounts of phenolics in solution, 1 mL samples of solution were placed into test tubes containing deionised water (4 mL) and mixed. A 0.5 mL aliquot of Folin-Ciocalteu reagent (Singleton and Rossi, 1965; Waterman and Mole, 1994) was added into each tube and mixed. After 7 minutes 1.5 mL of saturated Na_2CO_3 solution (20%, w/v) was also added. Three mL of deionised water was then added and the tubes mixed again. After 30 minutes the concentration of total phenolic compounds was determined using a spectrophotometer (Spectronic 1001, Milton Roy Co. Rochesterlaan, Belgium) set at 760 nm and the results expressed as coumaric acid equivalents.

Effect of Explant Age on Callus Induction (Experiment 1)

Immature (ca. 8 month-old) and mature (ca. 12 month-old) embryos were surface sterilised separately using 2 % (w/v) sodium hypochlorite for 15 minutes and rinsed twice

with sterile deionised water. Ten embryos were then placed into polycarbonate tubes (one per tube) containing 10 mL of a modified M1 containing 2,4-D (180 µM). The cultures were incubated under the standard conditions, with subculture every 4 weeks for a total of 12 weeks before embryogenic callus production and necrosis rate were determined.

Effect of Explant Slicing on Somatic Embryogenesis (Experiment 2)

Mature zygotic embryos were cut transversally and the haustorial tissue discarded. The remaining tissue was sliced longitudinally to produce 10 slices of equal thickness. The slices were then grouped according to their original position on the embryonic axis. Those from the middle of the axis were classified as group 1 while those from the edges as group 5. Ten slices from each of the five groups were then placed into Petri dishes containing 25 mL of M1. Intact, zygotic embryos were cultured under identical conditions as controls. Subculture of all explants was made 4 weeks later on further M1. The callus produced was then isolated and subcultured onto M2 and kept for another 4 weeks. At this time, the number of somatic embryos produced was counted then subcultured onto M3. Four weeks later they were subcultured onto M4. The number of shoots produced was counted 12 weeks later.

Effect of Explant Washing on Somatic Embryogenesis (Experiment 3)

Twenty-five embryo slices (from slice groups 1 and 2) were placed into glass jars (8 x 5 cm diameter) containing 50 mL of sterile deionised water and 2.5 gL^{-1} of activated charcoal. The jars were closed and incubated in the dark at 26 ± 1 °C while being shaken (90 rpm) for either 0, 5, 10, 15 or 25 hours. After each incubation period the explants were aseptically removed from the glass jars and blotted dry. The explants were then placed into Petri dishes (10 slices per dish) containing 25 mL of M1. The Petri dishes were closed with parafilm and incubated in the dark at 28 ± 1 °C. After 4 weeks incubation the explants were further subcultured onto fresh medium of the same composition. Following the initial incubation, the solution used to soak the sliced explants was sampled and the pH and total phenolic substance content determined.

Effect of Illumination on Somatic Embryogenesis (Experiment 4)

Ten embryo slices (from slice groups 1 or 2) were placed into Petri dishes containing 25 mL of M1. The Petri dishes were then incubated under illuminated conditions (PPFD ca. 120 µmol $m^{-2}s^{-1}$) at 28 ± 1 °C for 0, 3, 7, 14 or 21 days. After these treatments, the amount of water that had formed as condensation on the Petri dish lids was weighed and the cultures placed into the darkness for an additional 4 weeks of growth. After this they were assessed for the formation of embryonic callus.

Use of Activated Charcoal (Experiment 5)

Ten embryo slices (from slice group 1 or 2) were placed into Petri dishes containing 25 mL of M1 containing 0, 0.5, 1.0, 1.5, 2.5, 4.0, 7.0 or 10.0 gL^{-1} activated charcoal. The Petri dishes were closed with parafilm and incubated in the dark at 28 ± 1 °C. The explants were then subcultured onto fresh medium of the same composition at 4 weekly intervals. After 8 weeks, the callus produced was isolated and placed on M2 and kept at 28 ± 1 °C under a 12h photoperiod for 4 weeks followed by subculture onto M3 then 4 weeks later onto M4.

