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# Hyperhydricity in shoot cultures of *Scrophularia yoshimurae* can be effectively reduced by ventilation of culture vessels

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## Summary

An effective procedure for obtaining healthy shoots from nodal segments of *Scrophularia yoshimurae* is described. Nodal segments cultured on Murashige and Skoog's (MS) basal medium supplemented with 1.0 mg $L^{-1}$ benzyladenine (BA) and 0.2 mg $L^{-1}$ $\alpha$-naphthaleneacetic acid (NAA) induced multiple shoots in conical flasks that differed in the way they were closed and sealed. Hermitically sealed culture vessels resulted in high hyperhydricity/vitrification. High concentrations of ethylene and $CO_2$ were found to accumulate in these vessels. The hyperhydricity of the shoot cultures could be decreased by progressively ventilating the vessels. Exchange of gases was achieved by removing the Parafilm sealing without affecting sterility. This reduced the hyperhydricity rate and gave a good recovery of vitrified shoots, but resulted in decreased proliferation and a dehydration of proliferating nodal segments and the culture medium. The best number of normal shoots was observed when the parafilm was removed for gaseous exchange after four weeks of culture incubation. The results show that hyperhydricity in shoot cultures of *S. yoshimurae* could be prevented by sufficient gas exchange during culture.


*Abbreviations:* BA, benzyladenine; (MS) basal medium, Murashige and Skoog's basal medium; NAA, $\alpha$-naphthaleneacetic acid

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## Introduction

*Scrophularia yoshimurae* (Scrophulariaceae) is a herbaceous perennial plant used in traditional Chinese medicine (Chiu and Chang, 1998). In vitro-propagation is a valuable method for producing large numbers of genetically uniform, pathogen-free plants in a short time. A method for in vitro-propagation for *S. yoshimurae* has been established (Sagare et al., 2001). However, the high frequency of vitrified shoots was a major concern. Solving the problem of hyperhydricity would help in commercial production and conservation of the germplasm of this medicinally important species. Hyperhydricity, also known as vitrification, is a morphological, anatomical and physiological malformation that makes the plant tissue water-swollen (Pâques and Boxus, 1987). The phenomenon has been correlated to water availability, microelements and/or hormonal imbalance in the tissue culture medium (Kataeva et al., 1991). In addition to a high water content in leaf and stem tissues (Pâques and Boxus, 1987), vitrified plantlets have poor epicuticular wax production (Majada et al., 2001). These disorders are morphological responses to non-wounding stress conditions when explants are in unsuitable conditions (Kevers et al., 1984). The outcome is an unbefitting in vitro-culture micro-environment caused by the accumulation of ethylene and other volatiles in the headspace of the culture vessel, a condition distinctly different from the natural environment (Zobayed et al., 1999). The growth and development of plants in culture vessels is influenced by the nutrient medium (Tsay, 1998) as well as by the composition of the gaseous environment (Park et al., 2004). For gas exchange, conventional tissue culture systems depend on the type of culture vessel closure used. Any type of closure must maintain the sterility of the culture. Major concerns have been raised as to the adequacy of the ventilation achieved in closed vessels (Adkins, 1992; Armstrong et al., 1997). Kozai and Smith (1995) have described the conditions in culture vessels as generally constant in temperature, high in humidity, and variable in $CO_2$ content, accompanied by changes in medium composition and the accumulation of toxic materials.

Culture vessels can be considered as tiny greenhouses, and environmental conditions can be improved using techniques similar to greenhouse environmental control such as enhancing natural/forced ventilation. Controlled micropropagation systems, especially under forced ventilation, have been shown to produce morphologically superior and physiologically normal plants (Zobayed et al., 2000, 2001a). Net photosynthetic rates change with the type of ventilation and can strongly affect plant growth (Zobayed et al., 1999, 2002). The headspace of non-ventilated vessels accumulates components like ethylene, $CO_2$, acetaldehyde and ethanol. Of these, ethylene and $CO_2$ seem to be most critical and can be found in especially high concentrations in cultured cells and tissues (Righetti et al., 1990; Zobayed et al., 2001b). A promotive effect of ethylene inhibitors on shoot morphogenesis has been reported (Pua and Chi, 1993; Pua et al., 1996). In the present investigation, the concentrations of ethylene and $CO_2$ in culture vessels were measured weekly for two months and manipulated by removing the Parafilm sealing used to close the vessels. The aim of this study was to establish the influence of ethylene and $CO_2$, released by proliferating nodal segments of *S. yoshimura* during in vitro-culture, on shoot production and hyperhydricity and to find a simple culture modification that would prevent hyperhydricity.

