

## The effects of *in vitro* gaseous environment on the maturation of somatic embryo of black spruce (*Picea mariana* [Mill.] B.S.P.) and white spruce (*Picea glauca* [Moench] Voss)

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### Abstract

The objective of the current study was to determine the effects of the gaseous environment on conifer somatic embryos and to understand how the gaseous environment in the culture tissue system may influence the maturation. At the first time, the effect of confined and vented gaseous microenvironments (tightly sealed and vented containers) on the maturation of somatic embryo of white spruce was examined. The results showed that confined microenvironment promoted a higher production and uniformity of mature somatic embryos and prevented the precocious germination in comparison to vented environment. Reducing air exchange may be useful to synchronize the maturation stage. The headspace gas analyses of confined environment revealed a marked CO<sub>2</sub> accumulation (33.6 %), correspondent O<sub>2</sub> consumption (2.3 %) and C<sub>2</sub>H<sub>4</sub> accumulation (7 µl/l). To determine how the gaseous environment influenced the somatic embryo maturation, the effects of oxygen, and carbon dioxide were evaluated during the maturation stage of black spruce and white spruce. The results show that the addition of 5% CO<sub>2</sub> or 10% O<sub>2</sub> promoted somatic embryos in both spruce species. The beneficial effect of these gases on somatic embryo formation might be explained by their action on ethylene biosynthesis pathway since reduced oxygen concentration resulted in a marked increase of the endogenous levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in embryogenic tissues of white spruce. Abbreviations — ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; FID, flame ionization detector; GC, gas chromatography; HLM, half Litvay's medium.

**Key words:** *embryo maturation, Picea glauca, Picea mariana, somatic embryogenesis.*

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### Introduction

Since the description of an efficient protocol of somatic embryogenesis in Norway spruce was reported (Hakman *et al.* 1985) this technique has been used successfully for cloning several conifer species (Jain *et al.* 1995). Recently, Högborg *et al.* (1998) have discussed the potential and limitations of using this technique in conifer breeding programs. These authors indicated that the proliferation and maturation stages were the major steps of the technique.

Even though several chemical factors influencing the maturation of somatic embryo in conifers have been studied, relatively few studies have focused on the influence of *in vitro* gaseous environment. *In vitro* gas composition surrounding the cultures is one critical aspect of the culture microenvironment that can influence plant growth and development.

In conifers, Kvaalen and von Arnold, (1991) reported that low pO<sub>2</sub> and high pCO<sub>2</sub> stimulated somatic embryo maturation in Norway spruce. Previously, we showed that tightly sealed flask reduced gaseous exchange rate between the culture and the external environment and caused the accumulation of both carbon dioxide and

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ethylene and the depletion of oxygen. This confined gaseous microenvironment promoted the production of normal mature embryos and prevented precocious germination, yet without affecting the quality of somatic embryos of black spruce (El Meskaoui and Tremblay, 1999). Following up this work, we were interested in determining which gas was involved in the maturation of somatic embryos and what was the physiological process taking place.

Thus, the aims of the current study are: a) to correlate the gas composition in vessels to the production of white spruce somatic embryos; b) to investigate the effects of various levels of oxygen and/or carbon dioxide, on the maturation black spruce and white spruce somatic embryos; and c) to obtain more information regarding the effect of oxygen on the ethylene biosynthesis, endogenous 1-aminocyclopropane-1-carboxylic acid (ACC) an immediate precursor of ethylene.

## Materials and methods

### *Plant material*

Embryogenic tissues were induced from mature zygotic embryos of white spruce (*Picea glauca* [Moench.] Voss) (line G-316) and black spruce (*Picea mariana* [Mill.] BSP) (line M-67.5) as previously described (Tremblay 1990, Tremblay *et al.* 2005). The embryogenic lines were maintained by subculturing every 14 days on HLM-1 medium (Tremblay 1990). Somatic embryos were produced with 45  $\mu\text{M}$  abscisic acid (ABA), 6 % (w/v) sucrose and 0.4 % (w/v) Gelrite™ gellan gum (Schweizerhall Inc., South Plainfield, NJ, USA). Glutamine 0.1% (w/v), ABA and sucrose were filter-sterilized and added to the autoclaved medium.