Controlling the Effect of Ethylene (Experiment 6 to 8)

Ten embryo slices (from slice groups 1 or 2) were cultured onto M1 supplemented with one of three filter-sterilised additives: AVG (0 to 0.3 µM, Experiment 6, ACC (0 to 1500 µM, Experiment 7) or STS (0 to 20 µM, Experiment 8). The media were dispensed into glass culture vessels, sealed with Subaseal stoppers and incubated at 28 ± 1 °C in the dark for 4 weeks. At the end of this incubation period gas samples (1 mL) were removed from the headspace of the culture vessel and analysed for ethylene content. The rate of callus induction was determined by counting the number of explanted slices that had

produced embryogenic callus.

Effect of Polyamines on Callus Induction (Experiment 9 to 11)

Ten embryo slices (from slice groups 1 or 2) were placed on M1 to produce callus. About 100 mg of callus was then placed into glass culture vessels containing M2 (10 mL) supplemented with filter-sterilised putrescine (0.0, 0.5 or 5.0 mM, Experiment 9), spermidine (0.0, 0.5 or 5.0 µM, Experiment 10) or spermine (0.0, 0.05, 0.1, 0.2 or 5.0 µM, Experiment 11). The vessels were sealed with Subaseal stoppers and kept in the dark for 4 weeks. At this time the ethylene content in the headspace was measured and the mean number of somatic embryos counted.

Application of Osmotically-active Substances and Abscisic Acid (Experiment 12)

Young leaf sections (1 cm^3) were cut from 16 month-old Malayan Dwarf Coconut seedlings growing in a glasshouse. The sections were surface sterilised in sodium hypochlorite solution (1.5 % w/v) and induced to form callus on M1. Proliferated calli (about 100 mg fresh weight each) were placed in Petri dishes containing 25 mL of M2 supplemented with AbA (50 or 100 µM) and with PEG (9 mM: molecular weight 3,350; Sigma Chemical). Four weeks later, the callus was subcultured onto M3 but without supplements added and kept for a further 4 weeks. At this time, they were transferred to glass jars (8 x 5 cm diameter) containing 30 mL of M4 for germination under illumination. Somatic embryos were counted under a binocular microscope 8 weeks after the start of the treatment and the total number per container recorded.

Statistical Analysis

Each experiment was set up as a complete randomised design. The data sets were statistically analysed using SigmaStat (Jandel Corporation, San Rafael, USA). When appropriate, this was followed by mean comparisons according to the least significant different (P = 0.5). Square root transformations were undertaken when necessary prior to statistical analysis, but the data presented in tables are untransformed.

RESULTS

Effect of Explant Age on Callus Induction (Experiment 1)

Immature embryo explants produced callus after 4 weeks on M1, while the mature embryo explants required 4 weeks longer. The number of mature zygotic embryo explants producing embryogenic callus was significantly greater (50 %) than that of the mature ones (9 %) (Table 2). Necrosis was more intense in mature embryo explants (index value 2.0) than in immature ones (index value 1.5). The media became brown in colour, particularly under the mature embryo explants.

Effect of Explant Slicing on Somatic Embryogenesis (Experiment 2)

Slicing the mature zygotic not only increased their ability to form callus (Table 3) but also halved the time embryos took to form callus. During the first 4 weeks of culture, the intact zygotic embryos expanded, while the sliced tissues expanded and produced embryogenic callus. Eventually the intact zygotic embryos produced embryogenetic callus but at a much lower rate (5 % of explants) than sliced embryos. Slices taken from the centre of zygotic embryos (group 1) were the best for producing embryogenic callus (58 %). The number of embryogenic calli produced by this group was also significantly higher than those produced by any other slice group. The production of nodular callus significantly decreased as the source of the slice moved further away from the centre of the embryonic axis with the outer slices (group 5) producing no callus at all. The embryogenic callus produced by both intact and slices zygotic embryo explants developed into somatic embryos. Shoots were formed from these structures on M4 and shoots developed into plantlets. The greatest shoot production was on explants taken from the centre of the zygotic embryo (group 1).

