

Effects of *in vitro* culture types on regeneration and acclimatization of yellow poplar (*Liriodendron tulipifera* L.) from somatic embryos

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Abstract We compared germination efficiency for somatic embryos (SE) of *Liriodendron tulipifera* using semi-solid (SS), temporary immersion bioreactors (TIB), and continuous immersion bioreactors (CIB) to produce vigorous plants. The bioreactors were designed to be immersed in liquid media with plantlets with an adjustable immersion time. TIB and CIB improved germination rates up to 80.86% and 95.21%, respectively, however, CIB produced more hyperhydric plantlets than TIB. The height of plantlets in TIB was significantly higher than for those in CIB. Fresh weights of plantlets grown in CIB were significantly lower than for those grown in TIB. The lowest chlorophyll concentration was found in *in vitro* plantlets from CIB. We examined abnormally developed leaves, stems, and apical zones of *in vitro* plantlets that were produced in CIB.

Among the three types, SS showed the highest stomatal density and the shortest stomatal length in *in vitro* plantlets. After acclimatization, plants from CIB exhibited the lowest values in biomass, such as height, root collar diameter, leaf fresh weight, leaf length, leaf width, petiole length, petiole diameter, and leaf area. Photosynthesis and transpiration rates of *ex vitro* plants were not significantly different among the three culture types, but stomatal conductance was higher in TIB than in the SS and CIB. Therefore, the results suggest that TIB is the preferable bioreactor to improve *in vitro* plantlet regeneration of *L. tulipifera*. TIB-originated plants showed higher growth rate than SS and

CIB after transferring to soil.

Keywords Bioreactor, *Liriodendron tulipifera*, Regeneration, Somatic Embryogenesis, Temporary immersion

Introduction

Yellow poplar (*Liriodendron tulipifera* L.) was introduced from USA in the 1960s to support afforestation in the Republic of Korea (Ryu and Kim 2003). This species has a straight trunk and rapid growth when compared with other tall trees in the temperate region, e.g., pines (*Pinus* spp.), oaks (*Quercus* spp.), and firs (*Abies* spp.) (Wilcox and Raft 1969). It is also a highly adaptable species in terms of altitude and can be planted in various areas in Korea (Ryu et al. 2008). It provides good timber for furniture and veneer boards, and is a good source for bio-energy.

Clonal propagation is used to reduce variation and increase productivity in trees (Park 2002; Husen 2004; Bonga et al. 2010). Traditionally, cutting has been used as the main method; however, cutting has limited use because of the large requirement for plant material. Tissue cultures could be used as an alternative to this vegetative propagation technique. Among various techniques, somatic embryogenesis technology is the best method for efficient clonal plant multiplication (Park 2002; Bonga et al. 2010). Propagation techniques using somatic embryogenesis have been applied to overcome low productivity and genetic variability in spruce, conifer and oaks (Park 2002; Merkle 2005). Study of somatic embryos induction in *L. tulipifera* began with proembryogenic masses induction from zygotic embryos by Merkle and Sommer (1986). Induction and proliferation of proembryogenic masses (PEMs) of *L. tulipifera* was accomplished on solidified medium through a filtering process (Merkle et al. 1990). Protoplasts were isolated from suspension cultures of *L. tulipifera* and were regenerated on solidified media (Merkle and Somme 1987). This was similar to the traditional method

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in which semi-solid media was used. In Korea, somatic embryogenesis and plant regeneration of yellow poplar using immature seed material for yellow poplar has been attempted (Lee 2003; Son et al. 2005; Kim and Moon 2013); furthermore, An et al. (2010) attempted plant regeneration from somatic embryos using bioreactors and produced small, normal plants.

