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BRIEF COMMUNICATION

Improved growth and acclimatization of somatic embryo-derived *Oplopanax elatus* plantlets by ventilated photoautotrophic culture

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Abstract

To improve large-scale *in vitro* production of *Oplopanax elatus* Nakai, we cultured somatic embryo-derived plantlets under a heterotrophic condition (semi-solid culture with sucrose), photoautotrophic condition (semi-solid culture without sucrose), or modified photoautotrophic condition (liquid culture with forced ventilation). The plantlets grown under the modified photoautotrophic condition had more leaves as well as higher chlorophyll content, and higher net photosynthetic rate than those grown under the conventional conditions. Further, the photoautotrophically grown plantlets acclimatized better and sooner upon *ex vitro* transplantation than did the conventionally cultured plantlets. Consequently, a photoautotrophic culture method with forced ventilation is effective for enhancing the growth and acclimatization of *O. elatus*.

Additional key words: *ex vitro* transfer, medicinal plant, large scale culture.

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Oplopanax elatus Nakai (syn. *Echinopanax elatus* Nakai), commonly called Asian devil's club, is a highly endangered medicinal plant. Few restricted populations have been found in far southeastern Russia, north eastern China and the northern part of Korea (Fu 1992, Lee *et al*. 2002). The major bioactive components of this species are triterpenoid saponins, and four different types of saponins have been characterized (Connolly and Hill 2000). Conventional propagation of *O. elatus* is by seeds; however, poor seed setting and embryo dormancy restricts the propagation of this species. Therefore, *in vitro* propagation methods are used as alternatives, and the induction of somatic embryogenesis has been recently reported. The poor acclimatization of somatic embryo-derived plantlets is, however, a serious handicap (Moon *et al*. 2006). Recently, a photoautotrophic propagation methodology was applied to improve the physiological, anatomical, and morphological characteristics of *in vitro* grown plantlets (Mills 2009). Plants grown by this method showed better acclimatization to *ex vitro* transplantation (Kozai 1991a,b, Ziv 1995, Lian *et al*. 2002, Zobayed et al. 2004, Nguyen and Kozai 2005). Therefore, we conducted a series of experiments as a comparative study to ascertain the efficient method for large-scale propagation of *O. elatus*.

Somatic embryogenesis was induced *in vitro* from mature seeds of *O. elatus* by following the method of Moon *et al*. (2006). Somatic embryo-derived plantlets (2 cm in length) were used for this study. The plantlets were cultured under three different culture conditions: *1*) semi-solid culture with sucrose (conventional heterotrophic condition), in which the plantlets were cultured on half-strength Murashige and Skoog (MS, 1962) medium supplemented with 30 g dm^{-3} sucrose and solidified with 2 g dm^{-3} *Gelrite* (*Duchefa Biochemie*, Haarlem, The Netherlands); *2*) semi-solid culture without sucrose (photoautotrophic condition), in which the plantlets were cultured on half-strength MS medium without sucrose and solidified with 2 g dm^{-3} *Gelrite*; and *3*) liquid culture without sucrose and with forced ventilation (modified photoautotrophic condition).

In both semi-solid cultures, the conventional

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Abbreviations: MS - Murashige and Skoog; P_N - net photosynthetic rate; PPFD - photosynthetic photon flux density.
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heterotrophic and the photoautotrophic conditions, 10 explants were cultured per 400 cm^3 polypropylene culture vessel containing 100 cm^3 of culture medium. In the modified photoautotrophic condition, 30 plantlets were cultured in three plastic plug trays containing *Perlite* (sterilized by autoclaving). The trays containing the plantlets were kept in an acrylic container (60 × 20 × 30 cm) under sterile conditions and irrigated with half-strength MS medium for 15 min per day by using a programmable timer. The container was circulated with filter-sterilized forced air (2500 cm^3 min^{-1}) by using an air supply pump (*Dawkwang*, Seoul, Republic of Korea). All three types of cultures were maintained in tissue culture room for four weeks and under white fluorescent tubes providing photosynthetic photon flux density (PPFD) of 40 μmol m^{-2} s^{-1} during a 16-h photoperiod, temperature of 25 ± 1 °C and relative humidity of 70 %.

After four weeks of *in vitro* culture, the number of leaves, leaf area, petiole length, and root length were measured. The net CO_2 uptake of ten randomly selected plantlets from each culture was measured by using a portable photosynthetic system (*LI-6400*; *LI-COR Biosciences*, Lincoln, NE, USA). The chlorophyll content was measured according to Lichtenthaler (1987). In brief, chlorophylls were extracted from 0.5 g of young leaves with 80 % (v/v) cold acetone for 48 h; then, the absorbance was measured at 663.2, 646.8 and 470 nm and the chlorophyll content was calculated. For visual inspection of stomata, leaf samples were fixed in formalin, acetic acid and absolute alcohol (FAA), stained with acridine orange (0.2 mg in 1 cm^3 of 10 mM citrate-phosphate and 0.1 M NaCl) for 1 min and then examined under a fluorescent microscope (*Leica DMR*, Wetzler, Germany).

