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Headspace volatile markers for sensitivity of cocoa (*Theobroma cacao* L.) somatic embryos to cryopreservation

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Abstract The mechanisms that reduce the viability of plant somatic embryos following cryopreservation are not known. The objective of the present study was to evaluate the sensitivity of cocoa (*Theobroma cacao* L.) somatic embryos at different stages of an encapsulation–dehydration protocol using stress-related volatile hydrocarbons as markers of injury and recovery. The plant stress hormone ethylene and volatile hydrocarbons derived from hydroxyl radicals (methane) and lipid peroxidation (ethane) were determined using gas chromatography headspace analysis. Ethylene and methane were the only volatiles detected, with both being produced after each step of the cryogenic protocol. Ethylene production was significantly reduced following exposure to liquid nitrogen, but then increased in parallel with embryo recovery. In contrast, the production of methane was cyclic during recovery, with the first cycle occurring earlier for embryos recovered from liquid nitrogen and desiccation than those recovered from earlier steps in the protocol. These results suggest that loss of somatic embryo viability during cryopreservation may be related to the oxidative status of the tissue, and its capacity to produce ethylene. This study has demonstrated that headspace volatile analysis provides a robust non-destructive analytical approach for assessing the survival and recovery of plant somatic embryos following cryopreservation.

Keywords Cryopreservation - *Theobroma cacao* L. - Somatic embryos - Methane - Ethylene - Oxidative stress

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Introduction

Cryopreservation is increasingly used for the long-term preservation of plant genetic resources at −196°C. This technology is particularly important for the preservation of tropical species such as cocoa (*Theobroma cacao* L.), as the seed is recalcitrant to conventional seed storage protocols. However, tropical species often lack the mechanisms required to tolerate desiccation and cold, which can result in loss of viability following cryopreservation. The mechanisms causing this loss of viability are yet to be determined, although studies in *Ribes* spp, *Daucus carota*, *Brasicca napus* and *Oryza sativa* suggest that oxidative stress may be a contributing factor (Benson and Noronhadutra *1988*; Benson and Withers *1987*; Benson et al. *1992a*, *b*; Johnston et al. *2007*). The storage of in vitro plant cultures at low temperatures has been associated with the production of reactive oxygen intermediates (ROI) which include activated singlet oxygen ($^1O_2$), superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) (Benson and Withers *1987*; Fleck et al. *2000*). ROI are constantly produced in biological systems as a consequence of cellular O_2-consuming redox processes such as photosynthesis and respiration (Benson *1990*; Frahry and Schopfer *2001*; Schopfer et al. *2001*). The ·OH is particularly aggressive and can be generated non-enzymatically via Fenton and Haber–Weiss reactions (Benson *1990*), or enzymatically from reactions between peroxidases and ROI generated by plasma membrane NADH oxidase (Schopfer et al. *2001*, *2002*). Lipid peroxidation is one of the main results of free radical damage in biological systems and is initiated by free radical attack of polyunsaturated fatty acids located in membrane lipids. A lipid radical is formed which, upon addition of molecular oxygen, produces a lipid peroxyl radical. This radical reacts with a second fatty acid forming a lipid radical and lipid peroxide, resulting in a self-propagating chain (Sevanian and Hochstein *1985*). Lipid peroxides cause further damage when they break down to form toxic secondary lipid peroxidation products such as aldehydes and Schiff's bases (Esterbauer et al. *1988*; Frankel *1987*). Aldehyde breakdown products including malondialdehyde cross-link with proteins, enzymes and DNA (Benson et al. *1992a*, *b*; Wolff et al. *1986*), and are thus potential mutagens (Esterbauer et al. *1988*; Frankel *1987*). Oxidative stress is difficult to evaluate in many in vitro tissues as the mass is often small and of limited abundance, and many assays for oxidative damage are adversely affected by the carbohydrates and secondary metabolites that are abundant in in vitro tissues (i.e. thiobarbituric acid reactive substances; Benson and Roubelakis-Angelakis *1992*). However, headspace volatile analysis coupled with gas chromatography (GC) provides a non-destructive means to estimate stress-related volatiles during and following each step of the cryogenic protocol. The non-destructive nature of this methodology also allows direct comparisons to be made between the volatile profile and the recovery characteristic of the tissue. This methodology can be used to estimate ·OH formation, the biosynthesis of the stress hormone ethylene, and to detect end-products of lipid peroxidation such as ethane, ethylene, pentane and hexanal (Benson and Withers *1987*; Fleck et al. *2000*; Frankel *1980*). The ·OH is estimated through its reaction with dimethyl sulphoxide (DMSO), which in turn produces methyl radicals and subsequently methane which can be quantified (Benson and Withers *1987*; Fleck et al. *2000*).

Ethylene is a major plant hormone which is predominantly synthesized through the pathway from methionine to *S*-adenosylmethionine (SAM) via methionine adenosyltransferase, from SAM to 1-aminocyclopropane-1-carboxylate (ACC) via ACC synthase, and from ACC to ethylene via ACC oxidase (Yang and Hoffman *1984*). There is also some evidence that this hormone can also be produced via a free radical-independent pathway (Frankel *1982*). Plant tissues produce ethylene at most stages of growth and development, and are considered as an important promoter of stress responses and senescence (Yang and Hoffman *1984*). Stress-induced ethylene can be caused by a variety of environmental factors such as mechanical wounding, insect infestation, temperature, drought and chemicals. Stress ethylene production is associated with living tissue and with cell death generally halting ACC-dependent ethylene production. Variation in ethylene production has also been shown to be indicative of membrane integrity in a number of plant species exposed to chilling and freezing temperatures (Benson and Withers *1987*; Corbineau et al. *1990*), as a functional membrane is thought to be important for conversion of ACC to ethylene (Bouzayen et al. *1990*).

The development of a cryopreservation procedure for long-term storage of cocoa germplasm is important for the preservation of its biological diversity and genetic fidelity. The current procedure involves a sequence of treatments

that includes encapsulation, sucrose preculture, desiccation, liquid nitrogen storage and recovery (Fang et al. *2004*). Each step is a possible cause of stress to the stored somatic embryos, which could cause a concomitant reduction in embryo viability. The objective of the present study was to evaluate the role of ethylene and oxidative stress in the recovery of cryopreserved cocoa somatic embryos by non-destructively quantifying the formation of stress-related volatiles (methane, ethane and ethylene). Moreover, the hypothesis that oxidative stress is related to loss of embryo recovery will be further examined by supplementing the tissue culture medium with the free radical scavenger DMSO and the antioxidant quercetin.