Conventionally, tissue cultures are produced on solid media (George et al. 2008). *In vitro* plants are typically grown in conditions of high relative humidity, low light density, constant temperature, changing CO₂ concentrations, and limited space to produce controlled culture conditions (Lamhamedi et al. 2003). Recently, methods were introduced using bioreactors to promote culture efficiency, which might be the most efficient system and could be conducted using an automatic system (Paek et al. 2001). Bioreactors are classified into several types based on operational methods. The temporary immersion type increases somatic embryos and enhances the quality of embryogenic tissue (Etienne and Berthouly 2002). Additional advantage of reducing the costs by reducing the number of containers needed, reduces oxygen used, use efficient mixing, has limitative share levels, enables sequential media exchanges, and reduces contamination (Teisson et al. 1999). This method has been applied to numerous species. For example, it was used with somatic embryos of *Coffea* spp. (Etienne-Barry et al. 1999; Alberran et al. 2005) and cacao (*Theobroma cacao*) (Niemenak et al. 2008), for regeneration of strawberries (*Fragaria* spp.) (Hanhineva et al. 2005), and bud propagation of sugarcane (*Saccharum* spp.) (Lorenzo et al. 2001) and *Charybdis numidica* (Kongbangkerd and Wawrosch 2003).

The purpose of this study was to investigate physical conditions needed for somatic embryo germination of *L. tulipifera*, and to increase cultural efficiency using temporary immersion bioreactors.

Materials and methods

Plant material

To induce embryogenic calli, immature seeds were placed on Litvay (LM 1981) with 3% sucrose (w/v), 9.04 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1.11 μM 6-benzylaminopurine (BA), 0.8% glutamine (w/v), and 0.4% gelrite (w/v). After six weeks, cultured embryos were transferred to the medium without 2,4-D and BA for somatic embryo induction. After six weeks, the torpedo-stage somatic embryos were transferred to three culture types.

Culture types

Somatic embryos were cultured on three types of bioreactors for two weeks. The temporary immersion bioreactor (TIB) type was designed to immerse embryos temporarily in the medium. The cultures were immersed into the medium for one hour every four hours (Fig. 1). Air was supplied from a sparger at a flow rate of 1.0 vvm. Using the same bioreactor as in TIB, we also continuously supplied air to the medium by air pressure for the continuous immersion bioreactor (CIB) type. The semi-solid (SS) type was a gelled medium with 0.2% gelrite (w/v). All media based on 1/2 LM including 2% sucrose (w/v) were adjusted to a pH of 5.8. In TIB and CIB types, somatic embryos were inoculated with 6 g per 1 L of medium. Cultures were kept at 24 ± 1°C with a 16 h light and 8 h dark photoperiod under fluorescent lights. Observations were recorded for the cultures every two weeks. Germination rate, plantlet length, fresh weight, and hyperhydricity rate were measured. Hyperhydricity rate (%) was calculated as (hyperhydricity plantlet number / total plantlet number) × 100.

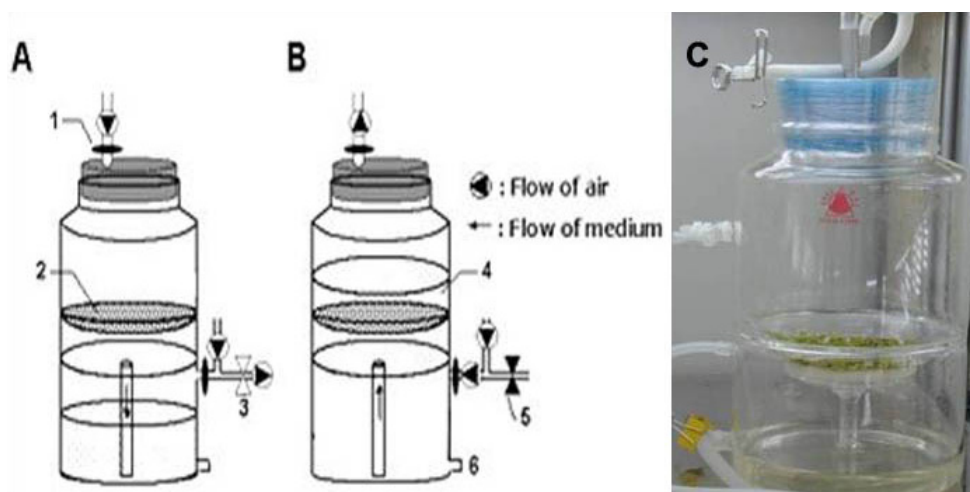


Fig. 1 Structure of the temporary immersion bioreactor. A withdrawal phase, B immersion phase, and C inoculated bioreactor. 1- Membrane filter, 2- Glass filter (sparger), 3- Solenoid valve (open), 4- Medium, 5- Solenoid valve (closed), and 6- Exchange medium line