### *Maturation of somatic embryos*

Effect of confined and unconfined micro-environment on the maturation of white spruce somatic embryos. This experiment was conducted on white spruce. The maturation was carried out in 500-ml Mason™ jars. The maturation medium solidified in jars laid horizontally. To provide either a confined or an open microenvironment during maturation, two types of closures were used on the jars. The open microenvironment was characterized by a gas exchange rate 140 times faster than the confined microenvironment according to El

Meskaoui and Tremblay (1999). The two conditions were compared to evaluate their effect on embryo maturation. The experiment followed a completely randomized design with 5 replicates per treatment and 6 pieces (80 mg  $\pm$  5 mg each) of embryogenic tissue per replicate.

### *Gaseous enrichment system*

In order to create different gaseous environments, specially designed growth chambers were built. Plexiglas chamber (65 cm x 46 cm x 30 cm) were equipped with a false floor (diffuser grill) to allow for circulation of gases. Petri dishes were placed in the four chambers and gases diffused in the Petri dishes through a microporous membranes fitted on the lid. The whole chamber was then enriched with the desired levels of gas. To avoid culture dehydration, the flux of gases was hydrated by bubbling in distilled water. The relative humidity inside the chambers was maintained at 90  $\pm$  5%. The gas flow passed through a 0.22  $\mu\text{M}$  air filter before injection in the growth chambers. Flux was maintained constantly day and night at a rate of 400 ml min<sup>-1</sup> in the system during the whole culture period. CO<sub>2</sub> and O<sub>2</sub> were injected from premixed calibrated cylinders (Produits Praxair Inc., Vanier, QC, Canada).

### *Effects of various level of oxygen and carbon dioxide on somatic embryo maturation of white spruce and black spruce*

The effects of gas enrichment on embryo maturation of black spruce (M-67.5) and white spruce (G-209) were studied through two independent experiments respectively comparing the effects: (1) of carbon dioxide (CO<sub>2</sub> tested: a) 5%, b) 10%, C) 20% of CO<sub>2</sub> balanced with air and d) a control containing air (to simulate the natural atmospheric composition) and (2) of oxygen (O<sub>2</sub> tested: a) 2% b) 5%, C) 10% of O<sub>2</sub> containing 0.035% CO<sub>2</sub> balanced with nitrogen and d) air. Each experiment was designed as randomized complete blocks with three replicates in time and ten Petri dishes per treatment per replicate. Six pieces of 80 mg ( $\pm$  5 mg) each of embryogenic tissue were cultured per Petri dish.

### *Effects of lengths of culture period on the maturation of somatic embryo of black spruce incubated under different concentration of carbon dioxide*

Four experiments were performed to examine the effects of the length of culture period on the production of somatic embryo matured under CO<sub>2</sub> enrichment in black spruce. For this purpose, somatic embryos were incubated for 1, 2, 3 and 4 weeks under various carbon dioxide concentrations. After incubation, cultures were transferred to normal air and maintained under this condition until the end of the experiment. Each experiment was a completely randomized design with 10 replicate per treatment, and 6 pieces (80 mg  $\pm$  5 mg each) of embryogenic tissue per replicate.

#### *Data collection*

After 5 weeks of maturation, the numbers of normal embryos (with 2 to 8 cotyledons symmetrically around the meristem) and abnormal embryos (fasciated, monocotyledonous or swollen, or with fused cotyledons) was determined.

#### *Determination of gas composition*

Concentrations of CO<sub>2</sub>, O<sub>2</sub> and C<sub>2</sub>H<sub>4</sub> in the head space of the culture vessels were determined at different time of maturation as described previously (El Meskaoui and Tremblay, 1999). Briefly, concentrations of CO<sub>2</sub>, O<sub>2</sub> and C<sub>2</sub>H<sub>4</sub> in the head space of the culture vessels were determined by sampling 2 ml of internal atmosphere and injecting them into a Perkin-Elmer gas chromatograph (GC) equipped with a 3.640 m x 3 mm O.D. Porapak Q column (80/100 mesh), a thermal conductivity detector (TCD) for CO<sub>2</sub> and O<sub>2</sub> analysis. Ethylene concentration was determined by sampling 1 ml of the internal atmosphere. It was injected into a GC equipped with a 1.82 m x 3 mm O.D. Porapak R column (100/120 mesh) and a flame ionization detector (FID). O<sub>2</sub>, CO<sub>2</sub> and C<sub>2</sub>H<sub>4</sub> identification was based on retention times relative to standards. Gas concentrations were determined using a Hewlett-Packard 3390A integrator. In all experiments, jars containing medium only were used as negative controls.

