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BRIEF COMMUNICATION

Effects of hypobaric growth conditions on morphogenic potential and antioxidative enzyme activities in *Saussurea involucrata*

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Abstract

Effects of reduced atmospheric pressure on morphogenic potential and antioxidative enzyme activities in regenerated tissues of *Saussurea involucrata* were evaluated. Leaf explants were cultured at atmospheric pressure 30, 60 or 101 kPa on Murashige and Skoog (MS) medium with several plant growth regulators (PGRs). Oxygen and carbon dioxide partial pressures were maintained at 21 and 0.038 kPa, respectively. At 60 kPa, 12 shoots per explant were recorded, which was 1.5 and 2.1-folds higher than at 101 and 30 kPa, respectively. A shooting frequency of 80 % was observed at 60 and 101 kPa. Rooted plantlets were obtained on MS medium with indoleacetic acid. At 30, 60 and 101 kPa, rooting of shoots was 49, 72 and 85.6 %, respectively. The rooted plantlets were successfully acclimatized to soil. Activities of all of antioxidative enzymes determined in present study were affected by hypobaric conditions.

Additional key words: auxin, catalase, peroxidase, shoot organogenesis, superoxide dismutase.

Saussurea involucrata Kar. *et* Kir., a member of *Asteraceae*, is a famous traditional Chinese medicinal plant (Guo *et al.* 2007). *S. involucrata* is a high altitude (3 800 m a.s.l.) plant found in Xinjiang province of China (Piao *et al*. 2001). It is reported to treat diseases like rheumatoid arthritis, gynopathy, *etc*. (Jia *et al*. 2005). Owing to over-exploitation of the wild plants for commercial purposes and the difficulty of cultivation, *S. involucrata* is threatened species and is listed as the second grade national protected wild plant in China (Fu 1992). Previously, a very few efforts were made for its conservation (Guo *et al*. 2007). Unfortunately, these regenerated plantlets could not acclimatize to normal atmospheric pressures.

Hypobaric conditions can affect morphology, physiology and biochemistry of plants (Corey *et al.* 2002, Richards *et al.* 2006). Recently, Paul *et al*. (2004) identified 200 genes involved in responses of *Arabidopsis* to low atmospheric pressure. In addition, pO_2/pCO_2 ratio played significant role in cultivating plants at higher altitudes (Musgrave *et al*. 1988, Schwartzkopf and Mancinelli 1991). Prolonged exposure to elevated CO_2 leads to offset of initial stimulation of photosynthesis (Wilkerson 2005, Levine *et al*. 2009). However, suppression of photosynthesis at long-term elevated CO_2 concentrations might be due to reduction in N-content of leaf.

Cost efficient production of *S. involucrata* can be achieved by acclimation this high altitude species to normal atmospheric pressure. However, the knowledge regarding effects of atmospheric pressure on growth and development parameters in this species is quite limited. Current report is an effort to understand the responses of this elite species to different atmospheric pressures.

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Abbreviations: BAP - benzylaminopurine; CAT - catalase; IAA - indoleacetic acid; NAA - naphthaleneacetic acid; PGR - plant growth regulator; POD - peroxidase; SOD - superoxide dismutase.
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Furthermore, involvement of antioxidative enzymes in morphogenic potential of *S. involucrata* was also evaluated.

Seeds of *S. involucrata* Kar. *et* Kir. were collected from Xinjiang province of China and identified by comparison to reference standards available at the Institute of Botany, Chinese Academy of Sciences, Beijing, P.R. China. Seeds were surface sterilized by rinsing in 70 % ethanol solution for 30 s, then immersed in 5.4 % aqueous solution of sodium hypochlorite for 20 min, followed by 3 rinses with sterile distilled water. Surface-sterilized seeds were germinated and maintained on Murashige and Skoog (1962; MS) solid medium for 30 d in a plant growth chamber with a 16-h photoperiod (irradiance of 30 - 40 μmol m^{-2} s^{-1}, cool-white fluorescent tubes) at temperature of 25 °C. Leaf explants (0.5 cm^2) were sectioned from the germinated seedlings and incubated in culture flasks (5 explants per flask) containing 50 cm^3 MS solid medium supplemented with 10 μM benzylaminopurine (BAP) and 2.5 μM naphthaleneacetic acid (NAA; Guo *et al*. 2007).