Measurement of chlorophyll contents

Levels of photosynthetic pigments (chlorophyll a, b, and carotenoids) were determined using the Lichtenthaler (1987) method. Fresh leaves were extracted with an 80% acetone solution (v/v). They were extracted in the dark at 4°C for 48 h. Pigment concentration was measured using a visible spectrometer (Uvikon-930, Kontron Instruments, Wurich, Switzerland) at 470 nm, 646.8 nm, and 663.2 nm.

Histological examination

To determine the development of the leaf, stem, and apical zone, regenerated plantlets were collected from the bioreactors of the three culture types. Samples were fixed with a solution containing 0.05 M glutaraldehyde and 1.6% paraformaldehyde buffer, prepared in 0.05 M phosphate buffer (pH 6.8), for 48 h. Next, they were dehydrated in a series of progressively more concentrated ethanol (30%, 50%, 60%, 80%, 90%, 95%, and 100%) and then embedded in glycol methacrylate (Technovit 7100, Kulzer, Germany) according to the protocol of Yeung (1999). Sections of 3 µm thickness were cut using an auto-cut rotary microtome (Leica RM 2165, Germany) and were mounted on glass slides. Mounted specimens were stained with 0.1% periodic acid, Schiff's solution, and 0.05% toluidine blue O, and examined under a light microscope (Leica D. M. R., Germany).

Free sugar analysis in the media

To examine carbohydrate levels, such as those for sucrose, glucose, and fructose in the medium from the three culture types, HPLC (TSP operating system) was used with a Prevail™ carbohydrate ES column (Alltech, 5 µm, 4.6 × 250 mm), gas flow of 2.2 L min⁻¹, injection volume of 20 µL, and detector tube temperature of 85°C. A mobile phase consisting of acetonitrile and water at 72 : 280 was used at a flow rate of 0.7 mL min⁻¹.

Stomata examination

Leaves were cut into 5 mm² sections and the abaxial sides were peeled off. Leaf segments were stained for 15 min in 0.01% acridine orange and washed with distilled water a minimum of three times. Stomatal density, length, and width were determined by fluorescent microscopic observation with a laser scanning system (IM50, Leica, USA).

Acclimatization

Plantlets from the three culture types were cultured on SS media for eight weeks. Next, plants were transferred to soil for acclimatization. The soil was a mixture of nursery bed soil : sand (1 : 1, v/v). The plants were grown at 21 ± 1°C. After four weeks, plant height, root collar diameter, leaf fresh weight, leaf size, petiole size, and photosynthesis capacity were recorded. Photosynthesis capacity was measured with the Li-6400 portable photosynthesis system (Li-cor. Inc., USA) at a relative humidity of 60% ~ 65%.

Statistical analysis

The experiments were performed using a randomized design. Each treatment consisted of five dishes with 10–15 explants per dish and was repeated twice. Data were analyzed using Duncan's multiple range test in the SAS program (SAS Institute, Cary, NC, USA).

Results

In vitro plantlets exhibited different features, as observed in somatic embryos (SEs) among the three culture types (Table 1, Fig. 2). The highest germination rate (95.21%) was served upon the CIB treatment. The highest ratio of normal plantlets without hyperhydricity was obtained using the TIB treatment, whereas TIB had a germination rate of 80.86%. Significant

Table 1 Germination rate and plantlet biomass of somatic embryos obtained from different culture types after 14 days

Type*	Germination rate (%)	Height (mm)	Fresh weight / normal plantlets (mg)	Hyperhydricity / normal plantlets (%)
SS	56.78	8.50 ^{b**}	11.35 ^{ab**}	32.14
TIB	80.86	11.09 ^a	8.19 ^b	15.22
CIB	95.21	10.15 ^{ab}	14.61 ^a	35.71
F value	-	3.66	4.75	-
p	-	0.03	0.01	-

*SS - semi-solid medium; TIB - temporary immersion bioreactor; and CIB - continuous immersion bioreactor.