Materials and methods

Plant material

Somatic embryos were initiated from floral explants collected at the University of Reading Intermediate Cocoa Quarantine Unit according to the procedure of Li et al. (*1998*). They were maintained in embryo development medium (ED) comprised of DKW basal salts, 100 mg l^{-1} *myo*-inositol, 2 mg l^{-1} thiamine-HCl, 1 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 30 g l^{-1} sucrose, 1 g l^{-1} glucose, and 2 g l^{-1} Phytagel, pH 5.7 (Li et al. *1998*). The cultures were maintained in 100 × 15 mm Petri dishes at 25 ± 2°C under a 16 h photoperiod at approximately 2,000 lux. Early-cotyledonary stage secondary somatic embryos (~2 mm in length and ~8.8 $gH_2O\ g^{-1}dw$) of the genotype AMAZ12 were used in all experiments.

Assessment of volatile hydrocarbon production and embryo survival throughout cryopreservation

Somatic embryos were subjected to the sequence of cryopreservation treatments as described in Fang et al. (*2004*): (a) encapsulation, within 3% calcium alginate beads; (b) preculture, encapsulation + preculture on ED medium enriched with 0.3 M sucrose for 3 days then on ED medium enriched with 0.75 M sucrose for another 4 days; (c) desiccation, preculture + dehydration over 40 g silica gel inside a 100 × 15 mm Petri dish for 4 h (moisture content of encapsulated embryos by 4 h drying is 0.18 ± 0.01 $gH_2O\ g^{-1}dw$); and (d) liquid nitrogen storage, desiccation + liquid nitrogen storage for 1 h and rewarming in a 35°C water bath for 5 min. Somatic embryos were recovered in dark at 25°C after each step of this protocol using sealable glass vials (3 replicates of 5 embryos per treatment) and Petri dishes (3 replicates of 10 embryos per treatment) containing 0.3 M sucrose-enriched ED medium for the first 3 days and on standard ED medium for the rest of the recovery period. The glass vials were used to profile volatile production during recovery, while the Petri dishes (100 × 15 mm; containing 25 ml of medium; headspace volume of ca. 50 ml) were included as positive controls for recovery on the basis that the Petri dishes are the standard recovery system used in this species.

The borosilicate glass vials (8 ml) and silicon–teflon seals/septa were sterilized before use in an autoclave at 115°C, 68,950 Pa, for 20 min, and ventilated for at least 2 h in a sterile laminar flow cabinet to remove contaminating volatiles and excess water. The culture medium (5.5 ml), a thin layer (250 μl) of 5% (v/v) DMSO on top of the medium, and five post-treatment encapsulated embryos (150 μl for each bead) were placed inside the vials. DMSO was added to the glass vials to scavenge hydroxyl radicals, with the resulting reaction detected as methane formation (Benson and Withers *1987*). DMSO was not added to the Petri dishes as it is not a standard medium additive for the recovery of this species. Volatile hydrocarbons were sampled from the glass vials at 24 h intervals for up to 12 days as described in Benson and Withers (*1987*) and Fleck et al. (*2000*). This approach is non-destructive and thus damage can be evaluated in the same tissues over the entire recovery period.

Headspace samples of 1 ml were withdrawn using a gas-tight syringe. The syringe was flushed out at least five times before removal to ensure complete mixing of the headspace. In between sampling different vials, the syringe was flushed out in the sterile air of the laminar flow cabinet. After the samples were taken, the vials were allowed to aerate for 30 min before resealing and returning to the culture room. Headspace samples were injected into a Pye Series 104 Gas Chromatograph, fitted with a Poropak R column, an injector at 200°C, a hydrogen/air flame ionization detector at 225°C, and an oven temperature of 60°C. Nitrogen was used as the carrier gas with a flow rate of 15 ml min^{-1}. Gas chromatographic peaks were identified using standard gas mixtures (15 ppm CH_4, C_2H_6, C_2H_4, C_2H_2, C_3H_8, C_3H_6, C_3H_4, C_4H_{10} in N_2, Supelco, UK) injected prior to each analysis. The peak area of the external standard was used to calculate the concentration of each volatile compound in the headspace samples. The amount of each volatile hydrocarbon (in nl) produced per 24 h per five embryos was calculated by multiplying the concentration of the volatile by the volume of the headspace. Hydrocarbon productions are expressed in volume (nl) and not in dry weight (DW) basis because it is important to keep the bead and tissue intact for non-destructive volatile assessment during recovery. Embryo-free control vials were incorporated everyday to determine the presence of any background volatiles evolved from media, vials and septa. As the background methane level was non-negligible in these vials and was found to derive from the ambient laboratory, all the data were daily corrected by subtracting the volume of methane in control vials from the samples.

The cumulative productions of the different hydrocarbons over the entire recovery period after each treatment in the cryogenic protocol were compiled and data were arcsine transformed prior to the analysis of variance (SAS Institute Inc., USA). Survival was also recorded 14 days following each treatment as the percentage of embryos exhibiting new tissue growth from any part of the embryo. Survival data from embryos in the vials and in Petri dishes were also submitted to the analysis of variance as described above.

Assessment of the effects of DMSO and quercetin on post-cryopreservation volatile hydrocarbon production and embryo survival

The effects of quercetin (dihydrate, Sigma) and DMSO (Sigma) incorporated in the sucrose preculture media (0.3 M for 3 days and 0.75 M for 4 days) at concentrations of 1, 10 and 50 μM and 1, 5 and 10% (v/v), respectively, on embryo post-cryopreservation recovery were determined. Both quercetin and DMSO were added to the medium before autoclaving. Embryo recovery was performed in glass vials (3 replicates of 5 embryos per treatment) and Petri dishes (3 replicates of 10 embryos per treatment) as described above, with a thin layer (250 μl) of 5% (v/v) DMSO included for volatile assessments in glass vials, and no DMSO added to Petri dishes to simulate normal recovery conditions. The evolution of volatile hydrocarbons was monitored on post-cryopreserved samples consecutively for 12 days after which embryo survival was recorded.