Tests were performed in three similar hypobaric chambers (0.15 m^3). They had sensors for CO_2 (*CPR-GD28*, *Kangerxing S&T Development Co*., Beijing, China), O_2 (*CPR-B4*), relative humidity (*SG-11510*, *MROS*, Beijing, China) and irradiance (*XYI-IV*, *Hangzhou Xiye Optoelectronic Engineer Co.*, Hangzhou, China). Explants were exposed to 30 and 60 kPa of total atmospheric pressure (the latter represents approximate natural growth pressure of *S. involucrata*, 3 800 m asl), while explants exposed to 101 kPa were taken as control. The partial pressures of O_2 and CO_2 were maintained at 21 and 0.038 kPa, respectively. These are equivalent to the present partial pressures of O_2 and CO_2 at 101 kPa. All chambers were placed in a growth cabinet with a 16-h photoperiod, irradiance of 30 - 40 μmol m^{-2} s^{-1}, temperature of 25 °C and relative humidity of 60 - 65 %. All three treatments were carried out simultaneously, and for each pressure treatment 3 replicates were used.

The frequency of shoot regeneration and the number of shoots per explant were recorded after 35 d in culture. Green and healthy regenerated shoots bigger than 40 mm were excised from mother tissues and cultured on half strength MS solid medium supplemented with 2.5 μM indoleacetic acid (IAA) under normal atmospheric pressure (101 kPa) for root induction. All media containing 0.6 % agar and 30 g dm^{-3} sucrose (pH 5.8) were autoclaved at 121°C for 18 min. The rooted plantlets were removed from the *in vitro* culture, rinsed in water to remove medium, and transferred to a mixture of peat, *Perlite* and *Vermiculite* (2:1:1; Guo *et al*. 2007). Each plantlet was covered with polyethylene bag in order to maintain high humidity (~90 %). After 21 d, the polyethylene covers were removed gradually and conditions for the plants were gradually changed until an adequate environment for growing directly in the soil was reached.

Supernatant of plantlet tissue extracted with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, and 4 % (m/v) insoluble polyvinylpolypyrolidone was used for enzyme assays. For determination of superoxide dismutase (SOD) activity, the reaction mixture (3 cm^3) consisted of 130 mM methionine, 0.75 mM nitroblue tetrazolium chloride (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8) and 0.05 cm^3 enzyme extract along with 0.02 mM riboflavin and test tubes were placed under irradiance of 80 μmol m^{-2} s^{-1} for 8 min to start the reaction. The above mixture without enzyme served as control. The absorbance was recorded at 560 nm. One unit of SOD activity is defined as 50 % decrease of the NBT reduction (Beauchamp and Fridovich 1971, Yu and Rengel 1999). For determination of guaiacol peroxidase (POD) activity, the reaction mixture (3 cm^3) consisted of 0.25 % (v/v) guaiacol and 3 mM hydrogen peroxide in 100 mM potassium phosphate buffer (pH 6.9). One unit of POD activity was defined as the increase in absorbance recorded at 470 nm (Hammerschmidt *et al*. 1982, Lee and Lin 1996). Catalase (CAT) was assayed by monitoring the decrease in absorbance at 240 nm. The reaction mixture (3 cm^3) consisted of 50 mM potassium phosphate buffer (pH 7.8) containing 12.5 mM H_2O_2 and 50 cm^3 enzyme (Beers and Sizer 1952, Tanida 1996).