**The same letter indicates there was no difference at the 5% significance level.

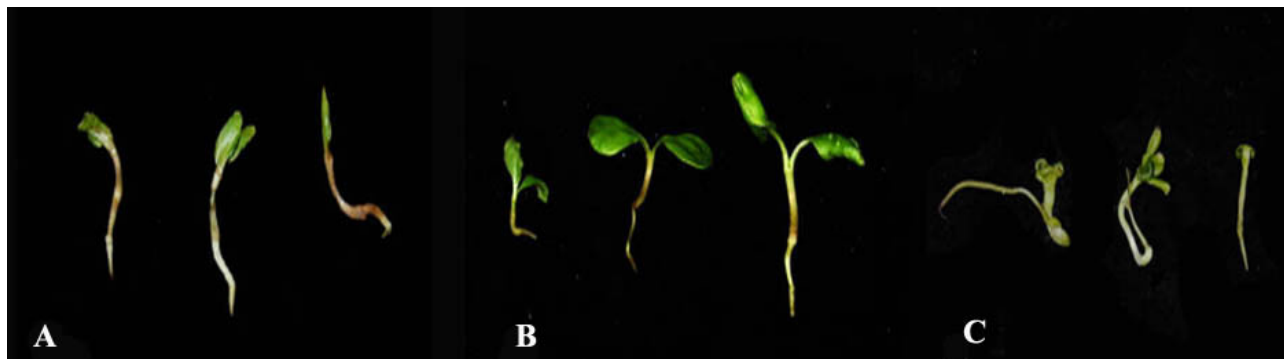


Fig. 2 Germination and plant conversion of somatic embryos from different culture types after two weeks. A- SS (semi-solid medium), B- temporary immersion bioreactor (TIB), and C- continuous immersion bioreactor (CIB)

differences were observed in plantlets heights in 11.09 mm of TIB, 10.15 mm of CIB and 8.50 mm of SS order (Table 1). In addition, the rate of hyperhydric plantlets formation was the lowest in TIB (15.22%) and the plantlets exhibited rapid vertical growth in the TIB treatment. CIB produced heavier plantlets (14.61 mg) than the other two culture types did (significantly different fresh weight as 11.35 mg (SS) and 8.19 mg (TIB)). This was likely caused by the higher hyperhydricity rate for CIB. In contrast, TIB treatment resulted the lowest number of hyperhydric plantlets and the smallest value (8.19 mg) in plantlet fresh weight, followed by SS (11.35 mg) and CIB (14.61 mg). The highest concentrations of chlorophyll a, b, and carotenoids of *in vitro* plantlets were from the TIB treatment (Fig. 3). CIB treatment produced the highest number of hyperhydric plantlets and the lowest concentrations of photosynthesis pigments.

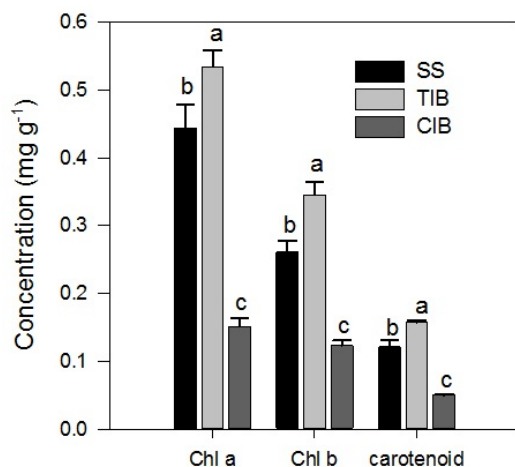


Fig. 3 Chlorophyll a, b, and carotenoid content in germinated plantlets as influenced by immersion time after two weeks. The same letter indicates there was no difference at the 5% significance level