#### *Assay of endogenous ACC*

Five pieces of embryogenic tissue (80  $\pm$  5 mg each) were cultivated on the maturation medium. At specific times, three replicates At specified times, 200 mg fresh weight of tissue were used for extraction in 6 ml 85% ethanol for 40 min, followed by a 20 min centrifugation at

13 000 g at 4°C. The supernatant was concentrated under vacuum at 35 °C and the residue was dissolved in 4 ml distilled water. ACC content in the extracts was assayed by conversion to ethylene according to Lizada and Yang (1979). Ethylene was determined by gas chromatography as described above. The transformation yield was determined for each extract.

#### *Environmental conditions*

Maintenance and maturation were carried out under a 16-h photoperiod at 23°C. A light intensity of 5-10  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , given by Gro-Lux WS (Sylvania, Ontario, Canada) fluorescent lamps, was used for maintenance and 10-15  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , given by Vita-Lite Plus (Duro-test, Houston, TX, USA) fluorescent lamps, was used for maturation.

#### *Statistical analyses*

For each experiment, homogeneity of variance was verified by the Bartlett's test (Sokal and Rohlf, 1995). Data from maturation were analyzed using the SAS GLM procedure (SAS Institute Inc., Cary, NC, USA) and means were compared using Bonferroni's multiple range test (Sokal and Rohlf, 1995) at  $P \leq 0.05$ .

## **Results**

#### *Gas concentration under sealed and vented microenvironments*

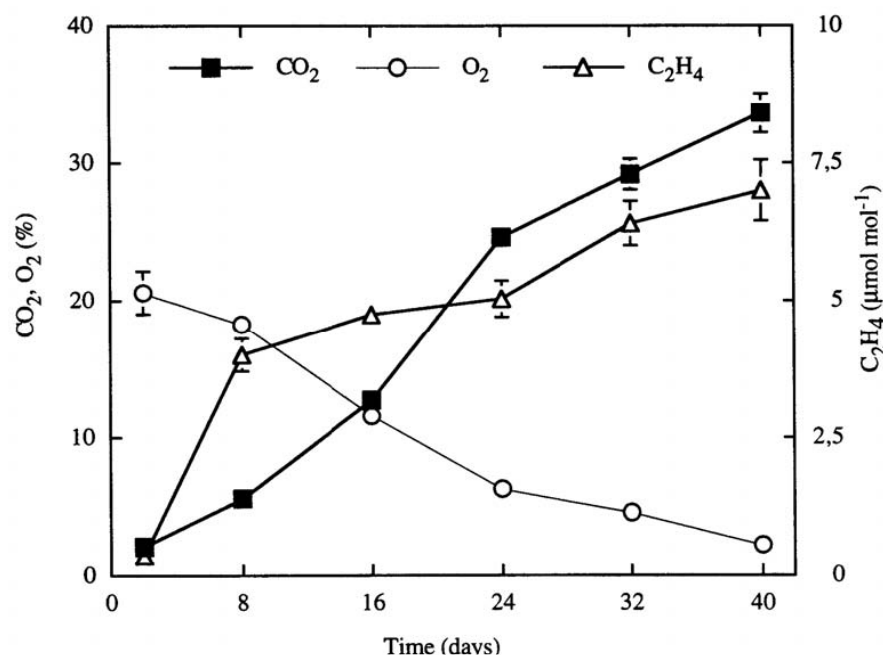
The evolution of gases in the headspace of vented microenvironment revealed that the C<sub>2</sub>H<sub>4</sub> level remained under the detection threshold with O<sub>2</sub> and CO<sub>2</sub> levels similar to those found in the ambient atmosphere. These results were similar to those obtained in absence of tissues (data not shown). However, in confined microenvironment ethylene and carbon dioxide levels increased respectively from about 1  $\mu\text{mol mol}^{-1}$  and 2% at day 2 to 7  $\mu\text{mol mol}^{-1}$  and 33.6% at day 40, while oxygen level decreased from 20.4% at day 2 to 2% at the end of the maturation period (Fig. 1).