## Materials and methods

Mature plants of *S. yoshimurae* YAMAZAKI were collected from Chitou, Nantou Country, Taiwan (altitude about 1300 m), replanted in 18-cm diameter pots in soil:peat:moss:vermiculite (1:1:1, v/v/v). Plants were maintained in growth chambers (Hotech Instruments Corp., Model 624 HD, Taipei, Taiwan) under light intensity of 100 $\mu$mol $m^{-2}$ $s^{-1}$, 16 h photoperiod, and 20/16 °C day/night temperature. Plants were irrigated once a week with tap water. A voucher specimen of the flowering plant (No. CMC SY 0111) has been deposited at the China Medical College Herbarium, Taichung, Taiwan. The shoot cultures were established using the published protocol developed in our laboratory (Sagare et al., 2001). Nodal segments from in vitro-grown shoots were used as explants in the present study. The explants were cultured in 500 mL conical flasks, each containing 100 mL of medium. The medium consisted of MS basal medium (Murashige and Skoog, 1962) with 1.0 mg $L^{-1}$ BA, 0.2 mg $L^{-1}$ NAA, 3% sucrose and 0.9% Difco Bacto agar (Difco Laboratories, Detroit, MI). The pH of all media was adjusted to 5.7 $\pm$ 0.1 with 1N NaOH or HCl before autoclaving at 121 °C and 105 kPa for 15 min. The cultures were incubated at 25 $\pm$ 1 °C under cool white fluorescent light at 38 $\mu$mol $m^{-2}$ $s^{-1}$ (Philips, Holland) with a 16-h photoperiod per day. Five flasks were cultured per treatment with twenty explants per flask. Six types of ventilation treatments were tested: (i) after

inoculation the culture vessels were closed with two layers of aluminium foil and sealed with two layers of Parafilm M®, which served as control; (ii) the vessels were closed using four layers of pharmaceutical dispense paper [9.5 × 9.5 cm, 0.046 mm thick, gas flow 0.5 mL $s^{-1}$, made from soft- and hard-wood fiber (50:50), Cheng Long Corporation, Taiwan] and sealed with two layers of Parafilm; (iii) same as (ii) but the culture vessels were ventilated by removing the Parafilm sealing after 1 week; (iv) same as (ii) with Parafilm removed after 2 weeks; (v) same as (ii) with Parafilm removed after 3 weeks; and (vi) same as (ii) with Parafilm removed after 4 weeks. The average number of shoots per explant and the percentages of vitrified shoots were recorded for 2 months at 7-day intervals.

The headspace of the culture vessels was analyzed weekly for its content of ethylene and $CO_2$. The gas (1 mL) was withdrawn at the end of the dark period and injected into a gas chromatograph (model GC-8AIT, Shimadzu, Kyoto, Japan)