Results

Volatile hydrocarbon production and embryo recovery throughout cryopreservation

Ethylene and methane were the only volatiles detected in the present study and their production from embryos recovered from each step of the cryogenic protocol is presented in Fig. 1. Somatic embryos were first recovered on 0.3 M sucrose medium for 3 days and thereafter transferred onto the standard culture medium. Somatic embryos subjected to alginate encapsulation were characterized by a decrease in ethylene production in the absence of methane during the first 3 days of recovery. From day 3 to 8, both ethylene and methane were present and their production peaked at day 6. From day 8 to 10, no methane was detected and a high level of ethylene which peaked at

day 9 was observed. This increase was associated with the regrowth of embryos starting at day 8. A similar trend was seen for embryos recovered after the sucrose preculture step, except that the first peak of methane production ended at day 7 and was followed by a short second peak of methane production at day 8. For embryos recovered from the desiccation step of the protocol, the first peak of methane was recorded as early as 2 days after transfer of embryos to the recovery medium. A second and final peak in methane production was observed at day 5 and no methane was observed beyond day 8. In contrast, ethylene continuously increased from day 3 to 10 and first embryo regrowth was recorded at day 9. For embryos recovered from the liquid nitrogen step of the protocol, methane fluctuations were more prevalent than all previous treatment steps with four consecutive peaks of methane production recorded, with the first occurring on the second day of recovery. The recovery of embryos following liquid nitrogen storage was slow compared with the other treatments (starting from day 12), which coincided with lower ethylene production and the continuous presence of methane after 8 days.

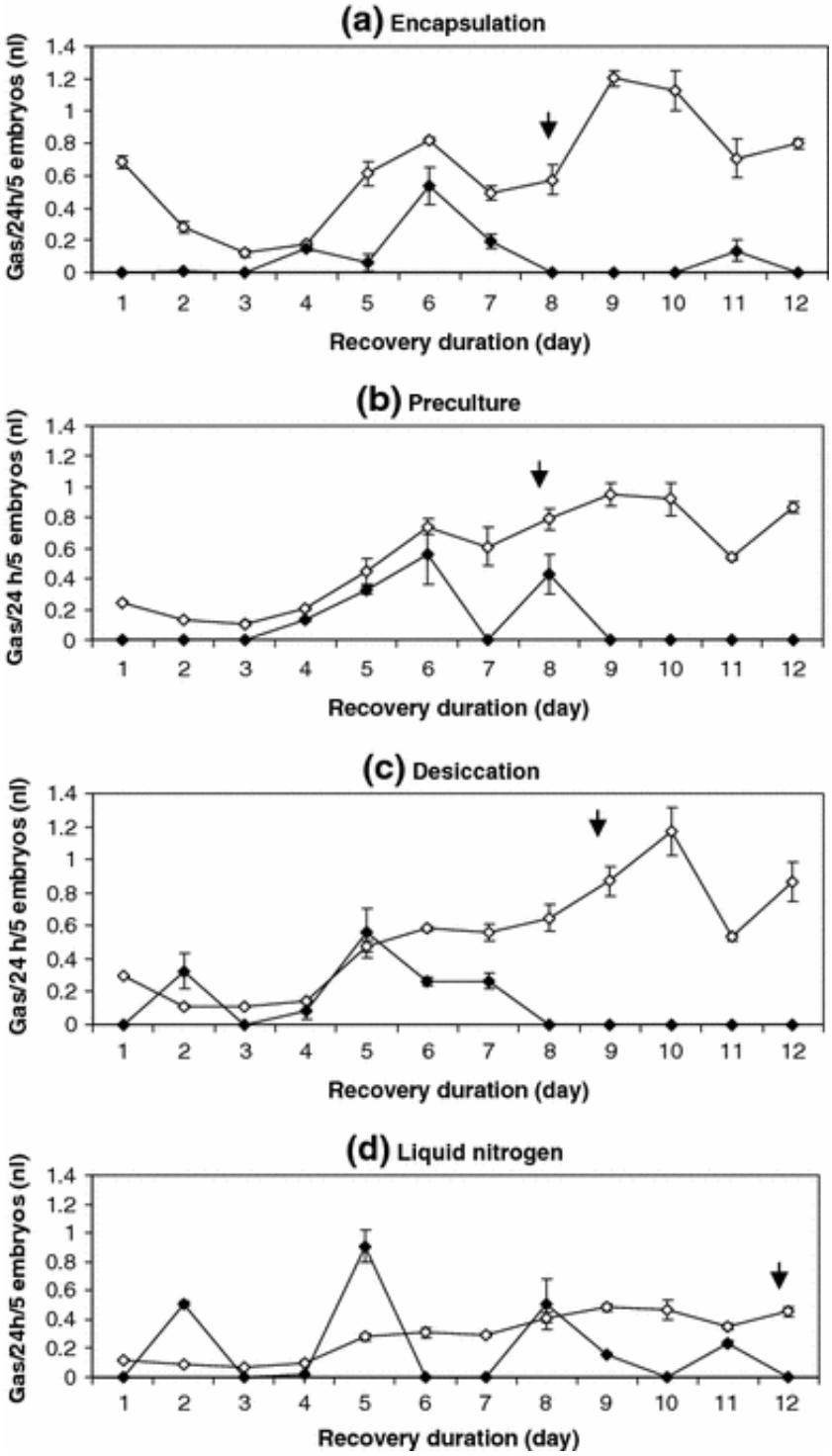

Fig. 1 Compilation of methane (*closed circles*) and ethylene (*open circles*) production during 12 days recovery of cocoa somatic embryos subjected to the different stages of cryopreservation: **a** encapsulation, **b** preculture, **c** desiccation and **d** liquid nitrogen storage. Somatic embryos were first recovered on 0.3 M sucrose medium for 3 days and thereafter transferred onto the standard culture medium. *Bars* represent SE. *Arrows* indicate the time at which embryo regrowth was first observed. Data represent the mean of three replicate vials of five embryos each

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Cumulative production of methane and ethylene over the 12-day recovery is summarized in Fig. 2 for embryos recovered from each step of the cryogenic protocol. Ethylene production was significantly lower following exposure to liquid nitrogen when compared to the three other treatments ($P = 0.0414$). In contrast, methane production was comparable for all treatments ($P = 0.5155$).