#### *Effect of sealed and vented microenvironments on black spruce somatic embryo maturation*

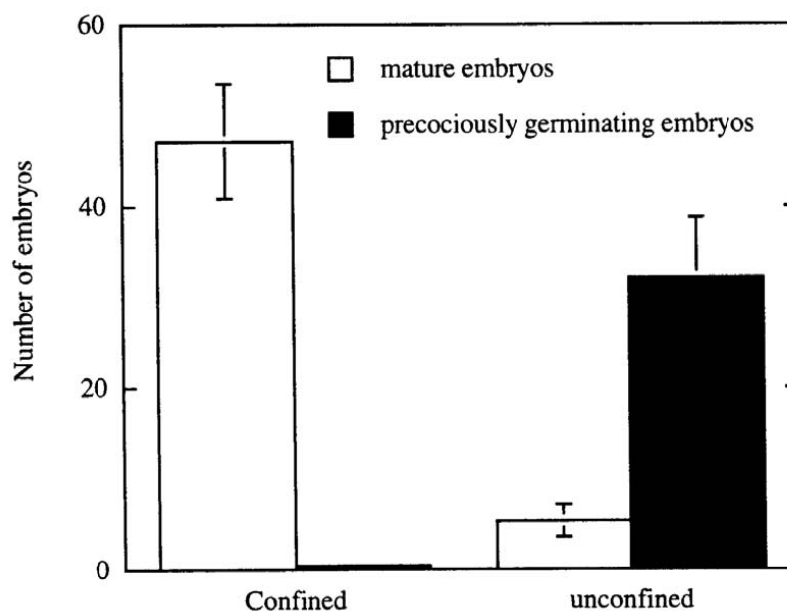
In white spruce, when the maturation was carried out under confined microenvironments, the production of normal somatic embryos significantly increased compared to vented microenvironments. Moreover, the type of microenvironment affected the quality of

embryo. Under the vented microenvironment, germinating white spruce somatic embryos were recorded whereas no precociously germinating embryos were observed under the confined

microenvironment (Fig. 2). Furthermore, the confined microenvironment limited the browning of embryogenic tissue, while the vented microenvironment favoured it.



**Figure 1.** Carbon dioxide, oxygen, and ethylene concentrations in the culture containers during maturation of white spruce somatic embryos in confined microenvironments. Data are means  $\pm$  SE,  $n=5$ .



**Figure 2.** Effect of confined and vented microenvironments on the maturation of white spruce (line G-209) somatic embryos. Data are means  $\pm$  SE,  $n=5$ .

*Effects of various level of carbon dioxide on somatic embryo maturation of white spruce and black spruce*

In preliminary tests, CO<sub>2</sub> at a concentration of 0.5, 1 or 2 % did not affect the production of somatic embryos. However, embryogenic tissues incubating under 5 % CO<sub>2</sub> showed a significant increase in the production rate of cotyledonary somatic embryos whereas incubation under 20% of CO<sub>2</sub> severely inhibited it in white spruce and black spruce (Table 1). Treatments with 5 and 10 % of CO<sub>2</sub> were found to be more effective for normal somatic embryo production as shown by the ratio normal/abnormal (Table 1).

*Effects of the duration of culture period on the maturation of somatic embryo of black spruce*

*incubated under different concentrations of carbon dioxide*

In black spruce embryogenic tissues incubated for different length of the culture period in 5% CO<sub>2</sub> and then transferred to normal CO<sub>2</sub> concentration, a long exposure (four weeks) to 5% CO<sub>2</sub> was favorable for maturation (Table 2). Furthermore, this treatment affected the quality of mature somatic embryos. More normal somatic embryos were produced as shown by the ratio normal/abnormal. However, a long exposure to 20% severely inhibited the production of mature somatic embryos whereas the production took place under a short exposure.