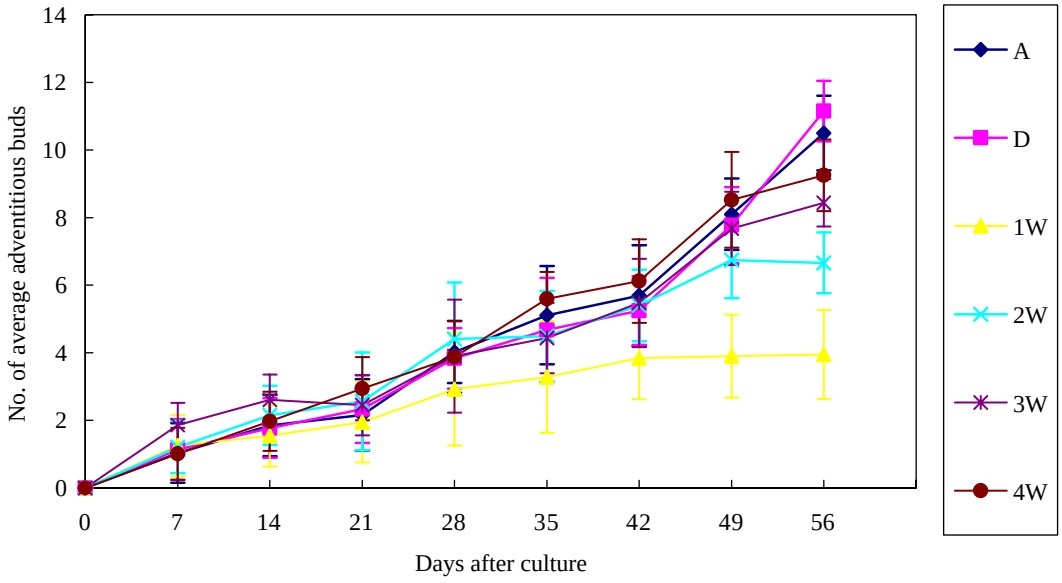


**Figure 1.** Average number of adventitious buds produced from nodal explants of *S. yoshimurae* when cultured in flasks with different closures. A: two layers of aluminium foil and sealed with Parafilm. D: four layers of dispense paper and sealed with Parafilm. 1W—4W: Same as D, except flasks were ventilated by removing the Parafilm at 1 week (1W), 2 weeks (2W), 3 weeks (3W) and 4 weeks (4W) after culture initiation.

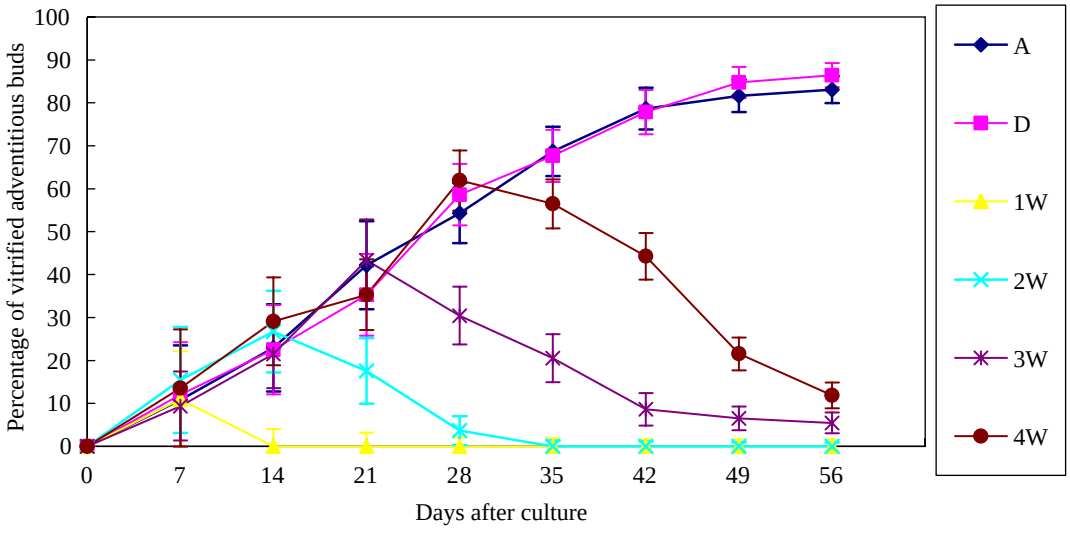


**Figure 2.** Percentage of vitrified adventitious buds produced from nodal explants of *S. yoshimurae* when cultured in flasks with different closures; A, D, 1W, 2W, 3W and 4W same as Fig. 1.

fitted with a flame-ionization detector for ethylene or model GC-8APF fitted with a thermal conductivity detector for $CO_2$ (Chen and Lee, 2002).

