African Journal of Agricultural Research Vol. 7(20), pp. 3111-3119, 26 May, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.1870
ISSN 1991-637X ©2012 Academic Journals

Full Length Research Paper

Effect of different carbon sources on the accumulation of carbohydrate, nutrient absorption and the survival rate of Chinese Ash (*Fraxinus mandshurica*) explants *in vitro*

Zhengzheng Deng[1,2*#], Jianmin Chu[3*#], Qingli Wang[4] and Lihua Wang[1]

[1]Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, People's Republic of China.
[2]Graduate University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China.
[3]Key Laboratory of Tree Breeding and Cultivation, State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, People's Republic of China.
[4]Kunming Branch, Chinese Academy of Sciences, Kunming 650204, People's Republic of China.

Accepted 23 April, 2012

In this study, we analyzed the effect of different carbon sources on: (i) carbohydrate and nutrient absorption in Chinese Ash (*Fraxinus mandshurica*) explants *in vitro*, and (ii) explant survival rate during *ex vitro* acclimatization. The explants were cultured under one of five conditions: (1) in a solid medium supplemented with 30 g l^{-1} sucrose (SM); (2) in a modified temporary immersion bioreactor (TIB) supplemented with sucrose at 0 (TIB0) or (3) 30 g l^{-1} (TIBS) concentration; (4) in TIB flushed with CO_2 at 700 to 900 µl l^{-1} (TIBC) with no sucrose; or in TIB flushed with CO_2 at 700 to 900 µl/l while simultaneously adding sucrose 30 g l^{-1} (TIBCS). The explants cultured under TIBCS exhibited the highest level of root induction and acclimatization survival rate. In addition, these explants exhibited maximized fresh weights (FW), dry weights (DW), and sucrose contents. Explants cultured in SM showed the highest rate of carbohydrate accumulation. At the end of the culture incubation period, absorption of nitrogen, phosphorus, potassium, magnesium, iron, zinc and manganese was also higher in explants cultured in SM than in TIB. This study revealed that the use of TIBCS provided optimum explant survival.

Key words: *Fraxinus mandshurica*, temporary immersion bioreactor, CO_2 enrichment, nutrient absorption.

INTRODUCTION

When *in vitro* explants are transferred from culture vessels to the green house, they show transition from a heterotrophic or photomixotrophic mode to a photoautotrophic mode (Van Huylenbroeck and De Riek, 1995; Van Huylenbroeck et al., 1998; Kozai et al., 2000). The energy source for *in vitro* explants is obtained from either sucrose in the growing medium, or from exogenous CO_2 enrichment. Different carbon sources will modify the micro-environmental conditions, thus affecting the photoautotrophic capacity and biomass accumulation of explants, in addition to their sucrose and ion absorption, and even their acclimation survival rate (Kozai et al., 1991; Arigita et al., 2010). A temporary immersion bioreactor (TIB) is an efficient propagation tool that improves the quality of explants (McAlister et al., 2005; Albarrán et al., 2005; Aragón et al., 2005). At present, however, the physiological response of the explants to CO_2 enrichment in TIB is not well known. In this study, we investigated how CO_2 enrichment and the application of nutrients contributes to the mineral nutrient uptake, carbohydrate accumulation and acclimation survival rate

*Corresponding author. E-mail: dengzz@iae.ac.cn. Tel: +86-24-83970360. Fax: +86-24-83970300. (Z. Deng). E-mail: jmchu@163.com; Tel: +86-10-62889604; Fax: +86-10-62889604 (J. Chu). #These authors contributed equally to this work and share first authorship.

Figure 1. Culture of Chinese Ash explants *in vitro*: A) microplants grown on gel, B) microplants grown in TIB conditions.

of high-quality Chinese Ash (*Fraxinus mandshurica*) explants cultured in controlled TIB atmospheres.Chinese Ash is a broadleaved tree that is an important component of northeast China's timber industry. It is also a key species of the Korean mixed pine-broadleaf forest. This fast-growing, highly adaptable species is valued for its wide range of uses, fine texture, and high economic value (Tan and Shen, 2003). However, a long history of overharvesting has led to a recommendation that the tree should be listed as a Grade 3 protected species in China (Fu, 1992). In order to meet the demand for wood, there have been numerous studies aimed at Chinese Ash tissue culture (Zhang and Luo., 2003; Tan and Shen, 2003; Deng et al., 2009). Nevertheless, the successful rooting rate of the species is still low (66%) (Zhang and Luo, 2003). In addition, there is no information available regarding the effect of differing levels of CO_2 in TIB on species growth, nutrient uptake during root induction, and acclimation survival rate. We chose Chinese Ash explants in transition from the heterotrophic or photomixotrophic phase to the photoautotrophic phase, and studied how different levels of CO_2 enrichment in TIB affect carbohydrate and mineral nutrient absorption, and photosynthetic characteristics during explant acclimation in the greenhouse. Our goal was to establish the optimal environment for the rooting and acclimation of high-quality Chinese Ash.