The developmental features of germinated plantlets by culture type were examined for leaf, stem, and apical zone differences among the three culture types (Fig. 4). Mesophyll and intercellular space of leaves grown in CIB were aggregated loosely compared with those from the other types. The epidermal and cortical layers were characterized as thin and underdeveloped. On the other hand, the cellular space of leaves grown in SS and TIB showed the regular arrangement and normal structures. The apical zone, including the apical meristem, grown in the CIB treatment was smaller than that of SS and TIB. The appearance of hyperhydric symptoms, such as low frequencies of conversion of SEs, excessive weight of plantlets, and production of abnormal plantlets were observed among plantlets grown in CIB.

Following the cultures, free sugar in the media was analyzed and was lower than 0.5% (w/v) for all three types (Fig. 5), although 2% of sucrose was included in all media initially. Glucose and fructose were below 0.3% (w/v) in both the TIB and CIB media, whereas it was not detected in the SS media.

Leaf stomata of plantlets from the SS treatment were most dense, whereas their length was the smallest among the three types (Table 2, Fig. 6). Stomata in SS were approximately twice as dense as those from TIB and CIB treatment, although stomatal density was similar for TIB and CIB treatments. Stomata length was shorter in SS than in the bioreactors (TIB and CIB), whereas their width was not significantly different among the three culture types.

Regenerated plants showed different morphological characters in height, root collar, leaf length, petiole length, petiole diameter, leaf fresh weight, and leaf area after they were transferred to soil (Table 3). The height, root collar diameter, leaf and petiole size were not in significant level between SS and TIB-originated plants, but the lower plants produced in CIB after growing in greenhouse for 8 weeks. TIB-originated plants demonstrated higher growth in all traits measured than CIB plants (Fig. 7). SS-derived plantlets also