## Results and discussion

In hermitically closed culture vessels, closed with two layers of aluminium foil or four layers of dispense paper and sealed with two layers of Parafilm, explants regenerated an average of 10.5 and 11.5 shoots (Figs. 1; 5A and B). Of these, more than 80% were vitrified (Fig. 2). The absolute number of adventitious buds and shoots as well as the percentage of hyperhydric shoots decreased on ventilating the vessels by removing the Parafilm, which was used for tight sealing (Figs. 1 and 2). Removal of the Parafilm also resulted in a rapid dehydration of plant tissues as well as culture media. Improved gas exchange increases evapotranspiration, which alters the physical properties of the culture medium. The change in water content of the culture medium may influence the morphogenetic response and could be instrumental in reducing hyperhydricity. However, the number of

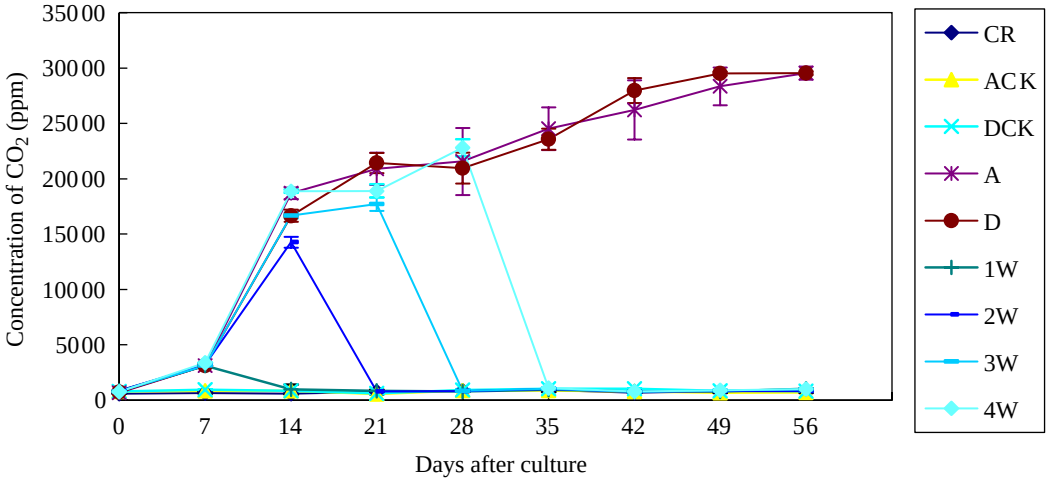


**Figure 3.** $CO_2$ content in vessels of caulogenic cultures from nodal explants of *S. yoshimurae*, cultured in flasks with different closures: A, D, 1W, 2W, 3W and 4W same as Fig. 1. CR: culture room. ACK: same as A, but medium without explants only. DCK: same as D, but medium without explants only.

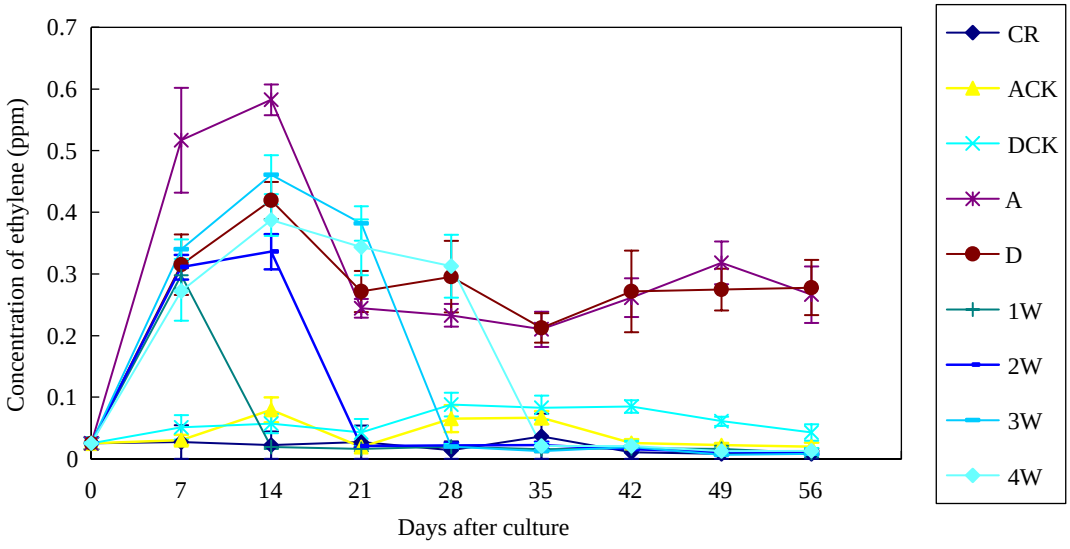


**Figure 4.** Ethylene content in vessels of caulogenic cultures from nodal explants of *S. yoshimurae*, cultured in flasks with different closures: CR, ACK, DCK, A, D, 1W, 2W, 3W and 4W same as Fig. 3.