MATERIALS AND METHODS

Plant material and cultural conditions

In vitro shoot tips (3 cm; Figure 1A) of Chinese Ash were excised from microplants grown on gelled (agar 0.7 g l^{-1}) woody plant medium (WPM) (Lloyd and McCown, 1980) supplemented with 30 g l^{-1} sucrose, 0.5 mg l^{-1} zeatin (ZT), and 0.1 mg l^{-1} α-naphthalene acetic acid (NAA). The materials were then transferred to the rooting medium, which was ½WPM (half strength concentrations of the major salts) either solid medium (SM) or a liquid TIB medium (Figure 1B) supplemented with 0.1 mg l^{-1} NAA. The growth regulators, after being adjusted to 5.8 pH, were sterilized together with medium at 120°C for 20 min. Six vessels were used in each treatment, and each experiment was repeated in triplicate. In each experiment, 300 ml of SM and liquid TIB medium cultures were used. Ten plants were inoculated in each of the 500-ml glass vessels used for each culture condition. The samples were placed in a growth room under the following conditions: 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic proton flux density (PPFD) and 25±2°C and a photoperiod of 16 h day^{-1}.

Description of the TIB

A modified TIB system (Escalona et al., 1999) was used in this study. Two separate vessels were used, the explant vessel and the liquid medium reservoir. The two containers were connected underneath with a silicone tube, which allowed the transfer of medium when the medium vessel was lifted above the explant vessel and vice versa. The top of each vessel contained a plug with two holes with silicone tubing. One tube was for the application of enriched CO_2. A 0.2 µm hydrophobic filter was connected to the silicone tubing, which guaranteed the sterility of the air entrance. The tube of the explants vessel was used for the delivery of enriched CO_2 or non-enriched filtered CO_2. Both holes of the liquid medium reservoir were connected by silicone tubing to a hydrophobic filter and ventilated with air (CO_2, 350 µl l^{-1}).

In TIB, the liquid medium was supplemented in one of four ways: with sucrose at 0 (TIB0) or 30 g l^{-1} (TIBS); without sucrose, flushed with CO_2 at 700 to 900 µl l^{-1} (TIBC) at a flow rate of 30 ml/min; or flushed with CO_2 at 700 to 900 µl l^{-1} at a flow rate of 30 ml/min while simultaneously adding sucrose 30 g l^{-1} (TIBCS). The reference, or control, group consisted of explants grown in non-ventilated vessels with 30 g l^{-1} sucrose in the solid medium (SM). The immersion cycle was 10 min every 6 h, which was regulated manually. The silicone tubes and vessels used were sterilized at 120°C for 20 min. Sensors connected to a computer monitored CO_2 levels inside the culture vessels.

Carbohydrate assay

Young leaves of explants (first and second leaves from the top) were excised from each culture vessel at the 7, 14 and 21th days, immediately frozen in liquid nitrogen (N_2), and stored at -80°C until use. Samples (200 mg fresh mass) were homogenized in liquid N_2 in a mortar, and sucrose was extracted as described by Stitt and

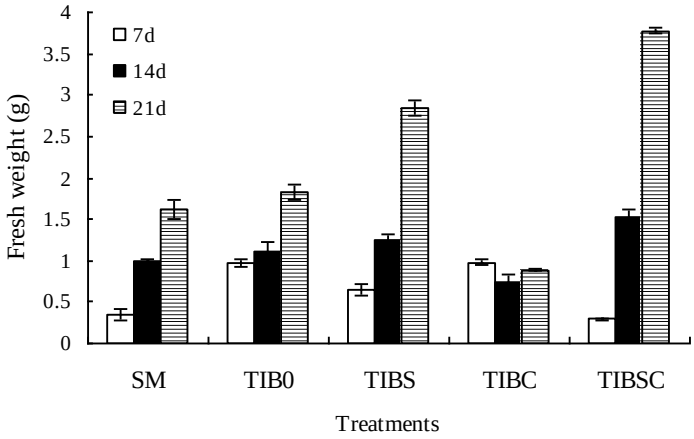

Figure 2. Explant fresh weight at 21 days of culture under varying CO_2 treatments.

Heldt (1985). Carbohydrate was assayed on the remaining pellet with the method described by Rufty and Huber (1983).