The design of all experiments was a complete randomized block. The average number of shoots per explant, the average number of roots per shoot, the mean shoot length, and the mean root length has been represented as mean of two replicates of 25 explants. All the experiments were repeated twice. The data were subjected to a one-way analysis of variance (*ANOVA*). Tukey-HSD test was used for calculation of significant differences by using *SPSS* for *Windows*, version *7.5.1* (*SPSS Inc.*, Chicago, USA), while $P \leq 0.05$

Table 1. Effect of atmospheric pressure on shoot morphogenesis in leaves explants of *S. involucrata* after 35 d of culture and root growth after transferring the shoots on rooting medium for another 35 d of culture. Means ± SE, $n = 50$. Means with the same letters are not statistically different at $P \leq 0.05$ according to Tukey's HSD test.

Atm. pressure [kPa]	Shoot number [explant^{-1}]	Responding explants [%]	Rooting [%]	Root number [shoot^{-1}]	Root length [mm]
101	8.04 ± 0.8^{b}	80.0 ± 9.8^{a}	72.0 ± 8.0^{b}	6.3 ± 0.9^{b}	35.5 ± 0.7^{a}
60	12.33 ± 0.8^{a}	82.6 ± 10.2^{a}	85.6 ± 9.0^{a}	8.5 ± 0.8^{a}	40.2 ± 2.3^{a}
30	5.86 ± 0.5^{c}	55.1 ± 8.1^{b}	49.0 ± 5.0^{cd}	4.6 ± 0.2^{c}	22.7 ± 0.5^{b}