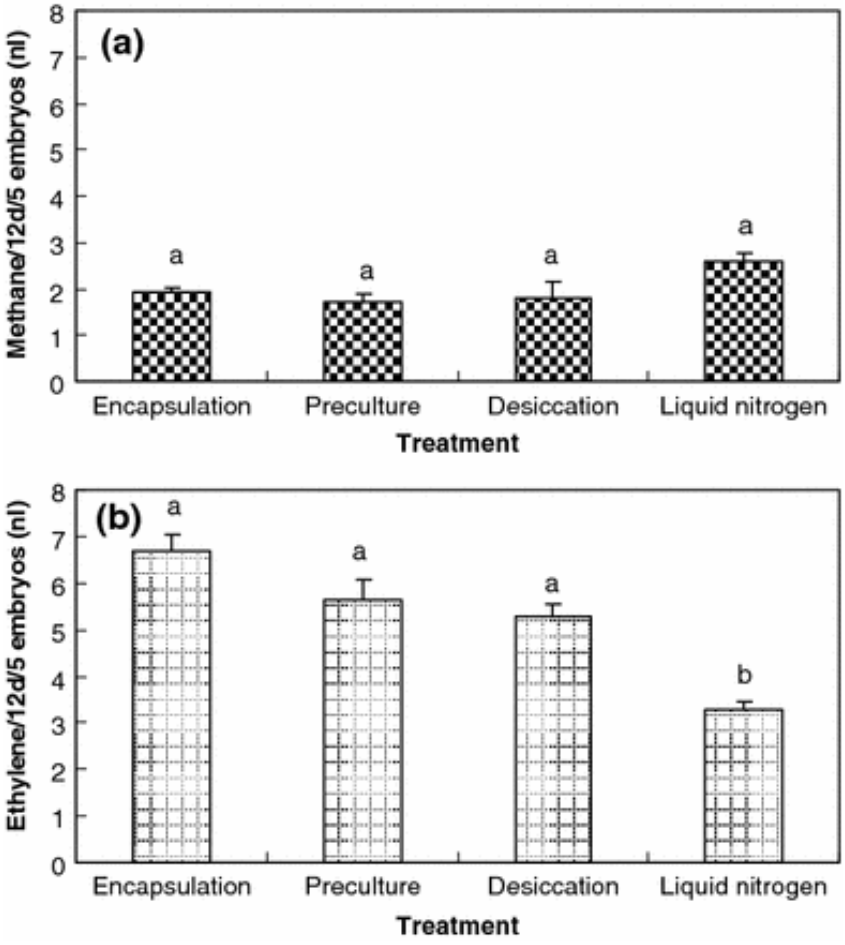


Fig. 2 Cumulative methane (**a**) and ethylene (**b**) productions of cocoa somatic embryos following each stage of the cryopreservation process over the 12 days recovery period. *Bars* represent SE. Data represent the mean of three replicates of five embryos and were ranked using Duncan multiple range test. Data with the same *letter* were not significantly different ($P = 0.05$)

Embryo survival in the GC vials was complete (100%) following encapsulation, preculture and desiccation. Only those embryos subjected to liquid nitrogen did not fully recover ($P = 0.0519$) (Fig. 3a). Survival of embryos which underwent the same treatments but recovered in Petri dishes exhibited a lower survival frequency after desiccation

and liquid nitrogen storage compared to the ones which were recovered in the GC vials (Fig. 3b). In both culture vessels, embryo regrowth was first observed following the encapsulation and preculture treatments, followed by desiccation and finally the liquid nitrogen treatment.

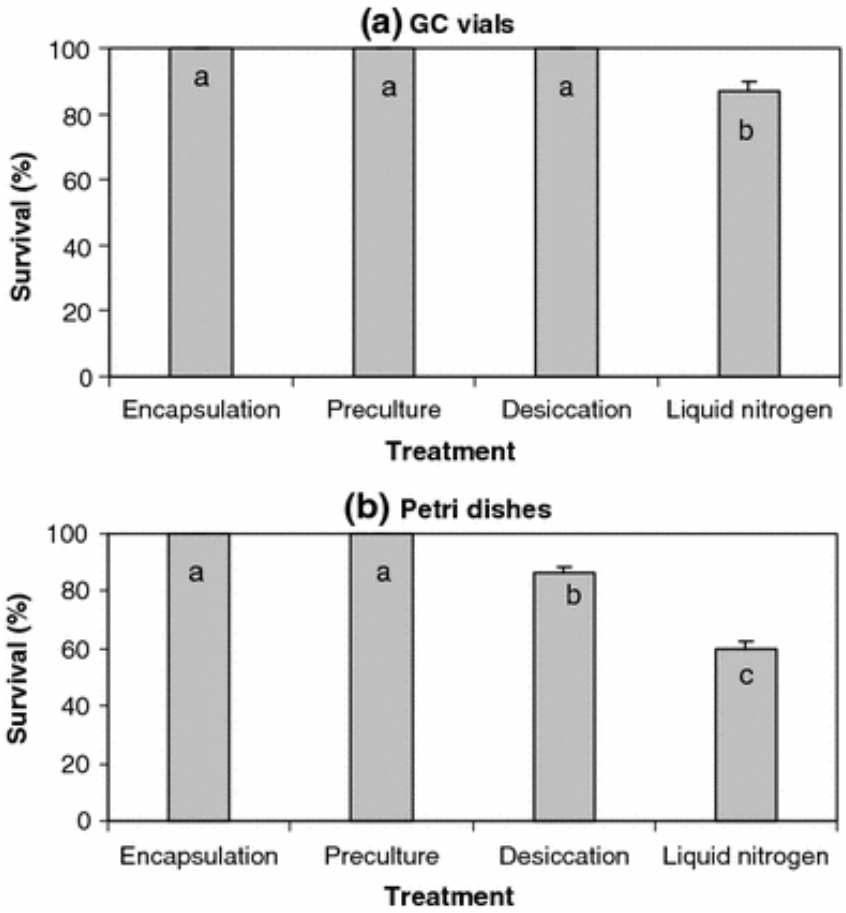
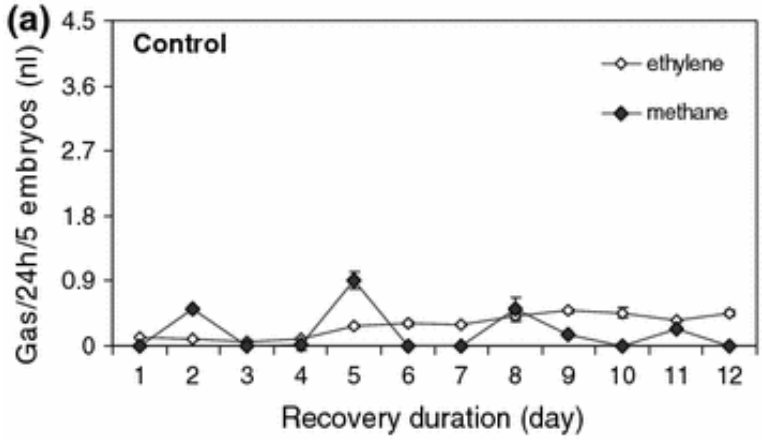
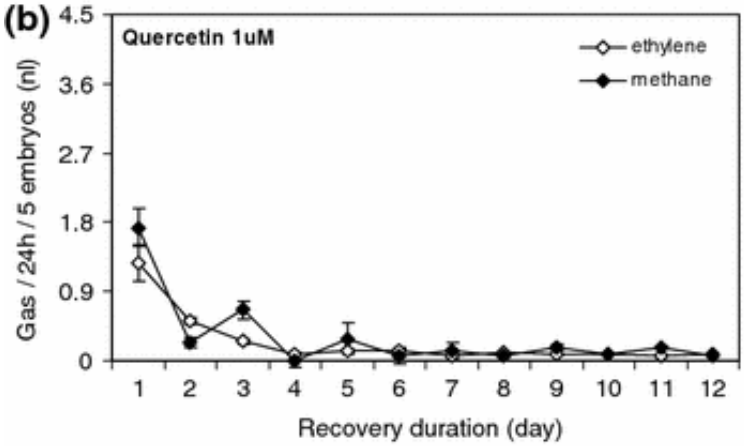