After the measurements, the plantlets were transplanted to plastic trays (50 × 30 cm) containing a sterilized mixture of peat moss and *Perlite* (1:1, v/v) and reared in a growth chamber for acclimatization (25 ± 2 °C, relative humidity 70 %, 16-h photoperiod, PPFD of 80 μmol m^{-2} s^{-1}). The survival rate and growth parameters were measured 4 weeks after transplantation.

In vitro propagation of plants requires the establishment of culture conditions to promote growth after achieving maximized multiplication. Culture conditions sustaining high multiplication rates have a major effect on the growth and physiology of developing plantlets (Ziv 1995). Leaves are the major organ affected during plantlet growth *in vitro* (Mills 2009); although the stem is also affected, it has (unless very malformed) a smaller immediate impact on plant water stress and *ex vitro* survival. In this study, the plantlets, grown under the photoautotrophic condition with forced ventilation, possessed more leaves (2.8 per plant) and larger leaf area (18.9 cm^2) than those cultured under the conventional and the photoautotrophic (without ventilation) conditions (Table 1, Fig. 1). Therefore, the growth and development of *O. elatus* plantlets can be greatly improved by using a photoautotrophic culture method with forced ventilation. These results are consistent with the findings of Nakayama *et al*. (1991) and Lian *et al*. (2002), who found better growth and higher photosynthetic rates with *in vitro* grown potato and statice plantlets, respectively, under photoautotrophic conditions with forced ventilation.

In vitro cultured plantlets have a divergent leaf anatomy and physiology compared with *ex vitro* grown plants (Radochová and Tichá 2009), such as reduced mesophyll differentiation with extensive intercellular spaces and decreased cuticle area as well as increased number and density of enlarged stomata with diminished function (Wetzstein and Sommer 1982, Donnely *et al*. 1985, Smith *et al*. 1986). Lian *et al*. (2002) have found higher stoma density in plantlets grown under heterotrophic conditions and less stomata in photoautotrophically grown plantlets. In agreement with such reports, we observed less stomata in the photoautotrophically cultured plants than in the heterotrophically cultured plants (data not shown).

The plantlets grown on the ventilated photoautotrophic culture exhibited about 1.5-fold higher

Table 1. Growth parameters of *O. elatus* plantlets under the conventional heterotrophic (CH), conventional photoautotrophic (CA) and photoautrophic with forced ventilation (AV) conditions. All parameters were measured *in vitro* before *ex vitro* transfer and after four weeks of *ex vitro* acclimatization. Means from 3 repetitions. The different superscript letters indicate significant differences at $P < 0.05$ according to Duncan's multiple range test.

		Survival [%]	Leaf number	Leaf area [cm^2]	Chl content [mg g^{-1}(f.m.)]	P_N [μmol(CO_2) m^{-2} s^{-1}]	Root length [cm]
In vitro	CH	100^a	1.4^b	2.5^c	0.28^b	2.05^b	8.6^a
	CA	100^a	1.6^b	8.8^b	$0.40^{a,b}$	2.72^b	8.8^a
	AV	100^a	2.8^a	18.9^a	0.43^a	4.45^a	5.9^b
Ex vitro	CH	33^b	3.2^b	7.3^c	0.34^b	4.07^c	11.4^a
	CA	75^b	3.0^b	15.1^b	$0.52^{a,b}$	6.32^b	11.0^a
	AV	92^a	5.1^a	30.6^a	0.60^a	8.20^a	8.7^b