**Table 1.** Effect of CO<sub>2</sub> enrichment on the number of black spruce and white spruce mature somatic embryos.

Treatment	Black spruce (line M-67.5)				White spruce (line G-209)			
	Total Embryo <sup>1</sup>	Normal Embryo <sup>1</sup>	Abnormal Embryo <sup>1</sup>	Normal/Abnormal <sup>1</sup>	Total Embryo <sup>1</sup>	Normal Embryo <sup>1</sup>	Abnormal Embryo <sup>1</sup>	Normal/Abnormal <sup>1</sup>
Control	64.6 <sup>b</sup>	40.4 <sup>b</sup>	24.2 <sup>ab</sup>	1.7 <sup>b</sup>	50.7 <sup>b</sup>	27.2 <sup>b</sup>	23.5 <sup>a</sup>	1.2 <sup>a</sup>
5% CO <sub>2</sub>	86.6 <sup>a</sup>	70.6 <sup>a</sup>	16 <sup>b</sup>	4.5 <sup>a</sup>	65.3 <sup>a</sup>	38.8 <sup>ab</sup>	26.5 <sup>a</sup>	1.5 <sup>a</sup>
10% CO <sub>2</sub>	62.6 <sup>b</sup>	50.8 <sup>b</sup>	11.8 <sup>b</sup>	4.8 <sup>a</sup>	78.2 <sup>a</sup>	44.8 <sup>a</sup>	33.3 <sup>a</sup>	1.7 <sup>a</sup>
20% CO <sub>2</sub>	n.d.	n.d.	n.d.	n.d.	10.8 <sup>b</sup>	6.3 <sup>c</sup>	4.5 <sup>b</sup>	2.4 <sup>a</sup>

<sup>1</sup>Data in columns displaying the same letters are not significantly different at  $P \leq 0.05$  according to Bonferroni's test. Each number is the mean of 3 replicates. n.d: not determined.

**Table 2.** Effect on maturation of black spruce somatic embryos (line M-67.5) of elevated CO<sub>2</sub>. After incubation in CO<sub>2</sub>, cultures were transferred to normal air until harvesting.

Incubation (days)	CO <sub>2</sub> (%)	Total number of somatic embryos <sup>1</sup>	Number of normal somatic embryos <sup>1</sup>	Number of abnormal somatic embryos <sup>1</sup>	Ratio Normal/Abnormal <sup>1</sup>
7	0.035	64.6 <sup>a</sup>	40.4 <sup>a</sup>	24.2 <sup>a</sup>	1.7 <sup>a</sup>
14	0.035	57.6 <sup>a</sup>	39.4 <sup>a</sup>	18.2 <sup>a</sup>	2.6 <sup>a</sup>
21	0.035	56.2 <sup>a</sup>	38.4 <sup>a</sup>	17.8 <sup>a</sup>	2.17 <sup>a</sup>
28	0.035	57.6 <sup>a</sup>	37.4 <sup>a</sup>	20.2 <sup>a</sup>	2 <sup>a</sup>
7	5	51.4 <sup>b</sup>	32.8 <sup>c</sup>	18.6 <sup>a</sup>	1.8 <sup>b</sup>
14	5	41.8 <sup>c</sup>	27.6 <sup>c</sup>	14.2 <sup>a</sup>	2 <sup>b</sup>
21	5	63.2 <sup>b</sup>	44.6 <sup>b</sup>	18.6 <sup>a</sup>	2.6 <sup>b</sup>
28	5	86.6 <sup>a</sup>	70.6 <sup>a</sup>	16 <sup>a</sup>	4.53 <sup>a</sup>
7	10	67.6 <sup>a</sup>	44.2 <sup>a</sup>	23.4 <sup>a</sup>	1.9 <sup>b</sup>
14	10	76.6 <sup>a</sup>	53.2 <sup>a</sup>	23.4 <sup>a</sup>	2.3 <sup>b</sup>
21	10	66.2 <sup>a</sup>	44 <sup>a</sup>	21 <sup>a</sup>	2.1 <sup>b</sup>
28	10	62.6 <sup>a</sup>	50.8 <sup>a</sup>	11.8 <sup>a</sup>	4.8 <sup>a</sup>
7	20	39.4 <sup>a</sup>	25.3 <sup>a</sup>	14.4 <sup>a</sup>	1.7 <sup>a</sup>
14	20	37.6 <sup>a</sup>	25.2 <sup>a</sup>	12.4 <sup>a</sup>	2.21 <sup>a</sup>
21	20	3.9 <sup>b</sup>	2.6 <sup>b</sup>	1.3 <sup>b</sup>	1.2 <sup>ab</sup>
28	20	00	00	00	00

<sup>1</sup>Within each treatment, data in columns displaying the same letters are not different at a level  $\alpha \leq 0.05$  according to Bonferroni's test. Each datum is the mean of 10 replicates. Each replicate was initiated with four pieces of 80 mg each of embryogenic tissue. Statistical analysis were applied separately on each experiment corresponding to [CO<sub>2</sub>].