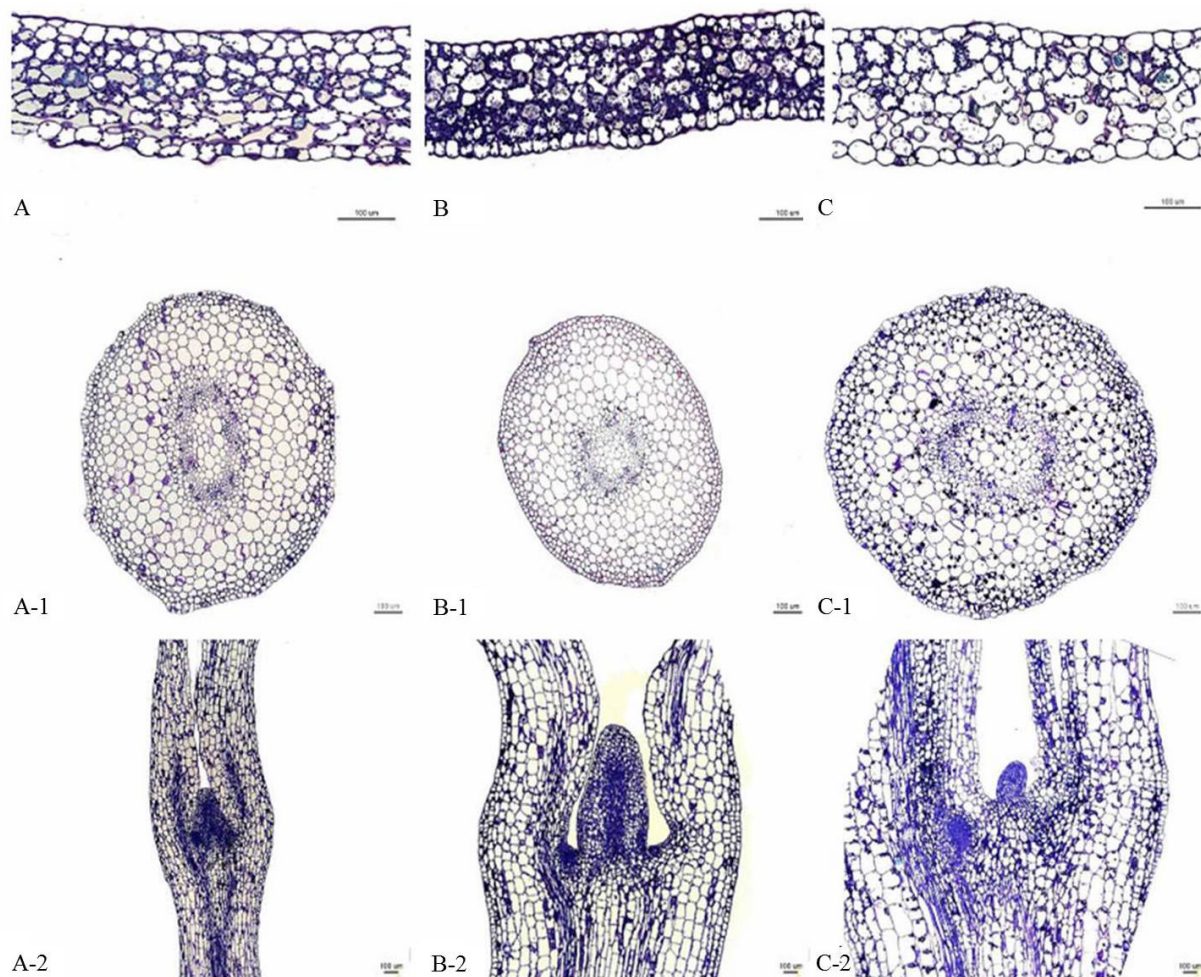


Fig. 4 Histological observations of leaf (A, B and C), stem (A1, B1 and C1), and apical zone (A2, B2 and C2) obtained from plantlets cultured on different culture types. A- semi-solid medium, B- temporary immersion bioreactor, and C- continuous immersion bioreactor. The magnification was 200X in A- C, 100X in D- E, and 200X in G- H

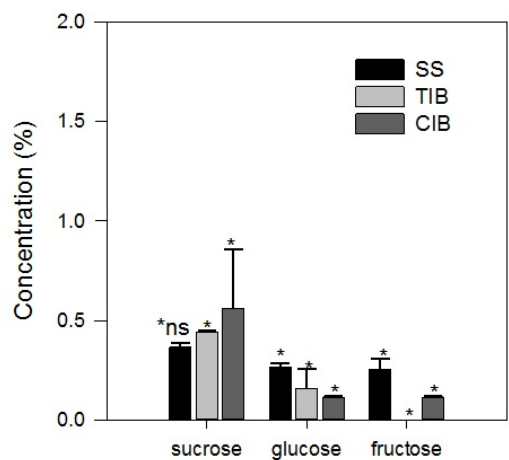


Fig. 5 Soluble carbohydrate content in media by culture type. *ns means that there was no difference at the 5% significance level

Table 2 Stomata characteristics of leaves in plantlets produced in different culture types

Type*	Density (mm ²)	Length (μm)	Width (μm)
SS	256 ^{**}	23.98 ^{b**}	16.96 ^{ns}
TIB	130 ^b	30.64 ^a	18.80 ^{ns}
CIB	113 ^c	30.84 ^a	17.14 ^{ns}
F value	1615.12	22.03	2.66
p	0.00	0.00	0.11

*SS - semi-solid medium; TIB - temporary immersion bioreactor; and CIB - continuous immersion bioreactor.

**The same letter indicates no difference at 5% significance level.

exhibited higher growth in all traits measured than CIB plants. Net photosynthetic rate was not significantly different among

the three culture types; stomatal conductance was higher in the TIB treatment than in the two other types; transpiration was higher in SS than in the two other types (Table 4).