Nutrient analysis

At different periods (7, 14 and 21th days) and culture conditions, the mediums were taken and used to quantify explant nutrient absorption. Ammonium nitrogen (NH^+_4) and nitrate nitrogen (NO_3^-) were assayed by continuous-flow autoanalysis (Auto AnalyzerIII Bran + Luebbe GmbH, Germany). Other elements, including K, Ca, Mg, Fe, Zn, B and Mn, were assayed by plasmaphoresis (ICP-AES, PE OPTIMA3000, USA). Phosphorus (P) content was assayed with capillary electrophoresis on a P/ACE MDQ system (Beckman Coulter). An uncoated fused silica capillary with a total length of 60 cm × 75 µm (effective length: 50 cm) was used. Samples were hydrodynamically injected into the capillary for 5 s at 0.5 psi at the detector side. Separations were carried out at -30 kV at 25°C using a 0.1 mM Tetradecyltrimethylammonium Bromide, 3.0 mM potassium dichromate and 3.0 mM boric acid buffer (pH 8.0). UV detection was carried out at 254 nm.

Growth parameters

Fresh weights (FW) of whole Chinese Ash explants were measured. Dry weights (DW) of whole explants were also measured after the samples were completely dried at 80°C. After 21 days of growth, the rate of rooting was recorded. The rooted *in vitro* explants were transferred to *ex vitro* conditions in sterile peat moss and vermiculite (1:1 proportion) and grown in pots. They were kept under 80 to 95% relative humidity, 0 to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD, photoperiod of 16/10 h (light/dark), and temperature of 25 ± 2°C. Photosynthesis rate was measured for one hour from 10:00 to 11:00 am by a portable LI-6400 (LI-COR Inc., Lincoln, Nebraska, USA), every 3 days for a period of 15 days. Explant survival rate was recorded at the end of the acclimatization stage.

Statistical analysis

All experiments were randomized. The one-way analysis of variance (ANOVA) test was used to analyze the data. Significant differences between means were measured using Duncan's multiple range test at the $P<0.05$ level.

RESULTS

Growth under different in vitro culture conditions

Explants *in vitro* were rooted for 7 days under SM and TIBCS conditions, and for 15 days under TIBS and TIBC conditions, and for 20 days under TIB0. Roots induced from SM were grown from callus; those rooted under TIB conditions were grown directly from the stem segment.

The FW of seedlings increased gradually over the course of culture time (Figure 2). After 21 days, the DW was higher in explants supplemented with sucrose than in seedlings supplemented in TIB with only CO_2 or nothing at all (Figure 3). The DW of explants cultured in TIBCS was 2 to 3.1 times higher than that of SM and TIB0, respectively. The DW of seedlings cultured in TIBC was the lowest. The rooting rate was highest in TIBCS, followed by SM, TIBS, and TIBC and TIB0, in order (Table 3).

Carbohydrate content

The content of sucrose in the explants increased gradually over the course of culture time in SM (Figure 4). In TIB, sucrose decreased over the first 7 days of culture in explants supplemented with CO_2, then subsequently increased. An exception to this trend was explants cultured in TIBS. After 21 days of culture, the content of sucrose was the highest in TIBSC and the lowest in TIB0. The content of starch of explants in SM after 21 days was highest (Figure 5). TIB supplemented with CO_2 or sucrose in the medium promoted the accumulation of starch but not as much as in SM.

Absorption of mineral nutrients

Absorption of NH_4^+-N and NO_3^--N was higher in SM than

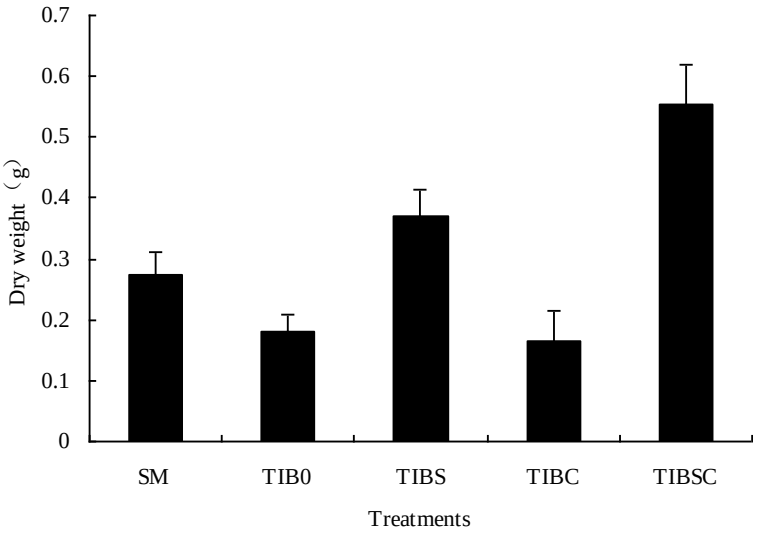

Figure 3. Dry weight of explants at 21 days of culture under varying CO_2 treatments.