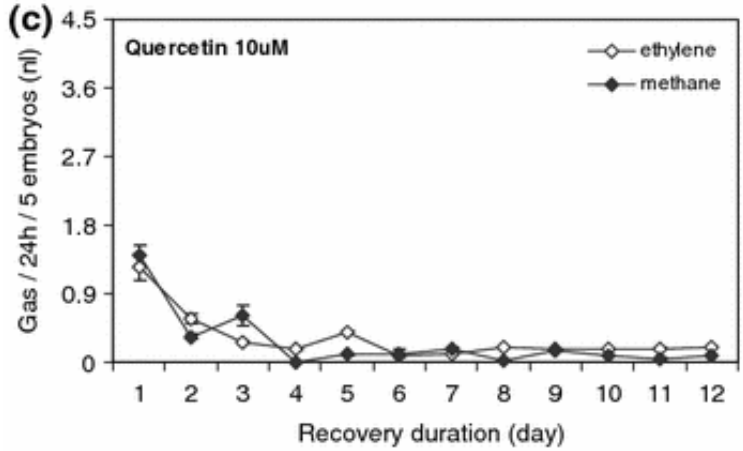
Fig. 3 Survival frequencies of cocoa somatic embryos recovered in GC vials (**a**) and in Petri dishes (**d**) following each stage of the cryopreservation process. *Bars* represent SE. Data were the mean of three replicates of five embryos in GC vials and three replicates of ten embryos in Petri dishes. Survivals were recorded 14 days after treatment and ranked using Duncan multiple range test. Data with the same *letter* were not significantly different ($P = 0.05$). *Bars* represent SE

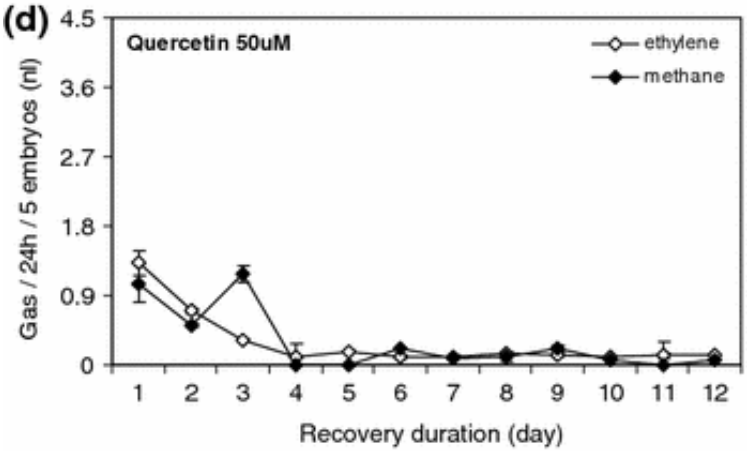
Effects of DMSO and quercetin on post-cryopreservation volatile hydrocarbon production and embryo survival

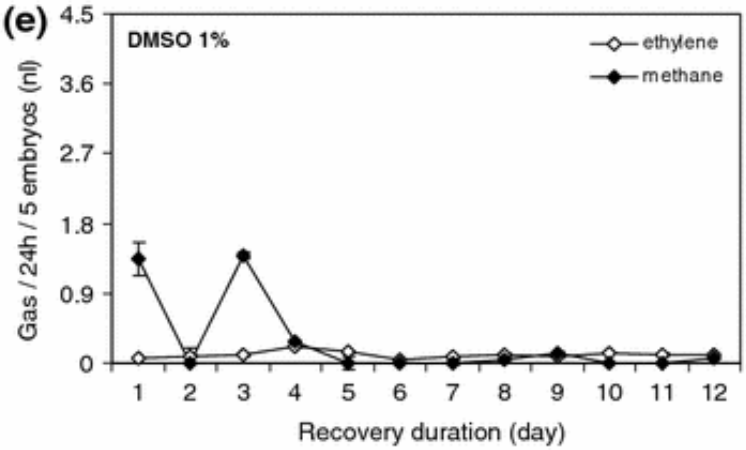
Similar trends in methane and ethylene evolution were observed for cryopreserved embryos exposed to the different pre-treatments supplemented with DMSO and quercetin (Fig. 4). Both methane and ethylene gradually diminished from day 1 to 4 and remained relatively constant thereafter, with the exception of the 1% DMSO treatment which showed a low level of ethylene production during the entire recovery period. Quercetin-treated embryos showed very similar level of ethylene and methane production regardless of the concentration used. The methane production was consistently higher in DMSO-treated samples compared with quercetin-treated samples but was comparable to the controls. Both methane and ethylene were observed in larger quantities during the first 4 days of recovery in DMSO- and quercetin-treated embryos compared to the controls, especially for the 5 and 10% DMSO treatments.