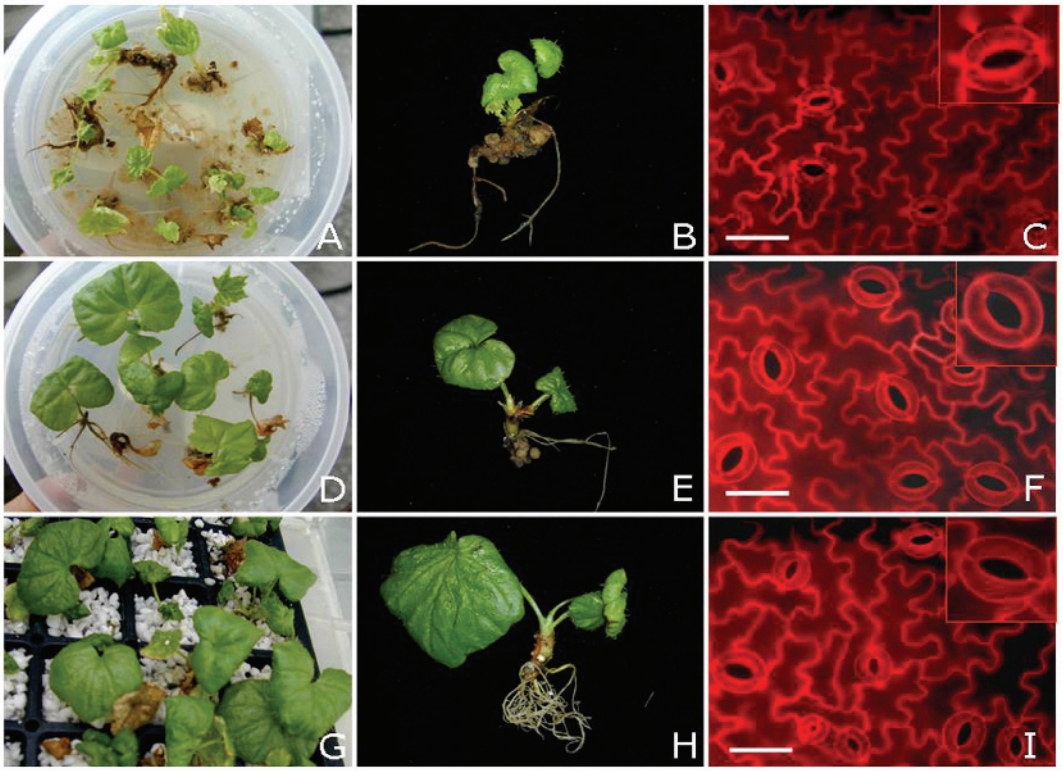

Fig. 1. *Oplopanax elatus* plantlets grown under different culture conditions for four weeks. *A,B,C* - Plantlets grown under the conventional heterotrophic conditions and their stomata. *D,E,F* - Plantlets grown under conventional photoautotrophic conditions and their stomata. *G,H,I* - Plantlets grown under the photoautotrophic conditions with forced ventilation and their stomata (*bar* =50 μm).

chlorophyll content and two fold higher net photosynthetic rate (P_N) than those grown on the semi-solid cultures with and without sucrose. In heterotrophic treatments, plantlets and explants experience atmospheric conditions with persistently low CO_2 and O_2 concentrations, and high humidity due to the limited ventilation of the vessel. Forced ventilation during micropropagation facilitates the gas exchange in the culture vessels, enriches the CO_2 concentration and in consequence P_N (Zobayed *et al*. 2004). Our results support these views, as the plantlets grown under the photoautotrophic condition showed a higher P_N (Table 1).

Micropropagated plants depend on the supply of sugars as an energy source during multiplication and acclimatization. *In vitro*-grown plantlets are usually heterotrophic and have limited photosynthesis during *in vitro* culture, which requires gradual transition to photoautotrophic growth during acclimatization. In order to provide better quality of plants prior to transplanting and to reduce plant losses, many researchers have advocated the *in vitro* growth of plants under photoautotrophic conditions (Kozai *et al*. 1992a,b, Pospisilova *et al*. 1992). On the other hand, Debergh (1991) and De Riek *et al*. (1991) have suggested that photoautotrophy is not necessary for good acclimatization, because heterotrophically grown plantlets can acclimate provided the right treatments. In tobacco culture, recently, abscisic acid (ABA) application to the last subculture improved plantlet acclimatization as they were less prone to wilting and better photoprotected (Pospisilova *et al*. 2009).

In the present experiments, the plantlets grown under the improved photoautotrophic condition (without sucrose and with forced ventilation) showed 92 % survival rate, and the survival rate of those cultured under the conventional heterotrophic and closed photoautotrophic conditions was 32 and 78 %, respectively. The plantlets grown under the photoautotrophic condition showed rapid development of new leaves after four weeks of *ex vitro* transplantation and greater growth parameters (Table 1). Zobayad *et al*. (2004) also documented that *in vitro* photoautotrophic culture may reduce the physiological changes necessary for shoot growth upon transfer to soil, thus improving the acclimatization and survival of plantlets.

In conclusion, our results suggest that a high rate of *in vitro* growth of *O. elatus* plantlets can be obtained by optimizing the culture conditions. The photoautotrophic method with ventilation is suitable for obtaining healthy plantlets that acclimate better and sooner on *ex vitro* transplantation than conventionally grown plantlets.

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