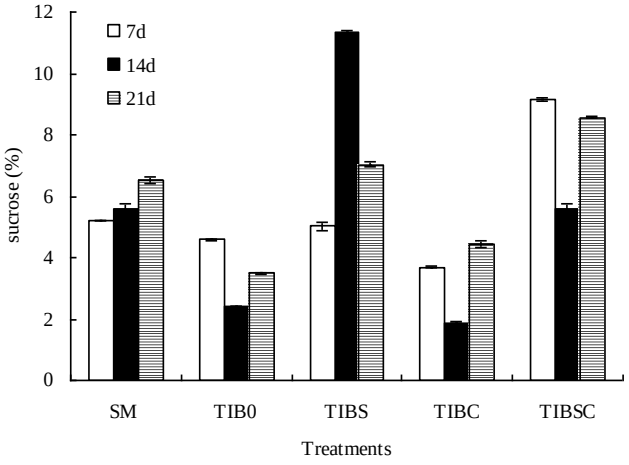

Figure 4. Variations of sucrose content under different CO_2 treatments.

in TIB (Table 1). After 21 d culture in SM, absorption of the NH_4^+-N was 7.68%, and the amount of NO_3^--N absorption was 7.53%. Compared with TIB0, the absorption of NH_4^+-N cultured under TIBC or TIBS was inhibited. However the amount of NO_3^--N absorbed under the different culture conditions was not statistically significant. Absorption of NH_4^+-N and NO_3^--N in TIBCS was higher than in the other TIB methods.

Absorption of other mineral nutrients is shown in Table 2. Explants grown under SM absorbed more phosphorus (P) than those grown under TIB. Over the course of culture time, P absorption under SM increased, with only 0.09 mg l^{-1} of P remaining in the medium at the end. Among explants cultured in TIB, P absorption was highest in TIBCS.Potassium (K) absorption was higher in SM than in TIB. In TIB0 and TIBC, K content did not increase in the explants, but increased in the medium.

Calcium (Ca) absorption in SM was less than in TIB. No CO_2 or sucrose supplementation promoted any Ca uptake. In fact, TIBC and TIBCS appeared to suppress Ca absorption. Under all assayed conditions, the absorption of magnesium (Mg) and iron (Fe) was always less than 0.25 mg l^{-1} or 0.56 mg l^{-1}, respectively.

Boron (B) absorption in SM was significantly less than in TIB. In TIB, more than 88% of B was absorbed during the first 7 d culture, and then subsequently decreased. It was noteworthy for SM that the content of B was reduced largely after autoclaving and far below the liquid medium.

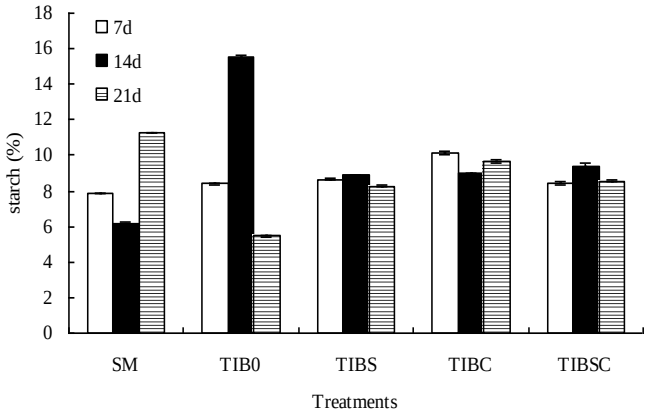

Figure 5. Variations of content of starch (%) alone with culture time under varying CO_2 treatments.

Table 1. NH_4^+-N and NO_3^--N concentration remaining in the growth medium and percentage of absorption of *in vitro* Chinese Ash after 21 days of culture under varying culture conditions.

Treatment	Culture period (days)	NH_4^+-N (mg/g)	NO_3^-N (mg/g)
	0	81.22±0.25	162.33±0.57
	7	78.31±0.41	160.39±0.50
SM	14	81.63±1.20	154.91±0.61
	21	74.98±0.79	150.11±0.56
		7.68% a	7.53% a
	0	82.41±0.84	161.05±1.64
	7	80.30±1.05	160.79±0.47
TIB0	14	79.51±0.07	156.54±2.54
	21	81.12±0.27	155.70±2.64
		1.57% c	3.32% c
	0	82.14±0.76	161.54±0.34
	7	80.40±0.89	161.87±1.93
TIBS	14	82.16±0.04	156.06±4.74
	21	81.07±0.51	157.38±0.31
		1.30% d	2.58% d
	0	82.41±0.42	161.05±0.38
	7	81.44±0.36	160.47±0.08
TIBC	14	82.95±0.39	158.66±0.30
	21	82.34±0.65	157.06±0.56
		0.08% e	2.48% d
	0	82.14±0.76	161.54±0.34
	7	80.02±2.43	161.47±1.45
TIBCS	14	81.05±0.29	157.37±0.07
	21	79.82±0.16	155.84±1.39
		2.82% b	3.53% b

Data are means ± SD of five data. Different letters indicate significant differences at P <0.05.