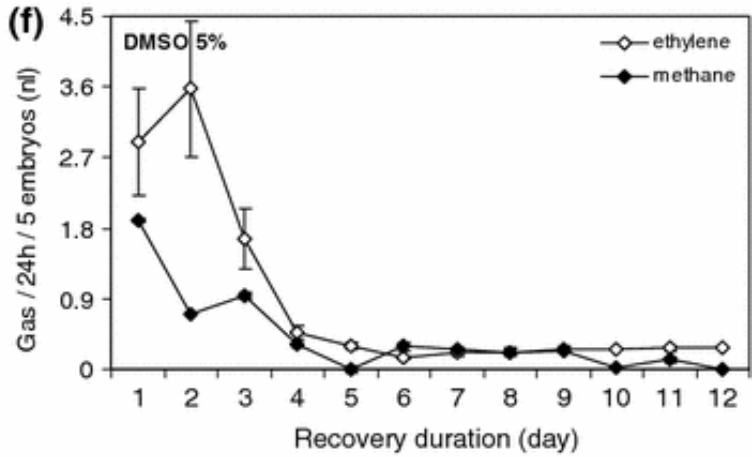

(a)
Control
ethylene
methane
Gas/24h/5 embryos (nl)
Recovery duration (day)


(b)
Quercetin 1uM
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)


(c)
Quercetin 10uM
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)


(d)
Quercetin 50uM
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)


(e)
DMSO 1%
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)


(f)
DMSO 5%
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)

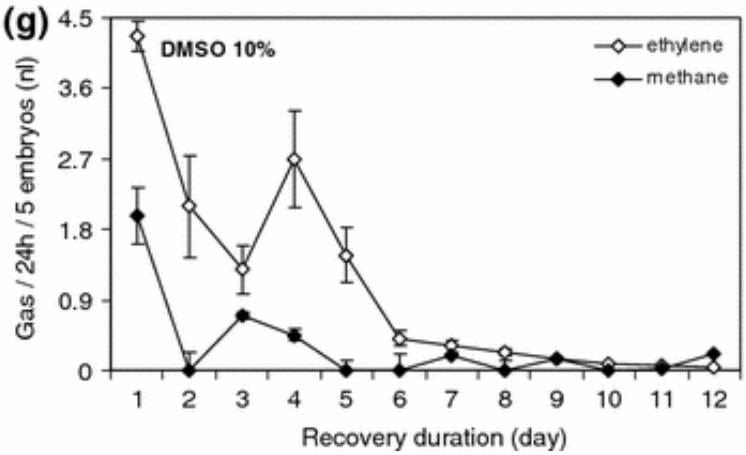

(g)
DMSO 10%
ethylene
methane
Gas / 24h / 5 embryos (nl)
Recovery duration (day)

Fig. 4 Time course of ethylene and methane productions in post-cryopreserved cocoa somatic embryos in the control (**a**) and following 1–50 μM quercetin (**b**–**d**) and 1–10% DMSO (**e**–**g**) treatment. Data were the mean of three replicates of five embryos each. *Bars* represent SE

Cumulative methane production over the entire recovery period was comparable in all the treatments ($P = 0.5892$) (Fig. 5a). In contrast, ethylene production was similar in all the quercetin treatments but variable between the DMSO treatments with a significantly higher production from the embryos which were treated with 5 and 10% DMSO ($P = 0.0374$) (Fig. 5b).

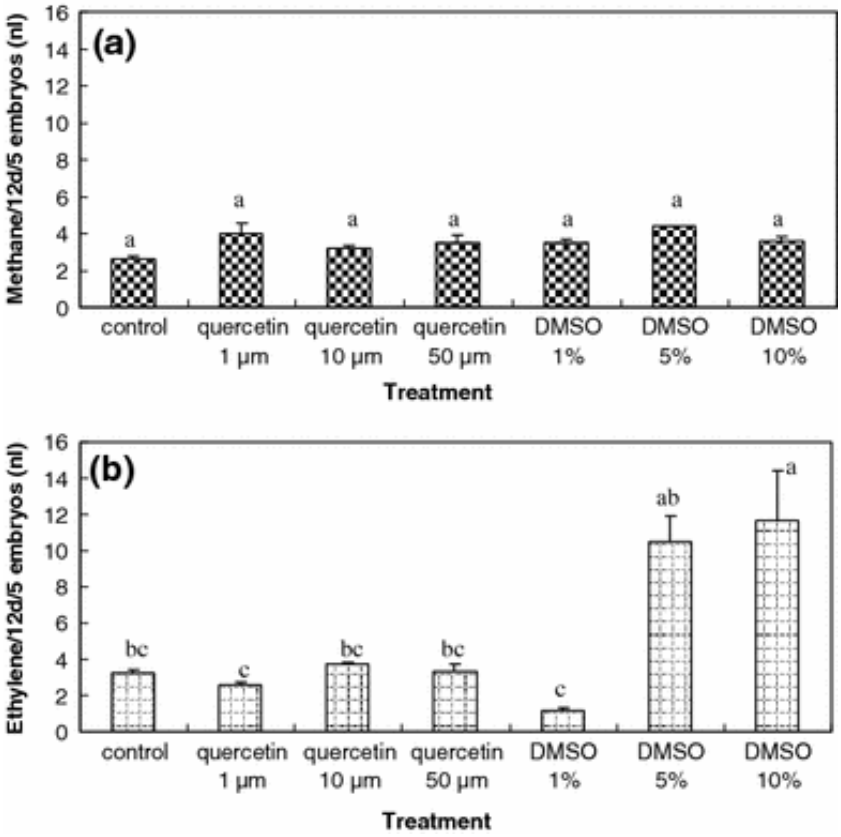


Fig. 5 Cumulative production of methane (**a**) and ethylene (**b**) in cocoa somatic embryos over the 12 days recovery period after preculture with 1–50 μM quercetin and 1–10% DMSO. Data represent the mean of three replicate of five embryos each. Data were ranked using Duncan multiple range test and those with the same *letter* were not significantly different ($P = 0.05$). *Bars* represent SE

In the GC vials, significant lower survival was observed in embryos precultured with 1 and 10% DMSO than the controls (Fig. 6a). In the Petri dishes, embryos incubated with 50 μM quercetin as well as with 5 and 10% DMSO showed a significantly lower viability compared to the controls (Fig. 6b).