shoots per explant decreased as the flasks allowed the diffusion of gases. By removing the Parafilm sealing after 1 or 2 weeks, the explants regenerated 4–7 shoots, all of them non-vitrified (Figs. 2 and 5C–F).

Ventilation also reduces the relative humidity in the culture vessel, accounting for the decrease of proliferation and hyperhydricity rate of plant tissue. A tissue culture vessel acts like a miniature greenhouse (Read, 1990), creating a microclimate according to the specific conditions applied. In a conventional tissue culture system, vessel closure is used to maintain sterility and to avoid excessive desiccation of the tissue and culture medium. This may cause the accumulation of volatile chemicals in the headspace, which in turn may reduce the availability of oxygen for transpiration, thereby generating an abnormal atmosphere that could interfere with growth and development (Gould and Murashige, 1985). Restricting gas exchange in culture vessels may slow tissue growth and cause undesirable morphogenetic and physiological changes (Debergh and Maene, 1984) such as vitrification (Ziv, 1991; Debergh et al., 1992). Carbon dioxide is often released in large amounts by growing tissue cultures. The high $CO_2$ content may significantly affect transpiration and photosynthesis and stimulate ethylene

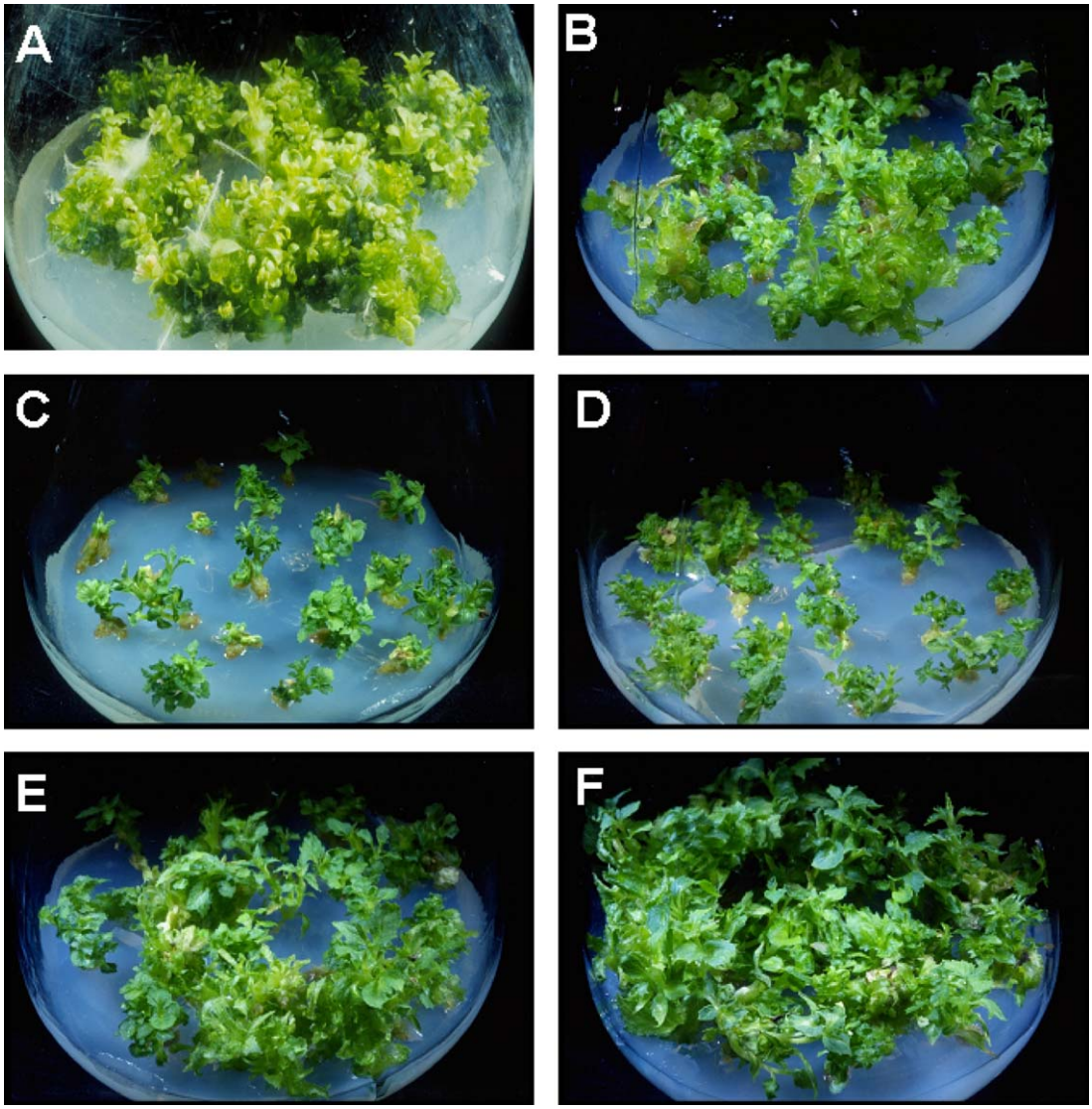


**Figure 5. (A–F)** Induction and proliferation of adventitious buds from nodal explants of *S. yoshimurae* cultured in flasks with different closures. A: two layers of aluminium foil and covered with Parafilm (picture shows condensation typical for non-ventilated vessels); B: four layers of dispense paper and sealed with Parafilm; C–F: same as B, except culture flask was ventilated by removing the Parafilm, at 1 week, 2 weeks, 3 weeks and 4 weeks after culture, respectively.

biosynthesis (Grodzinski et al., 1981) by enhancing the activity of the enzyme that converts 1-aminocyclopropane-1-carboxylic acid. The interaction between ethylene and $CO_2$ is rather intrusive and interferes with the differentiation of shoot buds (Kumar et al., 1987), either stimulating or inhibiting adventitious shoot formation (Kevers et al., 1992).