Effect of Explant Washing on Somatic Embryogenesis (Experiment 3)

Washing of the explants prior to culture increased callus formation (Table 4), particularly when they were washed for between 5 and 10 hours. Prolonged washing increased necrosis and decreased callus formation. All washing periods removed phenolic compounds from the explant slices but this could not be detected as a reduction in washing solution pH, except in the longest incubation time.

Effect of Illumination on Somatic Embryogenesis (Experiment 4)

Apart from a very short exposure to illumination (<3 days) embryogenic callus formation was progressively inhibited by illumination (Table 5). Transferring the cultures back to the dark could not reverse this inhibition. Intensity of tissue necrosis also increased as the illumination period increased (data not shown). In all illuminated cultures, condensation was produced on the lids of the Petri dishes. Such production did not occur with cultures placed in the dark. The longer the exposure time the greater the amount of condensation. Presumably this condensation is a result of water loss from the agar medium.

Use of Activated Charcoal (Experiment 5)

Activated charcoal significantly reduced explant necrosis (Table 6). All explants on M2 without or with low levels (1.0 gL^{-1}) of activated charcoal turned brown and died during early culture. Addition of high levels (>4 gL^{-1}) also caused the tissue to necrose, particularly at the callus proliferation stage. Callus formation only occurred when the media contained activated charcoal (1.5 to 4.0 gL^{-1}). The amount of callus formed was also increased by the addition of activated charcoal (up to 2.5 gL^{-1}), as was shoot production.

Controlling the Effect of Ethylene (Experiment 6 to 8)

Application of AVG to M1 significantly inhibited ethylene production and increased callus formation (Table 7). Addition of 1.5 µM AVG reduced ethylene production to 15 % of that seen in the control and doubled callus production. At higher concentrations AVG continued to decrease ethylene production but suppressed callus production. Application of ACC 100 µM increased ethylene production 3-fold and decreased callus production by 40 %. STS at low concentrations (1 to 5 µM) significantly stimulated callus induction but did not affect the amount of ethylene produced (except at 10 µM where ethylene production was significantly higher than in the control). This effect on ethylene production is not unexpected as STS reduces the effect of ethylene (not ethylene production) particularly at concentrations up to 5 µM with higher concentrations becoming toxic.

Effect of Polyamines on Callus Induction (Experiment 9 to 11)

Each of the three polyamines significantly increased somatic embryo production. The optimum concentrations for promotion were 0.5 µM spermidine, 0.5 mM putrescine and 0.01 µM spermine (Table 8). At these optimum concentrations for stimulation of somatic embryo production, spermidine and putrescine both significantly reduced ethylene production while spermine significantly increased its production.

Application of Osmotically-active Substances and Abscisic Acid (Experiment 12)

All combined AbA and PEG treatments produced significantly more somatic embryos than the control treatments of AbA alone (Table 9). The most effective combination was PEG (9 mM) with AbA (45 µM), at 50 µM 2,4-D, which depressed non-somatic embryogenic callus production. This treatment also promoted synchrony of embryogenesis.

DISCUSSION

The pathway for the formation of coconut somatic embryos and their subsequent proliferation is complex and affected by several factors. These include the explant type, its age and quality, the presence or absence of illumination, the accumulation of ethylene in the vessel headspace, secretion of phenolics into the medium, degree of culture necrosis and the way in which the somatic embryos are matured.

Of those explant types tested, immature zygotic embryo explants were best for callus production and somatic embryogenesis (Table 2). When using intact mature zygotic embryos as explants the rate of somatic embryogenesis was low. This may be because the outer cotyledonous tissues physically block the embryogenic tissue from coming into contact with the medium and this reduces the concentration of 2,4-D that is finally reaching the embryogenesis inducible tissues. Upon embryo slicing (Table 3), the embryogenic callus formation significantly increased and the time to produce this callus was half that of the intact zygotic embryo plants. Improvements in the rate of somatic embryogenesis by slicing have been reported before in soybean (Nadolska-Orczyk and Orczyk, 1994). It is possible that slicing increases the supply of 2,4-D to the important cells and subsequently promotes a higher rate of callus production and somatic embryogenesis.

It was noted that the rate of somatic embryogenesis production gradually decreased as the slices were taken further away from the centre of the embryonic axis suggesting that tissue close to the centre of the embryonic axis was most appropriate for initiating somatic embryogenic callus.