*Effects of various level of oxygen on somatic embryo maturation of white spruce and black spruce*

The production of mature somatic embryos was promoted under 5 or 10 % of oxygen in white spruce and black spruce (Table 3). Furthermore, using different embryogenic cell lines, we observed that low oxygen generally promoted maturation but the promotive concentrations varied between 2, 5 and 10% (Table 4) depending on the embryogenic cell lines. In black spruce, among four embryogenic cell lines, two showed a positive response to low-oxygen treatments. In white spruce, three embryogenic cell lines responded positively to low-oxygen treatments. Furthermore, the low-

oxygen treatments reduced the browning of embryogenic tissue similarly as observed in confined microenvironment

*Effects of reduced oxygen on endogenous ACC during somatic embryo maturation of white spruce.*

Analysis of endogenous ACC in embryogenic tissue of white spruce growing under modified atmosphere containing 10% of O<sub>2</sub> showed an increase in endogenous ACC levels compared to control (Fig. 3). Compared to control, the cellular level of ACC was increased by about 150 % at the beginning of period culture to reach a maximum about 450 % at during the last week of culture period.

**Table 3.** Effect of different levels of O<sub>2</sub> on the number of black spruce and white spruce mature somatic embryos.

Treatment	Black spruce (line M-67.5)				White spruce (line G-209)			
	Total Embryo <sup>1</sup>	Normal Embryo <sup>1</sup>	Abnormal Embryo <sup>1</sup>	Normal /Abnormal <sup>1</sup>	Total Embryo <sup>1</sup>	Normal Embryo <sup>1</sup>	Abnormal Embryo <sup>1</sup>	Normal /Abnormal <sup>1</sup>
Control	64 <sup>c</sup>	39.7 <sup>c</sup>	24.3 <sup>b</sup>	1.7 <sup>a</sup>	46 <sup>b</sup>	27.7 <sup>b</sup>	18.3 <sup>b</sup>	1.5 <sup>a</sup>
2% O <sub>2</sub>	89.2 <sup>bc</sup>	55.5 <sup>bc</sup>	33.7 <sup>ab</sup>	1.7 <sup>a</sup>	50.7 <sup>b</sup>	29.8 <sup>b</sup>	20.8 <sup>b</sup>	1.4 <sup>a</sup>
5% O <sub>2</sub>	105.7 <sup>ab</sup>	65.3 <sup>ab</sup>	40.3 <sup>ab</sup>	1.8 <sup>a</sup>	55.5 <sup>b</sup>	33.8 <sup>b</sup>	21.7 <sup>ab</sup>	1.6 <sup>a</sup>
10% O <sub>2</sub>	135.2 <sup>a</sup>	85.3 <sup>a</sup>	49.8 <sup>a</sup>	1.9 <sup>a</sup>	75.2 <sup>a</sup>	46.2 <sup>a</sup>	29 <sup>a</sup>	1.6 <sup>a</sup>

<sup>1</sup>Data in columns displaying the same letters are not significantly different at  $P \leq 0.05$  according to Bonferroni's test. Each number is the mean of 3 replicates.

**Table 4.** Effect of 10% O<sub>2</sub> on the maturation of total mature somatic embryos in several genotypes of black spruce and white spruce.