Table 2. Mineral nutrient concentration remaining in the growth medium *in vitro* culture of Chinese Ash and percentage of absorption at 21 d under varying culture conditions.

Treatment	period (days)	P (mg l^{-1})	K (mg l^{-1})	Ca (mg l^{-1})	Mg (mg l^{-1})	Fe (mg l^{-1})	Zn (mg l^{-1})	B (mg l^{-1})	Mn (mg l^{-1})
	0	2.32±0.32	47.69±0.15	9.82±1.38	2.30±0.01	0.77±0.06	0.25±0.01	0.04±0.00	0.63±0.00
	7	1.82±0.34	50.59±2.75	9.82±1.18	2.63±0.22	0.70±0.06	0.25±0.02	0.05±0.00	0.67±0.05
SM	14	0.80±0.36	49.09±1.75	9.50±0.1	2.38±0.08	0.28±0.24	0.16±0.02	0.04±0.01	0.65±0.01
	21	0.09±0.01	31.84±9.2	9.05±2.15	2.05±0.47	0.21±0.01	0.11±0.03	0.04±0.00	0.53±0.12
		96.12% a	33.24% a	7.84% c	10.87% a	72.73% d	56.00% a	-	15.87% a
	0	2.44±0.04	42.89±0.05	7.36±0.28	2.05±0.08	0.64±0.01	0.20±0.01	0.38±0.01	0.53±0.00
	7	2.14±0.03	46.39±0.85	5.24±0.60	2.26±0.21	0.25±0.01	0.20±0.00	0.03±0.01	0.46±0.01
TIB0	14	2.17±0.11	46.34±2.50	4.69±0.04	2.16±0.03	0.39±0.03	0.18±0.02	0.04±0.00	0.48±0.03
	21	2.05±0.04	52.64±5.20	5.34±0.96	2.20±0.28	0.22±0.09	0.17±0.03	0.03±0.00	0.45±0.03
		15.98% d	-	27.45% b	-	65.63% e	15.00% c	92.11% a	15.09% a
	0	2.46±0.06	43.44±0.30	6.90±1.94	2.04±0.09	0.64±0.04	0.20±0.00	0.43±0.30	0.53±0.01
	7	1.87±0.27	48.54±2.30	5.35±0.69	2.14±0.04	0.27±0.03	0.19±0.01	0.09±0.01	0.52±0.00
TIBS	14	2.13±0.00	43.64±1.20	4.32±0.07	1.96±0.04	0.29±0.04	0.16±0.01	0.06±0.02	0.49±0.00
	21	1.86±0.02	43.29±0.45	4.53±0.34	1.93±0.16	0.09±0.02	0.16±0.02	0.07±0.04	0.47±0.00
		24.39% c	0.35% c	34.35% a	5.39% b	85.94% a	20.00% b	83.72% c	11.32% b
	0	2.44±0.04	42.89±0.05	7.36±0.28	2.05±0.08	0.64±0.01	0.20±0.01	0.38±0.03	0.53±0.00
	7	2.32±0.12	45.39±0.75	6.97±0.17	2.05±0.04	0.55±0.18	0.24±0.05	0.02±0.00	0.53±0.03
TIBC	14	2.32±0.29	46.69±1.35	5.83±1.51	2.08±0.14	0.34±0.09	0.17±0.01	0.04±0.00	0.53±0.04
	21	2.48±0.04	45.69±0.95	6.86±0.15	2.03±0.02	0.17±0.05	0.18±0.00	0.03±0.01	0.51±0.01
		-	-	6.79% d	0.98% c	73.44% c	10.00% d	92.11% a	3.77% c
	0	2.46±0.06	43.44±0.30	6.90±0.23	2.04±0.09	0.64±0.04	0.20±0.00	0.43±0.30	0.53±0.01
	7	2.33±0.05	42.79±0.35	6.62±0.25	1.98±0.03	0.46±0.07	0.20±0.01	0.07±0.02	0.51±0.01
TIBCS	14	2.08±0.02	44.79±0.25	6.87±0.11	2.02±0.06	0.59±0.03	0.18±0.01	0.09±0.05	0.54±0.01
	21	1.47±0.05	42.04±1.10	7.46±0.29	2.18±0.06	0.13±0.04	0.18±0.02	0.04±0.02	0.51±0.02
		40.24% b	3.22% b	-	-	79.69% b	10.00% d	90.70% b	3.77% c

Data are means ± SD of five data. Different letters within a *column* indicate significant differences at P <0.05. "-" means the medium nutrients were not determined.