Another factor inhibiting callus formation was illumination (Table 5). Explants placed under illuminated conditions necrosed more rapidly and to a greater extend than those incubated in the dark. Similar results have been observed in other species (San-Jose and Vieitez, 1993) and were ascribed to the breakdown of some key metabolic products in the explants (Hangarter and Staginopoules, 1991). Alternatively, illumination may inhibit a key metabolic process in the explants (Seibert et al., 1975). In the present study, the inhibition was unlikely to be due to the breakdown of medium components since pre-illuminated media (without explants) did not adversely affect later callus formation (data not shown). Similarly, the removal of some water from the medium through condensation was not thought to be responsible. The light may have enhanced the oxidation of the phenolic compounds released by the tissues.

Explanted tissue necrosis in coconut is thought to be associated with the production of phenolic substances, which are then secreted into the tissue culture medium and influence the producing tissues (Verdeil and Buffard-Morel, 1995). Tissue necrosis was prevented in media with activated charcoal (Table 6) indicating that this substance may absorb some of the phenolic substances and prevent them from oxidising and causing damage to the tissue in the long term. Supplementation of the medium with activated charcoal was effective in reducing tissue necrosis but the concentrations used seemed to be very important. At either high (≥ 4 gL^{-1}) or low (<1.5 gL^{-1}) concentrations callus and shoot formation was significantly reduced. Since activated charcoal is known to absorb PGRs from the culture medium, high levels of AC may have reduced the amount of 2,4-D available for callus formation, while low levels of AC may not have been sufficient to absorb all of the toxic phenolic substances and PGRs applied may still be at high levels which could also enhance tissue necrosis. Washing explants prior to tissue culture may have removed some of the phenolic substances that were absorbed by the activated charcoal (Table 5). The phenolic substances produced lowered the medium pH.

Coconut explants and callus modify the atmosphere of the headspace of the culture vessel by producing ethylene. Production and accumulation of ethylene in the culture vessel headspace also occurs during rice callus growth (Adkins et al., 1993) and such accumulations have been shown to reduce somatic embryogenesis (Roustan et al., 1994). In an attempt to prevent such effects in coconut, an ethylene biosynthesis inhibitor, AVG, and a chemical which reduced the physiological action of ethylene (STS) were shown to promote somatic embryogenesis (Table 7). That ethylene was responsible for

inhibition of somatic embryogenesis was further indicated by the inhibition of this event by ACC, a chemical which stimulates endogenous ethylene production (Adams and Yang, 1979).

If the concentration of ethylene above a coconut culture is important then substances, which protect the tissues from ethylene stress and promote morphological development, should also promote somatic embryogenesis. The polyamines, spermidine, putrescine and spermine are known to delay plant senescence through their inhibition of ethylene action (Apelbaum et al., 1981) and reduction of ethylene production by competing for the use of SAM. When applied to coconut during the callus induction stage putrescine (7.5 µM), spermidine (0.5 µM), and spermine (0.01 µM) enhanced somatic embryogenesis. In two of the three cases (spermidine and putrescine) these improvements correlated with significant reductions in the amount of ethylene produced. However, addition of spermine increased ethylene production (Table 8). Since spermine is the product of combination of SAM and spermidine, addition of spermine may have a feed back mechanism to stimulate the use of SAM, mainly for ethylene production.

One of the major problems in coconut somatic embryogenesis is that the somatic embryos once formed fail to develop, mature and germinate normally (Kendurkar et al., 1995). In the present study, application of PEG (9 mM) in combination with AbA (45 µM) helped to overcome this problem (Table 9). The need for combination of AbA and osmotically active substances to mature somatic embryos has been shown before in several tree species (Li et al., 1997; Etienne et al., 1993). In the present study, the morphology of the somatic embryos was improved by combining PEG and AbA. This produced embryos that were more individual and more synchronous in their development. This improvement may relate to the function of osmotically active substances and AbA in promoting the expression of maturation-specific genes (Cuming et al., 1996). In addition, the osmotic potential generated by these substances may break cell interconnections, thus facilitating independent embryo development (Wethere, 1984).