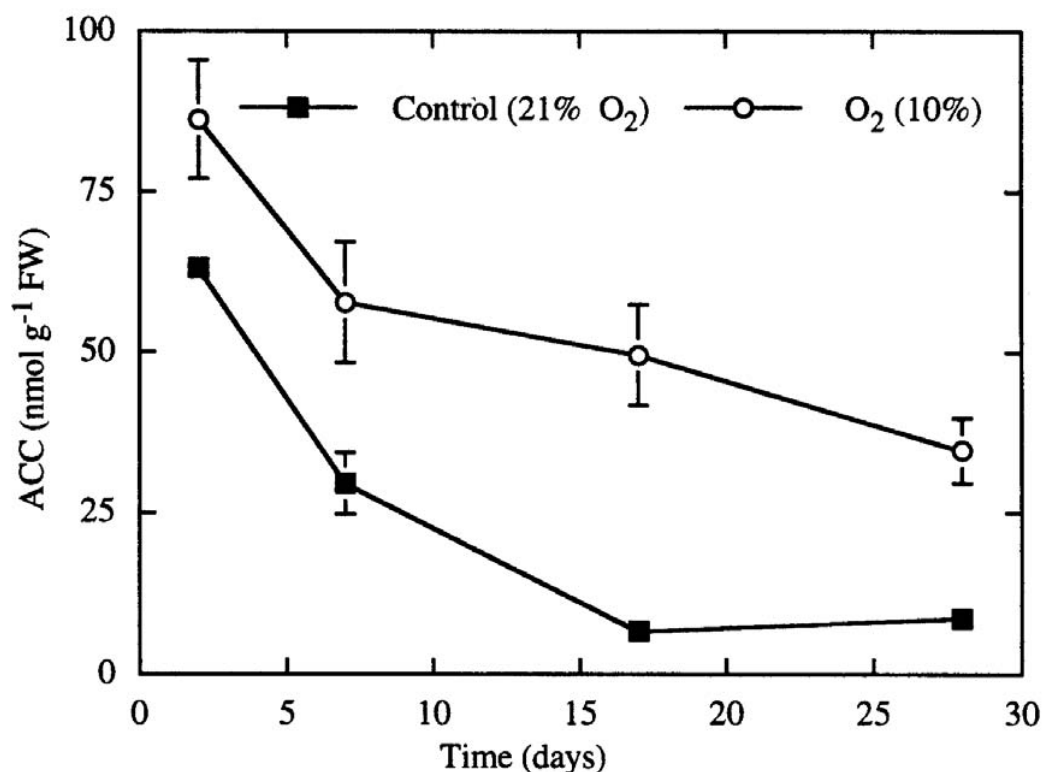
	Black spruce				White spruce		
Treatment O <sub>2</sub>	M-393.1	M-282	M-384	M-374	G-316	G-204	G-165
2%	+	0	+	0	+	+	–
5%	+	0	0	+	+	+	0
10%	0	0	0	+	+	0	+

+: Increase in production of mature embryos compared to controls; -: Decrease in production of mature embryos compared to controls; 0: No difference when compared to controls

## Discussion

The current study clearly demonstrates that gaseous environment influences the mature somatic embryo production. The results show that the incubation of embryogenic tissue under confined microenvironment promoted mature somatic embryo production and limited precocious germination in white spruce (Fig. 2). The confined environment synchronized the production of more uniform mature somatic embryos. The evolution of gases in the headspace of the culture vessel is given in (Fig. 1) and showed that ethylene and carbon dioxide levels accumulated while oxygen level

decreased during maturation period. The results obtained here were similar to those found in *Picea mariana* Mill. (El Meskaoui and Tremblay, 1999) and in *Selinum candolii* DC (Mathur, 1991). In the current study, we extended our investigation to separate the effect of individual gases (CO<sub>2</sub> and O<sub>2</sub>) on the response of mature somatic embryo production of black spruce and white spruce. The results show that incubation of embryogenic tissues under 5 % of CO<sub>2</sub> resulted in a significant increase in the number of cotyledonary somatic embryos whereas treatment with 20% of CO<sub>2</sub> severely inhibited it in white spruce and black spruce (Table 1).



**Figure 3.** Effect of reduced oxygen (10 %) on the levels of endogenous ACC during maturation of white spruce somatic embryos. Data are means  $\pm$  SE,  $n=3$ .

Compared to control, treatment with 5 and 10 % of CO<sub>2</sub> increased significantly the number of normal somatic embryos as shown by the ratio normal/abnormal (Table 1). Furthermore, a long exposure (four weeks) to 5% CO<sub>2</sub> was favorable for maturation (Table 2). A higher CO<sub>2</sub> level has also been found to have a positive effect on the production of mature somatic embryo in other species (Johannsson *et al.* 1982; Johannsson and Eriksson, 1984; Hohe *et al.* 1999). This beneficial effect may be due to the cellular acidification caused by the high concentration of CO<sub>2</sub> as observed in various plant systems (Guren *et al.* 1986; Lange and Kader, 1997). An other possibility is that elevated CO<sub>2</sub> interferes with ethylene. Elevated CO<sub>2</sub> inhibits ethylene production in many plant systems (Burg and Burg, 1967; Rothan and Nicolas, 1994; de Wild *et al.* 1999). However, in our experiment, the CO<sub>2</sub> was balanced with air, so an oxygen effect could be not excluded. In order to rule out this possibility, the effects of oxygen on maturation stage were studied using different concentrations of oxygen containing 0.03% of CO<sub>2</sub> and balanced with nitrogen. The results show that 10 % of oxygen