Manganese (Mn) absorption in SM was greater than in TIB. In TIB, TIB0 and TIBS promoted Mn uptake. Minimal Mn uptake occurred in enriched TIBC and TIBCS.

Absorption of zinc (Zn) in SM was 3.33 to 7.94 times higher than in TIB. In TIB, the range of absorption was 0.018 to 0.043 mg l^{-1}.

Photosynthesis characteristics and acclimation survival rate

After 21 days of culture, rooted explants were transferred to a greenhouse for *ex vitro* acclimation. There was a significant difference in survival rate of explants cultured under the different assayed conditions (Table 3). Explants cultured in TIBCS displayed the highest survival rate, 27% higher than those cultured in SM. TIB0 resulted in the lowest acclimation survival rate .There was also a significant difference in photosynthesis in explants that received CO_2 enrichment (Figure 6). Photosynthetic rate of leaves in explants cultured in SM, TIB0 and TIBS was negative at first, then gradually transitioned to autotrophic after 6 (SM and TIBS) and 12 days (TIB0). Explants cultured in TIBC and TIBCS displayed positive a positive photosynthetic rate at first, and subsequently continued to increase. After 15 days acclimation, the photosynthetic rate in TIBCS was three times higher than in any other culture method.

DISCUSSION

The results of this study show that plant culture microenvironment plays an important role not only for *in*

Table 3. Rates of explants rooting and acclimation survival under different carbon source conditions.

Treatment	Rooting rate (%)	Survival rate (%)
SM	90a	75d
TIB0	32b	46e
TIBS	85a	80c
TIBC	45b	84b
TIBSC	93a	95a

Values are the means of three replicates. Means within a row followed by different letters are significantly different at 5% level in Duncan's multiple range test.

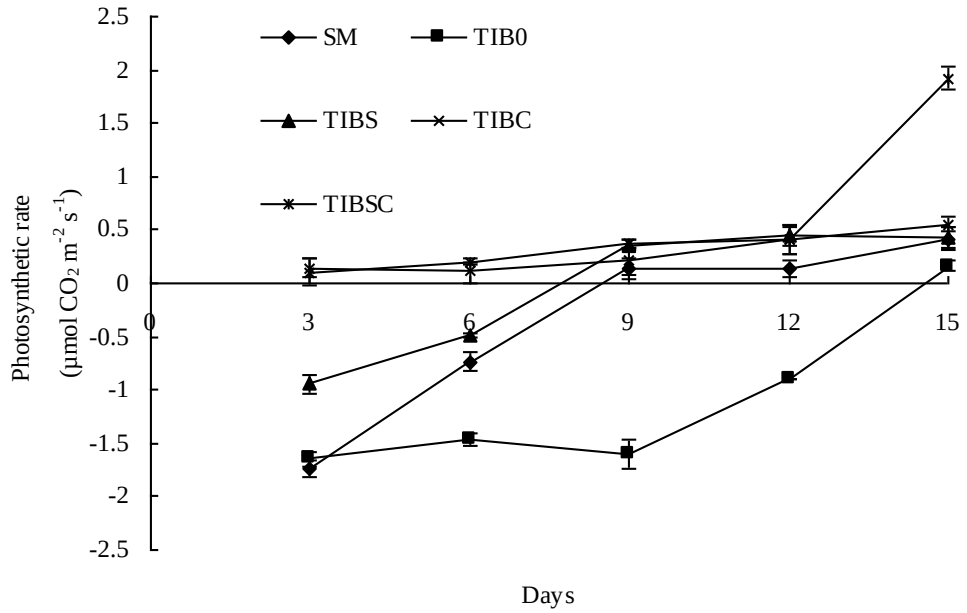

Figure 6. Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1}) during the acclimation of explants.

vitro carbohydrate accumulation, but also for *ex vitro* survival rate. SM and TIBCS increased the explants DW and reserves of carbohydrates, which enhanced acclimation survival rate. These results are in line with other studies (Van Huylenbroeck et al., 1995; Wilson et al., 2001). Explants that absorbed excessive amounts of starch *in vitro* display an *ex vitro* decrease in photosynthesis, which results in a decrease in chloroplast light uptake, and an increase in resistance to CO_2 diffusion (Neales and Incoll, 1968). In our study, when explants cultured in SM and TIBS were transferred from *in vitro* to *ex vitro* conditions, the photosynthetic rate changed gradually from negative to positive; these explants needed 6 d acclimation to transition from photoheterotrophy to photoautotrophy.