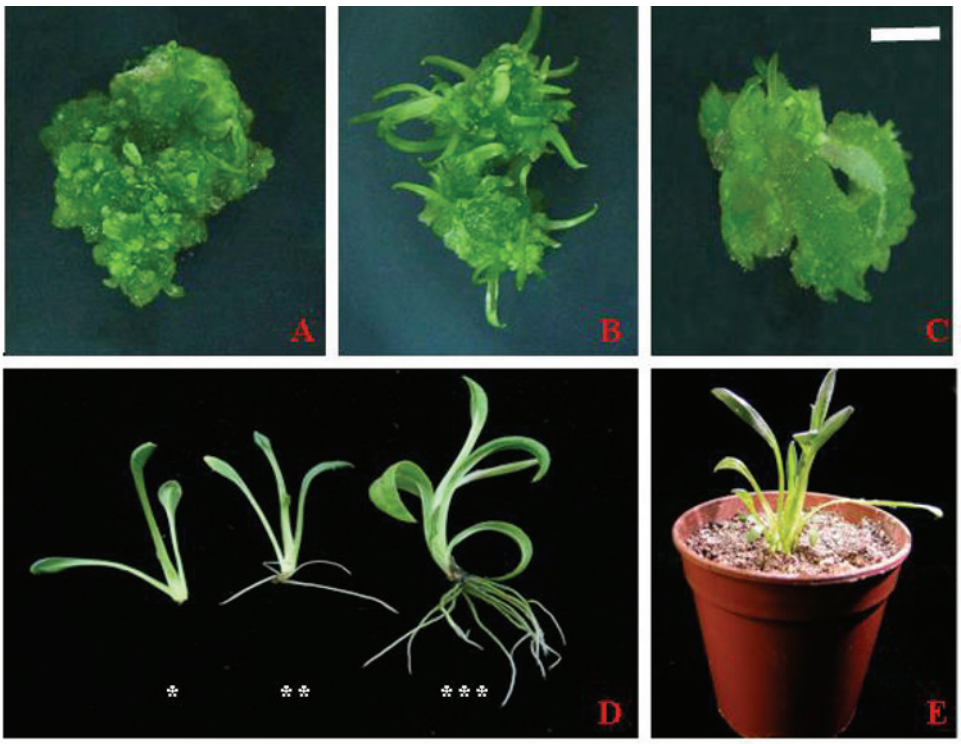

Fig. 1. Shoot morphogenesis induced by the combination of 10 μM BAP and 2.5 μM NAA from leaf explants under diverse atmospheric pressure treatments determined after 35 d: *A* - under 101 kPa; *B* - under 60 kPa; *C* - under 30 kPa (*bar* = 0.6 mm). *D* - rooting of regenerated shoots under 101 kPa treatment on half-strength MS medium supplemented with 5 μM IAA (* indicates shooting; ** indicates rooting after 21 d; *** indicates rooting after 35 d). *E* - micropropagated plants in pots after 45 d of *ex vitro* transfer.

value was regarded as significant.

After 10 d of culture in different atmospheric pressures, the compact light green callus appeared on MS medium supplemented with 10 μM BAP and 2.5 μM NAA, which subsequently produced shoots. Period of callus formation was similar under diverse atmospheric pressures; however the time of shoot formation was different. Several reports are available regarding effects of hypobaric conditions on physiological changes in crop plants (Mansell *et al*. 1968, Andre and Massimino 1992, Massimino and Andre 1999, Goto *et al*. 1995, 1996, He *et al*. 2009a, Levine *et al*. 2008, Stanghellinni and Bunce 1993), but effects on regeneration potential in plants was not yet reported. The regenerated shoots appeared after 18 d of culture at 30 kPa and 14 d at 60 and 101 kPa. Significantly more shoots were observed on leaf explants exposed to 60 kPa than to other treatments. Furthermore, 80 % of shoot regeneration frequency was observed at 60 and 101 kPa, but significantly lower at 30 kPa (only 55 %; Table 1). Contrarily, He *et al*. (2009b) reported that hypobaric conditions of 25 kPa did not significantly affect gas exchange. However, Laurin *et al*. (2006) reported higher moisture loss from cucumbers exposed to 71 kPa than from those exposed to 101 kPa due to effects of reduced pressure on stomatal opening. We hereby generalized that hypobaric conditions played significant role in morphogenesis of high altitude plants. This report endorses previous study, where regenerated *Saussurea* plants only survived at high altitude region of TianShan mountains (Guo *et al*. 2007).

The regenerated shoots of about 40 mm size were transferred to rooting medium and grown under normal atmospheric pressure (101 kPa; Fig. 1*D*) and the rooting indexes were recorded after 35 d (Table 1). The previous different atmospheric pressure treatments significantly affected the date of rooting, number of roots per shoot and root length. Small white spots appeared on shoots collected from 60 or 101 kPa, which developed into roots. However, the shoots developed from 30 kPa treatment had shown delayed rooting (data not shown). After 35 d of culture, 8.5 roots per shoot were obtained reached for 60 kPa treatment, which was 1.4-fold higher than for 101 kPa treatment. However, fewer and smaller roots (4.6 shoot^{-1}) were induced in the shoot developed at 30 kPa. All these plantlets were transferred *ex vitro* and survival rate of 80.2 % was recorded after 45 d of transfer (Fig. 1*F*). Effects of hypobaric conditions on plant morphogenesis are not fully known. Nonetheless, increased ethylene production and protein and saccharide contents were reported in plantlets grown in hypobaric conditions (He *et al.* 2003, Guo *et al.* 2008). Li *et al.* (2006) reported that hypobaric storage delayed the post-

Table 2. Effect of atmosphric pressure on the activities [U g^{-1}(f.m.) min^{-1}] of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) during the shoot morphogenesis of *S. involucrata* (35 d of culture). Means ± SE, $n = 6$. Means with the same letters are not statistically different at $P \leq 0.05$ according to Tukey's HSD test.