A new protocol has been developed for the preparation of quality explant tissues, formation of somatic embryogenic callus, its subsequent proliferation and the formation of somatic embryos, their maturation and development. This new protocol has put emphasis on improvements the in vitro ecology of the explanted and callus tissues. The improved protocol is able to regenerate plantlets more efficiently than previously published protocols.

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Tables

Table 1. The composition of the culture media used in the in vitro studies of coconut. Each medium contained Eeuwens (1976) Y3 mineral nutrients, Morel and Wetmore (1951) vitamins, 2.5 g L^{-1} activated charcoal and 7 g L^{-1} agar.

Components	Medium			
	Induction (M1)	Proliferation (M2)	Maturation (M3)	Regeneration (M4)
2,4-D (µM)	125	125	50-10	0
BA (µM)	5	5	5	15
2iP (µM)	5	5	5	0
IAA (µM)	0	0	0	15
Sucrose (g L^{-1})	30	40	40	40

Table 2. The somatic embryogenic response of immature and mature zygotic coconut embryos (Experiment 1). The rate of culture necrosis is also indicated where 0 was no necrosis and 4 was 75 to 100% necrosis. Values within treatments followed by the same letter are not significantly different at P = 0.05 level.

Explant/embryo age (months)	Necrosis index	Explants producing embryogenic callus (%)
Immature (8)	1.5 ± 0.5 a	50 ± 8 a
Mature (12)	2.0 ± 0.5 a	9 ± 5 b

Table 3. The tissue culture response of sliced mature zygotic embryo explants. The embryo tissue (without haustorium) was sliced longitudinally into five parts (Experiment 2). These were grouped according to their position within the embryonic axis. Slices from the mid region were referred to as 1 while the exterior sections were referred to as 5. Intact zygotic embryos were used as controls and referred to as 0. Values within treatments followed by the same letter are not significantly different at P = 0.05.

Slice position	Explants producing embryogenic callus (%)	Number of somatic embryos produced per explant	Number of shoots produced per explant
0	0 ± 0 a	1.3 ± 0.8 a	1.5 ± 0.6 a
1	57.5 ± 4.8 b	12.8 ± 2.0 b	9.0 ± 3.1 b
2	22.5 ± 2.5 c	2.8 ± 0.5 a	1.7 ± 0.9 a
3	7.5 ± 7.5 a	0.8 ± 0.5 a	0.0 ± 0.0 a
4	2.5 ± 2.5 a	0.0 ± 0.0 a	0.0 ± 0.0 a
5	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a

Table 4. Effect of explant (embryo slices) pre-washing on necrosis index (where 0 is no necrosis, 4 is full necrosis), explant percent producing somatic embryogenic callus (Experiment 3). Values within treatments followed by the same letter are not significantly different at P = 0.05.

Time (h)	Necrosis index	Total phenolics (mg g FW^{-1})	Solution pH	Explants producing embryogenic callus (%)
0	3.7 ± 0.1 a	0.0 a	7.0 ± 0.1 a	30 ± 3 a
5	2.9 ± 0.2 b	0.2 b	7.1 ± 0.0 a	51 ± 6 b
10	2.8 ± 0.3 b	0.2 b	7.1 ± 0.0 a	45 ± 7 b
15	3.4 ± 0.3 a	0.2 b	7.0 ± 0.1 a	43 ± 4 b
25	3.5 ± 0.1 a	0.3 c	6.0 ± 0.1 b	20 ± 0 a

Table 5. Embryogenic callus produced on sliced mature zygotic embryo explants incubated in illumination for between 0 and 21 days before being transferred to the dark (Experiment 4). The amount of water condensed on the lid of the Petri dish was weighed after the illumination period was complete. Values within treatments followed by the same letter are not significantly different at P=0.05.

Incubation (h)	Water condensation (g)	Explants producing embryonic callus (%)
0	0.0 ± 0.0 a	30 ± 6 b
3	0.1 ± 0.1 a	40 ± 3 b
7	0.3 ± 0.1 b	17 ± 3 a
14	0.6 ± 0.1 c	13 ± 8 a
21	1.5 ± 0.3 d	10 ± 4 a

Table 6. The effect of activated charcoal (AC) in the culture medium on the rate of tissue necrosis, somatic embryo production and shoot regeneration (Experiment 5). Values within treatments followed by the same letter are not significantly different at P = 0.05.