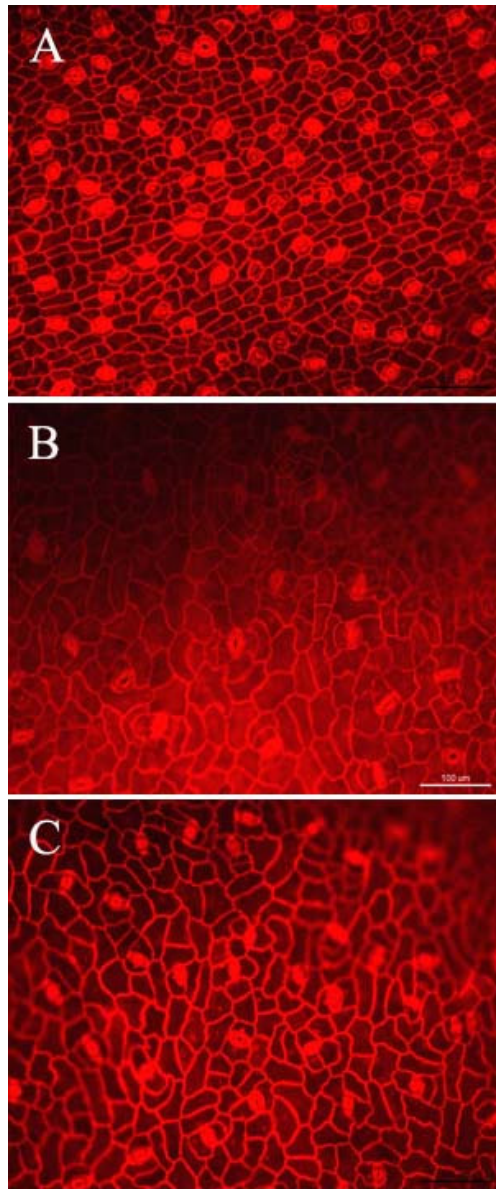


Fig. 6 Different shapes of stomata in germinated plantlets obtained from different culture types. A- semi-solid medium, B- temporary immersion bioreactor, and C- continuous immersion bioreactor

Discussion

We can reduce time and cost effectively by suspension culture, in which we can renew media without using new containers, provide relatively regular culture conditions by medium asepsis using a micro-filter, and remove the container easily (Etienne and Berthouly 2002). CIB exhibited the highest germination rate (95.21%), which was probably related to the extended immersion time. Suspension cultures, including traditional bioreactors have been reported to induce hyperhydricity. Conversely, *in vitro* plantlets were regenerated slowly in the SS treatment, which did not have enough aeration to provide full contact with nutrients (Escalona et al. 1999). TIB was reported to increase the germination rate of SEs up to 60% and reduced the abnormal plantlets to half that of SS (Etienne and Berthouly 2002). The present study showed that healthy plants conversed in TIB system followed by germinated efficiency. Hyperhydricity plants were investigated lower in TIB than the other types (SS and CIB). So, we are proposing the TIB systems for somatic embryo conversion stage of *L. tulipifera*.

Because the TIB type supplies sufficient oxygen, it appears to offer advantages in the reduction of hyperhydricity and increase in stimulatory growth substances (Alister et al. 2005; Zhao et al. 2012). Plant tissue culture techniques have been employed to propagate many species but acclimation is still a problem for successful plant production (Preece and Sutter 1991; Kozai 1999). The problems observed are related to relative lower humidity, lack of stomata control, and moisture loss caused by an abnormal cuticle layer and necrosis (Brainerd and Fuchigami 1982; Estrada-Luna et al. 2003). The results of our trials were similar to that of earlier reports. Many CIB plantlets were did not develop normally, and the results of *in vitro* conditions did not support the production of healthy plants. For this reason, many researchers have used the TIB system to increase the multiplication ratio and root



Fig. 7 *Ex vitro* growing plants obtained from different culture types. SS- semi-solid medium, TIB- temporary immersion bioreactor, and CIB- continuous immersion bioreactor

Table 3 *Ex vitro* growth and biomass of plants derived from different culture types