in white spruce and black spruce improves the production of mature somatic embryos (Table 3). Furthermore, using different embryogenic cell lines, we observed that low oxygen promoted maturation but the concentration varied among 2, 5 and 10% (Table 4). Thus, a low oxygen had a promotive effect on the production of mature somatic embryos. These results agree with other studies, which showed that low O<sub>2</sub> stimulates both somatic and zygotic embryogenesis (Kessell and Carr, 1972; Norstog and Klein, 1972; Carman, 1988, 1990; Mavituna and Buyukalaca, 1996). In contrast, the low O<sub>2</sub> inhibited somatic embryo production in alfalfa (Stuart *et al.* 1987) and in carrot (Jay *et al.* 1992). However, the current study showed that the embryogenic cells of both spruces are able to produce mature somatic embryos under hypoxia and that CO<sub>2</sub> is not a growth-limiting factor. It has been proposed that hypoxia during culture might simulate the in ovulo environment normally encountered during zygotic embryo development and consequently might enhance somatic embryo production (Carman, 1988).

The precise mechanism by which O<sub>2</sub>

influence somatic embryogenesis is not clear but it is possible that the beneficial effect of O<sub>2</sub> levels in promoting the maturation of somatic embryos probably may be related to ethylene metabolism. Indeed, it is well documented that anaerobic or low-oxygen environments inhibit ethylene biosynthesis and led to the ACC accumulation in several varieties of tissues (Abeles *et al.* 1992). In our study, analysis of endogenous ACC in embryogenic tissue of white spruce growing under modified atmosphere containing 10% of O<sub>2</sub> revealed an increase in ACC levels compared to the control (Fig. 3). This increase of endogenous levels of ACC may due to a reduction of ethylene biosynthesis through a reduction of ACC oxidase activity. It is now known that oxygen is required for ethylene production since it participates directly in the conversion of ACC to ethylene throughout a mechanism in which ACC oxidase binds first to O<sub>2</sub> and then to ACC (Yip *et al.* 1988).

On the other hand, it is well known that the polyamines and ethylene biosynthetic pathways share a common precursor, S-adenosyl-methionine (Yang and Hoffman, 1984) and that ethylene and polyamines may regulate each other's biosynthesis and action (Minocha and Minocha, 1995). Kramer *et al.* (1989) reported previously that low oxygen inhibited ethylene biosynthesis and increased endogenous polyamines. The polyamines promote growth and delay the senescence, whereas ethylene may accelerate senescence in tissue culture. According to our observations, the low oxygen treatments led to reduced senescence in both embryogenic cell lines whereas the application of higher levels of oxygen increased this phenomenon. It is well recognized that low oxygen could reduce the synthesis of oxidative enzymes and could stimulate the protective enzymes. Ethylene also may be implicated in senescence of tissue since a reduction of oxygen inhibits ethylene biosynthesis and delayed senescence. Furthermore, it is well demonstrated that the ethylene metabolism was implicated in somatic embryogenesis and that its inhibition promoted the somatic embryos maturation both in black spruce (El Meskaoui and Tremblay, 2001) and in white spruce (Kong and Yeung, 1994; El Meskaoui *et al.* 2000).

In conclusion, this study shows the

importance of the gaseous microenvironment during maturation of conifer somatic embryos. The confined microenvironment promoted the production and the uniformity of mature somatic embryos, and reduced precocious germination. This can be explained by the higher CO<sub>2</sub> accumulation and/or low oxygen, which alter ethylene metabolism.

## Acknowledgments

Thanks are extended to the Ministry of Natural Resources of Quebec (Ste-Foy, Que, Canada) for providing control pollinated seeds. This research program was supported by a grant from the Ministry of Industry, Trading, Science and Technology (Synergy program) in partnership with BECHDOR Inc., PAMPEV Inc. and CPPFQ Enr. to F.M. Tremblay.

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