Culture [d]	Atm. pressure [kPa]	SOD	POD	CAT
0	101	160.9 ± 6.26^{d}	1342 ± 77.3^{c}	253.3 ± 30.55^{cd}
	60	160.9 ± 6.26^{d}	1342 ± 77.3^{c}	253.3 ± 30.55^{cd}
	30	160.9 ± 6.26^{d}	1342 ± 77.3^{c}	253.3 ± 30.55^{cd}
7	101	166.7 ± 2.54^{cd}	1344 ± 40.7^{cd}	247.8 ± 13.88^{cd}
	60	179.4 ± 8.42^{c}	1392 ± 116.5^{c}	282.2 ± 17.76^{c}
	30	158.4 ± 8.60^{d}	1592 ± 116.7^{b}	222.2 ± 27.76^{d}
14	101	192.3 ± 4.22^{bc}	1246 ± 18.4^{d}	233.3 ± 35.26^{cd}
	60	213.3 ± 5.02^{b}	1365 ± 78.4^{c}	315.6 ± 22.25^{b}
	30	162.7 ± 7.10^{d}	1681 ± 107.8^{ab}	203.2 ± 14.18^{de}
21	101	229.6 ± 8.81^{b}	$1124 \pm 36.0d^{e}$	213.3 ± 26.67^{cd}
	60	252.3 ± 3.22^{a}	1255 ± 98.2^{d}	381.1 ± 26.83^{a}
	30	177.1 ± 5.79^{cd}	1722 ± 45.3^{ab}	182.1 ± 26.25^{e}
28	101	230.0 ± 4.19^{b}	998 ± 58.4^{e}	189.3 ± 15.28^{de}
	60	256.3 ± 1.93^{a}	1094 ± 101.3^{de}	370.1 ± 39.14^{de}
	30	173.3 ± 8.62^{cd}	1774 ± 68.3^{a}	189.3 ± 21.22^{a}
35	101	220.8 ± 2.48^{b}	1058 ± 89.5^{de}	192.1 ± 18.35^{de}
	60	242.4 ± 5.22^{ab}	1070 ± 56.4^{de}	349.0 ± 23.70^{de}
	30	166.5 ± 8.62^{cd}	1674 ± 171.6^{ab}	201.4 ± 12.49^{ab}

harvest senescence in plants.

In order to elucidate the mechanism of the effect of various atmospheric pressures on shoot morphogenesis, their effects on the activities of antioxidant enzymes in plant tissues were investigated. SOD activity was severely affected by hypobaric growth. The highest activity was found at 60 kPa and the lowest at 30 kPa. SOD activity at 60 and 101 kPa increased during cultivation (Table 2). Gradual increase in SOD activity after *ex vitro* transfer was observed in plantain plantlets cultured in temporary immersion bioreactors (Aragón *et al.* 2010). SOD performs conversion of superoxide radical to H_2O_2, which were further metabolized by CAT (Alscher *et al*. 1997). CAT activity was also highest at 60 kPa and lowest at 30 kPa. However, CAT activity at 101 and 30 kPa decreased during cultivation, while CAT activity at 60 kPa firstly increased to maximum at day 21 and then decreased (Table 2). Conversely, 30 kPa treatment significantly enhanced POD activity, but for 60 kPa treatment this activity was comparable to control. This showed that different antioxidative enzymes responded differently to similar atmospheric conditions. Cui *et al*. (1999) reported that somatic embryo production in *Lycium* was correlated with reduced CAT activity. Totipotency in tobacco protoplast was associated with the expression of CAT isoenzyme (Libik and Konieczny 2005). H_2O_2 is considered as major player in induction of oxidative stress (Foyer *et al*. 1997). This H_2O_2 is further metabolized and neutralized by set of antioxidative enzymes (Scandalios *et al*. 1997). However, during root regeneration in *Crocus*, high CAT activity was detected by Vatankhah *et al*. (2010). Therefore, the variations of antioxidant enzyme activities were not consistent with diverse developmental stages in different plant species. POD was the key antioxidant enzyme associated with plant tissue response to 30 kPa in current report. However, shoot organogenesis was associated with activities of all of antioxidative enzymes in current report.

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