In TIBC, explant sucrose content and DW increased, but to a lesser degree than in TIBS; additionally, TIBC displayed a decreased rooting rate compared to TIBS. It is possible that TIBC produces environmental stress, which suppresses explants growth. Therefore, when these explants, which were cultured in enclosed, high-humidity vessels, were transferred to a ventilated, low-humidity environment enriched with CO_2, they do not acclimate well to the new environment and grow more slowly. Other findings (Arigita, 2010) indicate that shoot length and DW decrease and mortality increases when explants are cultured in glass boxes flushed with CO_2 at 300–2000 μmol·mol^{-1}. So, when explants are flushed with CO_2, sucrose supplementation in the medium is necessary for explants growth, at least early in explants culture (Arigita et al., 2010). Rooted explants cultured in TIBC display a positive photosynthetic rate in acclimation. Similarly, explants rooted in TIBCS also display positive photosynthesis. However, the more sucrose in the medium during *in vitro* culture, the less the Rubisco activity and CO_2 absorption (Hdider and Desjardins, 1994). Arigita et al., (2010) suggests that explants cultured at 600 μmol·CO_2·mol^{-1} in 20 g l^{-1} of sucrose for the first 20 days culture far better when transferred to a sucrose-free medium. This contributes to the

development of the efficient photosynthetic machinery needed to adapt to new conditions (Van Huylenbroeck and Debergh, 1996). Our results demonstrated that the degree of autotrophy of Chinese Ash cultures was affected by sucrose supplementation and CO_2 concentration. In TIB0, due to no exogenous carbon source, there was a decrease in all parameters that contributed to explants acclimation and survival.

Ca and B absorption in SM was less than in TIB. It isprobable that more Ca and B are required for root growth. After sterilization at 120°C for 20 min, the content of B in SM explants was less than in the liquid medium. The absence of B in the SM growth medium affects root growth (Tanaka and Fujiwara, 2008). At the end of our experiment, there were no nutrients completely absorbed in TIB.

There was a small residual amount of P in SM. The absence of P restricts plant growth (Pareilleux and Chaubet, 1981), and this was possibly the limiting element in many tissue cultures (Lumsden et al., 1990). In our study, B and P in SM were the main limiting factors affecting *in vitro* plant growth.

TIB provides a better *in vitro* gas and nutrition environment than SM (Roels et al., 2006). Ventilation in the culture vessels probably affects the quality of the root system (Jackson, 2003). Zhang et al. (2009) reports that, compared to photomixotrophic micropropagated *Siraitia grosvenorii* explants grown on SM without forced ventilation, photoautotrophic micropropagated explants grown with forced CO_2 ventilation have a more developed root system, higher quality shoots and no callus at the shoot base. Our study proved that TIBCS contributed positively to explants root growth and increased rate of acclimation survival.

ACKNOWLEDGEMENTS

This work was supported by the Important Directive Project of the CAS Knowledge Innovation Program (N°. SCX2-YW-N-031).

Abbreviations

SM, Solid medium; **TIB,** temporary immersion bioreactor; **TIB0,** temporary immersion bioreactor supplemented with sucrose at 0 g l^{-1}; **TIBS,** temporary immersion bioreactor supplemented with sucrose at 30 g l^{-1}; **TIBC,** temporary immersion bioreactor flushed with CO_2 at 700–900 µl l^{-1}; **TIBCS,** temporary immersion bioreactor flushed with CO_2 at 700–900 µl l^{-1} simultaneously added sucrose 30 g l^{-1}; **ZT,** zeatin; **NAA,** α-naphthalene acetic acid; **WPM,** woody plant medium; **DW,** dry weight; **FW,** fresh weight.

REFERENCES

Albarrán J, Bertrand B, Lartaud M, Etienne H (2005). Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica*) somatic embryos. Plant Cell Tiss. Org. Cult., 81: 27-36.

Arigita L, Cañal MJ, Tamés RS, González A (2010). CO_2-enriched microenvironment affects sucrose and macronutrients absorption and promotes autotrophy in the in vitro culture of kiwi (*Actinidia deliciosa* Chev. Liang and Ferguson). In Vitro Cell Dev. Biol. - Plant, 46: 312-322.

Aragón CE, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal MJ, Sandoval J, Roels S, Debergh P, González-Olmedo JL (2005). Photosynthesis and carbon metabolism in plantain (*Musa* AAB) plantlets growing in temporary immersion bioreactor and during *ex vitro* acclimatization. In Vitro Cell Dev. Biol. – Plant, 41: 550-554.

Deng ZZ, Wang LH, Wang QL (2009). Effects of plant growth regulators on growth and endogenous hormones of *in vitro* plantlets of Manchurian Ash. J. Northeast For. Univ., 37(12): 10-13.

Escalona M, Lorenzo JC, González B, Daquinta M, González JL, Desjardins Y, Borroto CG (1999). Pineapple (*Ananas comosus* L. Merr) micropropagation in temporary immersion systems. Plant Cell Reports, 18: 743-748.