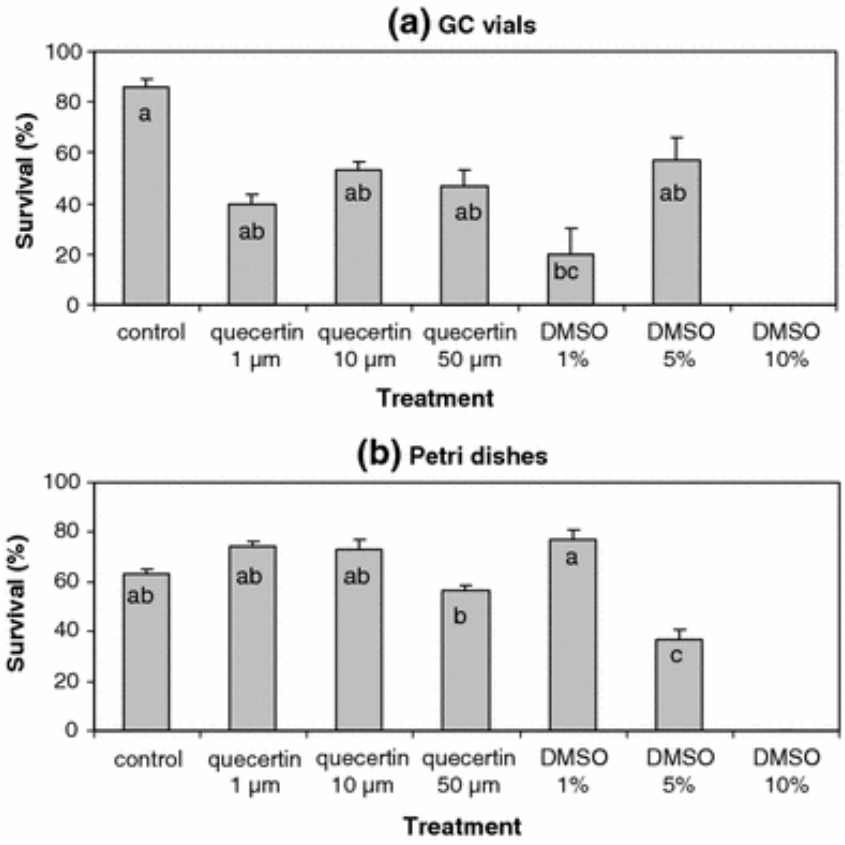


Fig. 6 Post-cryopreservation survivals of somatic embryos recovered in GC vials (**a**) and in Petri dishes (**b**) after preculture with 1–50 µM quercetin and 1–10% DMSO. Data represent the mean of three replicates of five embryos in GC vials and three replicates of ten embryos in Petri dishes. Survival was recorded 14 days after cryopreservation and was ranked using Duncan multiple range test. Treatments with the same *letter* were not significantly different ($P = 0.05$). *Bars* represent SE

Discussion

The use of volatile hydrocarbons as markers for recovery of cocoa somatic embryos from the different stages of the cryogenic protocol provided new insights into the roles of the hydroxyl radical (detected as methane in the presence of DMSO) and ethylene in the recovery process. Methane production was characterized by several peaks during the recovery period. These peaks were found to occur earlier and more often for embryos recovered from the desiccation and liquid nitrogen than from the encapsulation and sucrose preculture steps of the protocol; two steps which also resulted in greater loss of embryo viability. The early peaks of methane production probably reflect bursts in ROI production attributed to desiccation and liquid nitrogen stress, with later peaks reflecting ROI bursts associated with sustained stress or the initiation of recovery processes such as embryo germination. This suggests that excessive hydroxyl radical formation during the first 2–3 days of recovery may be detrimental to long-term embryo recovery, while in contrast those radicals produced later in the recovery process maybe an inevitable and essential component of the metabolic processes required for embryo germination. Therefore, strategies using antioxidant supplements to reduce the potential deleterious effects of hydroxyl radicals in cocoa would be of most benefit during the first 2–3 days of recovery. Studies with *D. carota* and *Euglena gracilis* also showed that hydroxyl radical formation was associated with reduced survival (Benson and Withers *1987*; Fleck et al. *2000*). The concept that oxidative stress could be detrimental for recovery was strengthened in further studies that demonstrated that antioxidant supplements could improve post-thaw survival rates (Benson et al. *1995*; Fleck et al. *2000*).

The time course of methane and ethylene production in the post-treatment recovery period could be divided into three phases and associated with embryo recovery. During the early stage of recovery (days 1–3), a decrease in ethylene production often coincided with the appearance of methane. Reduced ethylene production in all the treatments may indicate that the embryos were under stress due to the space restriction inside the vials. The low level of ethylene in the encapsulated embryos may be associated with a decrease in respiration rate as observed in encapsulated carrot somatic embryos (Kersulec et al. *1993*), whereas low levels of ethylene observed in the preculture, desiccation and liquid nitrogen treatments may reflect treatment-induced stress. This was further supported by the production of methane in the desiccation and liquid nitrogen treatments. Alternatively, sucrose-precultured embryos may have reduced ethylene production due to osmotic stress-induced membrane disruption in some cells of the embryos as indicated by our microscopic analyses (unpublished data). The second phase (days 3–8) was marked by an increase in both ethylene and methane, suggesting that tissue recovery was assisted by the free radical scavenging property of DMSO. At this stage, the embryos were recovered on standard ED medium (containing only 0.08 M sucrose), and were thus liberated by the constraint of the cryoprotectant and were able to resume ethylene synthesis. The third phase (days 8–12) showed an opposite trend to the phase I as increased ethylene coincided with a decline in methane. During this phase, embryos may have overcome treatment-induced stress and started to restore the antioxidant status and metabolism. This was associated with the recovery of embryos as evidenced by tissue regrowth.