Type*	Height (mm)	Root collar diameter (mm)	Fresh weight of leaf (g)	Leaf length (mm)	Leaf width (mm)	Petiole length (mm)	Petiole diameter (mm)	Leaf area (cm ²)
SS	47.89 ^{a**}	2.96 ^{a**}	0.19 ^{b**}	35.46 ^{a**}	59.26 ^{a**}	33.19 ^{a**}	0.65 ^{a**}	21.16 ^{b**}
TIB	48.24 ^a	3.28 ^a	0.32 ^a	40.27 ^a	65.24 ^a	36.49 ^a	0.73 ^a	27.45 ^a
CIB	37.47 ^b	2.01 ^b	0.10 ^c	24.21 ^b	38.30 ^b	23.54 ^b	0.55 ^b	10.14 ^c
F value	8.54	4.18	21.43	15.62	14.00	7.46	9.91	16.44
<i>p</i>	0.00	0.04	0.00	0.00	0.00	0.01	0.00	0.00

*SS - semi-solid medium; TIB - temporary immersion bioreactor; and CIB - continuous immersion bioreactor

**The same letter indicates there was no difference at the 5% significance level.

Table 4 *Ex vitro* photosynthesis capacity of leaves in plants derived from different culture types

Type*	Photosynthesis (umol CO ₂ m ⁻² s ⁻¹)	Stomatal Conductance (mol H ₂ Om ⁻² s ⁻¹)	Transpiration (nmol H ₂ Om ⁻² s ⁻¹)
SS	5.75 ^{ns}	0.12 ^{b**}	1.61 ^{ns}
TIB	7.16 ^{ns}	0.20 ^a	2.38 ^{ns}
CIB	6.67 ^{ns}	0.11 ^b	1.39 ^{ns}
F value	1.97	51.12	3.12
<i>p</i>	0.19	0.03	0.09

*SS - semi-solid medium; TIB - temporary immersion bioreactor; and CIB - continuous immersion bioreactor.

**The same letter indicates there was no difference at the 5% significance level.

initiation from *in vitro* plantlets (Yan et al. 2011; Niemenak et al. 2013; Akdemir et al. 2014; Ramos-Castella et al. 2014; Gao et al. 2015). Relatively high humidity seemed to result in high stomatal density in the semi-solid medium. Similarly, SEs of black spruce (*Picea mariana*) did not germinate because they could not pass the pre-germination stage in non-ventilated containers; however, they developed into normal plantlets when cultured in ventilated containers (Abdelmalek and Francine 1999).

Sucrose is widely used in tissue culture as an *in vitro* energy source and osmoticum. Various carbohydrates are used for these purposes, such as sucrose, glucose, and fructose. When used in tissue cultures, sucrose is hydrolyzed to glucose and fructose, which are utilized for plant growth (Vishnevetsky et al. 2000; George et al. 2008). Sucrose content was decreased to 0.5% in all treatments after two weeks, and therefore it is necessary to supply sucrose with a salt compound for long-term cultures. Through our trials using different culture conditions, we determined that hyperhydricity, observed either histologically or morphologically, resulted in poor mesophyll development, low chlorophyll and protein content, and disorder of the grana arrangement (Wetzstein and Sommer 1982; Ziv et al. 1983; Lee et al. 1988; Capellades et al. 1991).

Plantain (*Plantago* spp.) plants from TIB cultures had a higher survival rate than those from gelled medium. Photosynthetic rate at the midpoint of acclimatized plants grown in TIB cultures was significantly higher than that of plants grown in gelled media (Aragon et al. 2014). Chlorophyll content of leaves is indirectly related to nitrogen content, and carotenoids increase antioxidant activity with leaf growth (Zhao et al. 2005). Photosynthetic rate was not significantly different among the three culture types we studied. Shoot meristem culture was effective for producing healthy plantlets and TIB helped to increase efficiency in the case of a high loss rate for seeds and / or vegetative organs in yam (*Dioscorea* spp.) (Balogun et al. 2014). Furthermore, TIB may be utilized as an alternative for the above constituents, such as isomangiferin and iriflophenone 3-C-β-glucoside, which are expensive and difficult to obtain (Kokotkiewicz et al. 2015).

Embryogenesis is still limited in forestry as a commercial technique because of low acclimatization rate and high cost (Lamhamedi et al. 2003; Thompson 2014). Thus, we suggest the use of TIB to increase efficiency of embryo germination and improve acclimatization of *in vitro* plants.

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