Fu, LG (1992). Chinese Plant Red Book: rare and endangered plants, Beijing Science Press, China, 1: 736.

Hdider C, Desjardins Y. (1994). Effects of sucrose on photosynthesis and phosphoenolpyruvate carboxylase activity of *in vitro* cultured strawberry plantlets. Plant Cell Tiss. Org. Cult., 36: 27-36.

Jackson MB (2003) Aeration stress in plant tissue cultures. Bulg. J. Plant Physiol., Special issue: 96-109.

Kozai T, Iwabuchi K, Watanabe K, Watanabe I (1991). Photoautotrophic and photomixotrophic growth of strawberry plantlets *in vitro* and changes in nutrient composition of the medium. Plant Cell Tiss. Org. Cult., 25: 107-115.

Kozai T, Kubota C, Zobayed S, Nguyen QT (2000). Developing a mass-propagation system of woody plants. In: Watanabe K, Komamine A et al. Challenge of plant and agriculture sciences to the crisis of biosphere on the Earth in the 21st century. Landes Biosphere Georgetown, 293-306.

Lloyd GB, McCown BH (1980). Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Proc. Int. Plant Propagators Soc., 30: 421-437.

Lumsden PJ, Pryce S, Leifert C (1990). Effect of mineral nutrition on the growth and multiplication of *in vitro* cultured plants. In: Nijkamp, HJJ, Van Der Plas, LHW, Van Aartijk, J, et al. Plant cell and molecular biology. Kluwer Acad. Pub. Dordrecht, 108-113.

McAlister B, Finnie J, Watt MP, Blakeway F (2005). Use of the temporary immersion bioreactor system (RITA®) for production of commercial Eucalyptus clones in Mondi Forests (SA). Plant Cell Tiss. Org. Cult., 81: 347-358.

Neales TF, Incoll LD. (1968). The control of leaf photosynthesis rate by the level of assimilate concentration in the leaf: A review of the hypothesis. Bot. Rev., 34(2): 107-125.

Pareilleux A, Chaubet N (1981). Mass cultivation of *Medicago sativa* growing on lactose: kinetic aspects. European J. Appl. Microbiol. Biotechnol., 11: 222-225.

Roels S, Noceda C, Escalona M, Sandoval J, Canal MJ, Rodriguez R, Debergh PC (2006). The effect of headspace renewal in a temporary immersion bioreactor on plantain (*Musa* AAB) shoot proliferation and quality. Plant Cell Tiss. Org. Cult., 84: 155-163.

Rufty TW, Huber SC (1983). Changes in starch formation and activities of sucrose-phosphate synthase and cytoplasmic fructose-1,6-bisphosphatase in response to source-sink alterations. Plant Physiol., 72: 474-480.

Stitt M, Heldt HW (1985). Control of photosynthetic sucrose synthesis by fructose-2-6-bisphosphate. Planta, 179: 188-204.

Tan SY, Shen HL (2003). Tissue culture and plantlet regeneration from hypocotyl of *Fraxinus mandshurica*. Plant Physiol. Commun., 39(6): 623.

Tanaka M, Fujiwara T (2008). Physiological roles and transport mechanism of boron: Perspectives from plants. Pflugers Arch.-Eur. J. Physiol., 456: 671-677.

Van Huylenbroeck JM, Debergh PC (1996). Impact of sugar concentration *in vitro* on photosynthesis and carbon metabolism during *ex vitro* acclimatization of *Spathiphyllum* plantlets. Physiologia Plantarum, 96: 298-304.

Van Huylenbroeck JM, De Riek J (1995). Sugar and starch metabolism

during ex vitro rooting and acclimatization of micropropagated *Spathiphyllum* 'Petite' plantlets. Plant Sci., 111: 19-25.
Van Huylenbroeck JM, Piqueras A, Debergh PC (1998). Photosynthesis and carbon metabolism in leaves formed prior and during ex vitro acclimatization of micropropagated plants. Plant Sci., 134: 21-30.
Wilson SB, Heo J, Kubota C, Kozai T. (2001). A forced ventilation micropropagation system for photoautotrophic production of sweetpotato plug plantlets in a scaled-up culture vessel: Ⅱ. Carbohydrate status. HortTechnology, 11: 95-99.
Zhang HJ, Luo FX (2003). Study on *in vitro* culture of immature embryo of *Fraxinus mandshurica*. Scientia Silvae Sinicae, 39(3): 63-69.
Zhang MJ, Zhao DD, Ma ZQ, Li XD, Xiao YL (2009). Growth and photosynthetic capability of *Momordica grosvenori* plantlets grown photoautotrophically in response to light intensity. HortScience, 44(3): 757-763.