Methane is associated with the free radical scavenging property of DMSO and the production of hydroxyl radicals (Benson and Withers *1987*). The persistent production of methane indicated that DMSO was continuously acting as a free radical scavenger during the entire recovery period. DMSO initially employed to assist the GC assay appeared to confer some desiccation and cryopreservation tolerance to cocoa somatic embryos. Although concentrations of 10% (v/v) and greater are the usual rates of application required for DMSO to act as cryoprotectant, it was shown that DMSO applied at 0.22% v/v (=250 μl of 5% v/v solution added per GC vial) in the present assay appeared to protect embryos from desiccation and cryopreservation injuries.

Since the exposure of embryos to desiccation and liquid nitrogen was shown to be associated with a burst of methane production in the first 2–3 days of recovery, attempts have been made in the present study to assist post-cryopreservation recovery of embryos by the timed application of anti-oxidative compounds. The antioxidants were added during the preculture phase to allow sufficient time for antioxidant uptake, and to ensure their availability before the ROI burst associated with the final two steps of the cryogenic protocol. Similarly for *Citrus*, the supplementation of tissue culture medium with antioxidant was most beneficial for post-thaw recovery when applied during the preculture step of the protocol (Wang and Deng *2004*). A number of antioxidants have been applied to in vitro cells and tissues stored at low temperatures and were shown to have beneficial properties. For example, DMSO was employed in increasing the recovery of cryopreserved *D. carota* and it has been shown to scavenge ·OH for up to 7 days after thawing (Benson and Withers *1987*). Post-cryostorage survival of *E. gracilis* and *O. sativa* was significantly enhanced when the chelating agent desferrioxamine was included in the recovery medium (Benson et al. *1995*; Fleck et al. *2000*). The properties of desferrioxamine have been attributed to iron sequestration and thus the prevention of ·OH generation. Reduced glutathione significantly improved shoot tip survival after cryopresevation of *Citrus* (Wang and Deng *2004*). In the present study, DMSO and quercetin were tested for their protective effects on cocoa somatic embryos. Quercetin is a flavonoid capable of directly scavenging free radicals, chelating reactive elements such as iron and copper, preventing lipid peroxidation and inhibiting oxidative enzymes (Mira et al. *2002*; Terao et al. *1994*). It has been reported to protect against lipid peroxidation when model phospholipids bilayers were exposed to aqueous oxygen radicals (Terao et al. *1994*). The present study is the first to determine the effect of quercetin on plant tissue cryo-tolerance.

Viability of cocoa somatic embryos recovered in the Petri dishes was significantly lowered by the presence of DMSO at 5 and 10% in the preculture medium in comparison to other treatments. These DMSO concentrations are reflective

of those used in cryopreservation protocols where it is used as a cryoprotectant (Benson and Withers *1987*; Fleck et al. *2000*). In the GC vials, only 10% DMSO treatment resulted in a significantly lower embryo survival. Generally, the survival rate of embryos in the GC vials was lower than in the Petri dishes except for the controls, and this suggests that the exposure of embryos to quercetin or DMSO during preculture and subsequently to DMSO in the vials was harmful for their recovery. DMSO is known to enhance the intracellular penetrative capacity of many compounds (Williams and Barry *2004*), and the presence of DMSO in the GC vial may have resulted in higher intracellular uptake of residual quercetin and/or other residual components of the pre-treatment medium (e.g. sucrose). The negative effects of DMSO and quercetin were also evidenced by the inhibition of ethylene production during most of the recovery period. The toxicity of DMSO may relate to its potential to enhance membrane fusion and phase separation (Yu and Quinn *1998*), and that of quercetin to its capacity for chelating metal ions (e.g. copper) which are involved in ethylene synthesis (Yang and Hoffman *1984*).

The concentration at which the antioxidant was applied to the embryos may be important for their post-cryopreservation recovery. For example, post-cryopreservation embryos exposed to 0.22% DMSO in the vials exhibited a higher recovery (86%) than embryos precultured in the presence of 5% DMSO (37%) in the Petri dishes. It was therefore possible that cocoa embryo recovery could be assisted by a lower concentration of DMSO. Further testing using different DMSO concentrations in the preculture medium could be helpful to confirm the above hypothesis.

Ethylene was continuously detected following all stages of cryopreservation but varied in quantity according to the step of the protocol from which the embryos were recovered. The lowest level of ethylene was detected following exposure to liquid nitrogen, which was also the step of the protocol which caused the greatest reduction in embryo viability. Similar results have also been observed for *Ribes* spp. and *D. carota* (Benson and Withers *1987*; Johnston et al. *2007*). The reduction in ethylene production following exposure to liquid nitrogen probably reflects an increased proportion of dead cells and/or a diminished capacity for ethylene biosynthesis in live cells from impaired membrane function. While these results showed that low ethylene was associated with low recovery in the first experiment, this relationship did not occur in the subsequent experiment using high concentrations of DMSO (5–10%). Reasons for this response are not known, but a similar response has also been found for *Olea europea* somatic embryos (Siddika et al. unpublished data) and *Ribes* shoot-tips (Green et al. unpublished data). Future experiments are required to ascertain if the ethylene produced by high DMSO concentrations and if the suppressed ethylene biosynthesis by liquid nitrogen, are via the same pathway (i.e. derived enzymatically from ACC or non-enzymatically from lipid peroxidation).

Three conclusions could be drawn from the present study: (a) volatile hydrocarbon assessment using GC was a useful tool to evaluate stress-related markers at different stages of the cryopreservation procedure for cocoa somatic embryos; (b) methane production fluctuated during recovery, with the fluctuations more prevalent and occurring earlier following steps of the cryogenic protocol which resulted in loss of viability (i.e. desiccation and liquid nitrogen storage); and (c) ethylene production was associated with embryo recovery in cryogenic systems free from high concentrations of DMSO. The physiological importance of these markers for embryo recovery needs to be determined in experiments with inhibitors of ethylene biosynthesis and/or action, and through the strategic application of antioxidants at particular stages of the cryogenic protocol.

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