In the present study, we observed a strong correlation between the number and quality of adventitious shoots and the accumulation of ethylene and $CO_2$ in the headspace of the culture vessel. The concentrations of ethylene and $CO_2$ varied with the type of closure used. In vessels without explants there were no significant changes in the $CO_2$ and ethylene contents. An exponential increase in the concentration of $CO_2$ was recorded in the tightly sealed culture vessels while the concentration of ethylene did not increase significantly within 2 weeks (Figs. 3 and 4). Removal of the Parafilm sealing caused drastic changes to headspace composition. The concentrations of ethylene and $CO_2$ declined. There was a negative effect on the number of shoot buds developed on the nodal segments while a beneficial effect was observed on the quality of shoot developed from these buds (Fig. 5). Plants of *Tagetes erecta* cultivated in vitro in ventilated containers exhibited healthier shoot growth than plants cultivated in tightly sealed containers (Aguilar et al., 2000). Effective ventilation has also resulted in minimizing vitrification in carnations (Jo et al., 2002) and potato (Zobayed et al., 2001b; Park et al., 2004).

Our simple technique of closing culture vessels with dispense paper and removing the Parafilm used for tight closure improved the quality of the shoots. Contrary to the findings of Zobayed et al. (2001b), better ventilation did negatively affect the number of shoot buds formed. Apparently, in *S. yoshimurae*, the conditioning of the headspace by the differentiating tissue has also a positive morphogenetic effect combined with a phenopathic one, leading to vitrification. Nevertheless, this study emphasizes the importance of ventilation to flush out ethylene, $CO_2$ and other volatile components accumulated in the headspaces of culture vessels to achieve non-hyperhydric shoot morphogenesis. Furthermore, our study suggests that ventilation can be time-sensitive. Ventilating the flask by removal of Parafilm after 4 weeks gave the best compromise regarding quality and quantity. This protocol could be used in the establishment of large numbers of uniform and healthy *S. yoshimurae* plants through tissue culture for pharmacological studies or for replanting in the natural habitat.

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