AC (gL^{-1})	Necrosis index	Somatic embryos produced per explant	Shoots produced per explant
0.0	4.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
1.0	4.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
2.0	3.2 ± 0.2 b	4.8 ± 2.4 b	0.0 ± 0.0 a
2.5	3.3 ± 0.2 b	8.2 ± 2.0 b	4.0 ± 1.8 b
4.0	1.0 ± 0.2 c	0.8 ± 0.8 c	0.2 ± 0.2 a

Table 7. Effect of AVG, ACC and STS on ethylene production and callus initiation (Experiment 6, 7 and 8). The explants were slices taken from zygotic embryos which were then placed in glass culture vessels containing M1 and sealed with Subaseal stoppers. After 4 weeks culture gaseous samples were removed from the headspace and measured for their ethylene content. At the same time callus initiation was determined. Values within treatments in the specified experiment followed by the same letter are not significantly different at P = 0.05.

Treatment		Ethylene ($\mu L\ g^{-1}\ FW^{-1}$)	Callus induction (%)
Experiment 6			
AVG (μM)	0.0	6.2 ± 0.8 a	28 ± 3 a
	0.5	5.0 ± 0.9 a	35 ± 4 b
	1.0	1.9 ± 0.6 b	50 ± 10 c
	1.5	1.2 ± 0.3 b	48 ± 5 c
	3.0	1.0 ± 0.1 b	18 ± 3 a
Experiment 7			
ACC (μM)	0.0	4.8 ± 0.2 a	25 ± 3 a
	50.0	10.8 ± 1.0 b	18 ± 6 ab
	100.0	13.0 ± 1.0 c	14 ± 4 b
	500.0	9.8 ± 0.4 b	10 ± 3 b
Experiment 8			
STS (μM)	0.0	7.2 ± 1.8 a	38 ± 8 a
	1.0	10.2 ± 2.4 ab	62 ± 8 b
	2.0	7.8 ± 1.0 a	62 ± 6 b
	5.0	10.4 ± 2.6 ab	60 ± 8 b
	10.0	12.4 ± 1.2 b	46 ± 8 a
	20.0	7.2 ± 1.4 a	32 ± 4 a

Table 8. Effect of three polyamines on somatic embryogenesis and ethylene production (Experiment 9,10,11). Values within treatments in the specified experiment followed by the same letter are not significantly different at P = 0.05.

Treatment		Ethylene (µL g^{-1} FW^{-1})	Somatic embryogenesis
Experiment 9			
Putrescine (mM)	0.0	4.7 ± 0.8 b	14.7 ± 2.0 a
	7.5	2.1 ± 0.2 a	30.3 ± 3.8 b
	15.0	6.7 ± 0.5 c	14.7 ± 4.4 a
Experiment 10			
Spermidine (µM)	0.0	5.0 ± 0.8 b	10.5 ± 1.8 a
	0.5	1.9 ± 0.3 b	16.0 ± 1.0 b
	5.0	10.1 ± 2.2 bc	12.0 ± 1.1 a
Experiment 11			
Spermine (µM)	0.0	4.2 ± 1.1 a	7.3 ± 0.5 a
	0.01	5.7 ± 0.9 ab	27.0 ± 4.2 b
	0.1	7.7 ± 1.1 b	20.5 ± 3.3 ab
	1.0	7.5 ± 1.6 b	21.0 ± 3.1 ab
	5.0	8.8 ± 1.1 ab	14.0 ± 3.8 ab

Table 9. The effect of 2,4-D, AbA and PEG on the number of somatic embryos and their subsequent development into plantlets from an initial 100mg clump of callus derived from leaf explants (Experiment 12). Values within treatments followed by the same letter are not significantly different at P = 0.05.

Additive			Somatic embryos produced per leaf-explant	Plants in vitro
2,4-D (µM)	AbA (µM)	PEG (mM)		
0	0	0	2.3 a	0
50	45	0	4.8 ab	0
100	45	0	2.8 a	0
50	45	9	8.5 c	1
100	45	9	6.5 bc	0

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