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Influence of ventilation closures on plant growth parameters, acclimation and anatomy of leaf surface in *Scrophularia yoshimurae* Yamazaki - a medicinal plant native to Taiwan

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ABSTRACT. Different ventilation closures, including aluminum foil (AF) and a varying number of dispense papers (DP) had different effects on leaf growth parameters, *in vitro* rooting, survival rate and the anatomical features of the leaf surface of *in vitro* and *ex vitro* acclimated plants of *Scrophularia yoshimurae*—an important medicinal plant. The lowest plant growth parameters and *ex vitro* acclimation rate (<7.0%) were obtained using AF as ventilation closure. A scanning electron microscopy (SEM) study of leaf surfaces of plants derived from different ventilation closure treatments showed that parameters—including density and size of epidermal cell and stomata, size of guard cells, and stomata aperture-differed significantly among various treatments, and this in turn affected plant survival rate. Leaves derived from AF treatment had higher epidermal cell (15094 cells/mm^2) and stomata (38/mm^2) densities than DP treatments. Well-ventilated container closures, such as with DP, improved the morphological characteristics of leaves and in turn enhanced the survival rate during *ex vitro* acclimation (maximum rate being 66.7%). The present study not only provides an improved micropropagation method of *S. yoshimurae* but also gives scientific reasons for the different acclimation rates obtained with various container closures.

Keywords: Hyperhydricity; *In vitro* culture; Scanning electron microscopy; *Scrophularia yoshimurae*; Stomata; Ventilation closure.

Abbreviations: AF, aluminum foil; **BA,** benzyladenine; **DP,** dispense paper; **MS,** Murashige & Skoog's medium; **NAA,** α-naphthaleneacetic acid; **SEM,** scanning electron microscopy.

INTRODUCTION

Scrophularia yoshimurae Yamazaki (Family - Scrophulariaceae) is a perennial herb, native to Taiwan. In traditional Chinese medicine in Taiwan, it is called "Xuanshen", a substitute for *Scrophularia ningpoensis* (Chiu and Chang, 1998). The species is used for treatment of inflammation, laryngitis, tonsillitis, abscesses of carbuncles (Reid, 1996), and constipation. It can lower blood pressure and blood sugar levels and also has antibacterial and anti-oxidant properties. Small doses of it have been reported to be cardiotonic (Reid, 1996). Populations of *S. yoshimurae* are distributed in the central mountain range of Taiwan, and they are adapted to a narrow set of environmental conditions (Liu, 1998). Hence, it is very difficult to locate plants in the wild. In Taiwan, the processed roots of *S. ningpoensis* are imported from China and used as a crude drug since *S. yoshimurae* is not cultivated on a commercial scale, and roots collected from natural habitat are insufficient to meet the local demand. Hence it became imperative to search for alternative propagation methods. *In vitro* culture techniques have been used successfully for propagation of many medicinally important plant species (Tsay, 1992; 1999; Nalawade et al., 2003; Mulabagal et al., 2004).

To maintain the sterility of *in vitro* cultures, it is essential to cover culture vessels with ventilation closures (sealing). Different types of ventilation closures are commonly used. They sometimes cause restriction of gaseous exchange between the vessel atmosphere and outside environment (Buddendorf-Joosten and Woltering, 1994) and result in poor aeration and hyperhydric culture conditions. Hyperhydricity is a morphological, anatomical, and physiological abnormality very often observed in micro-propagated plants (Kevers et al., 1984; Debergh et al., 1992). Anatomical features of the leaf surfaces in tobacco and cauliflower have been reported to be affected

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by the type of ventilation closure used and were linked to hyperhydricity (Zobayed et al., 1999a, 1999b, 2001).

A method of *de novo* regeneration of *S. yoshimurae* has been established in our laboratory (Sagare et al., 2001); however, the high frequency of hyperhydric shoots was a major problem. Hence, the aim of the present study was to evaluate the influence of different ventilation closures on the growth parameters of leaf, root, and plant survival rate and also to carry out a scanning electron microscopy (SEM) study of the leaf surfaces of *S. yoshimurae* plants derived from these different ventilation closures. The results in the present study should be of immense help in understanding the underlying scientific principles behind different rates of acclimation achieved with different container closures and also boost the commercial production and conservation of *Scrophularia yoshimurae.*

MATERIALS AND METHODS

In vitro culture establishment and culture conditions

In vitro shoot multiplication in *S. yoshimurae* was achieved by culturing shoot-tip explants on MS basal medium (Murashige and Skoog, 1962) supplemented with BA (1.0 mg l^{-1}) + NAA (0.2 mg l^{-1}) in a 500-ml glass flask (TAIWANGLASS, Taiwan) containing 100 ml medium as reported earlier (Sagare et al., 2001). The pH of all media was adjusted to 5.7 ± 0.1 with 1 N NaOH before autoclaving at 121°C, 105 kPa for 15 min. Culture vessels were capped with two layers of aluminum foil (AF) (Reynolds Consumer Products, Alcoa Inc., Richmond, Virginia, USA) before autoclaving. After inoculation, culture vessels were capped with two layers of aluminum foil (hereafter referred as AF) or 2, 3, or 4 layers of dispense paper [9.5 × 9.5 cm, 0.046 mm thick, gas flow 0.5 mls^{-1}, made from soft- and hard-wood fiber (50:50), Cheng Long Corporation, Taiwan] as ventilation closure (hereafter referred as DP2, DP3, and DP4, respectively). All cultures were incubated for 8 weeks at 25 ± 1°C under cool white fluorescent light at 38 μmol m^{-2} s^{-1} (Philips, Holland) with a 16-h photoperiod per day.

Influence of ventilation closure on growth parameters of leaf and root

For rooting, *in vitro* shoots derived from DP3 treatment were cultured on MS basal medium devoid of growth regulators (GR). Culture vessels were capped either with two layers of aluminum foil or 2, 3, or 4 layers of dispense papers as described in the previous section and incubated for 8 weeks. Each treatment had eight explants with four replicates.

Influence of ventilation closure on *ex vitro* acclimation rate

In vitro plants after pre-trimming (about 4-cm-long shoot and 3-cm-long root) were immersed in 1000X dilute 50% Benlate (Dupon, USA) solution for 1 h. After that, they were transplanted into a seedling pot (56 × 34 × 4 cm, 60 wells, each well of 5 cm in diameter × 4 cm in depth) containing an autoclaved mixture of BioMix:vermiculite: perlite (1 : 1 : 1) ratio. The seedling pot was placed inside a transparent polycarbonate (PC) box (56 × 34 × 9 cm) with a cover (56 × 34 × 9 cm) to maintain high relative humidity. The PC box was kept inside a growth chamber (Hotech, Model 624 HD, Taiwan) with a light intensity of 100 μmol m^{-2} s^{-1} and a 14-h photoperiod at a 22/18°C day/night temperature for 4 weeks. After that, the PC box was taken from the growth chamber and kept outside at an ambient room temperature for 4 weeks. Each treatment had six rooted plantlets in three replicates.

Scanning Electron Microscopy (SEM) study of leaf surfaces of *in vitro* plants and *ex vitro* acclimated plants

For SEM study, 8-week-old (4 weeks in growth chamber and 4 weeks under room condition), *ex vitro* acclimated plants and 4-week-old *in vitro* plants derived from different ventilation closures treatments were used. Leaves from the same position in the plant (2^{nd} or 3^{rd} pair from apex) of the same approximately size were sampled and frozen in liquid nitrogen immediately. SEM (JEOL-JSM-6330F, Japan) examination of both abaxial and adaxial surface of leaves was carried out. For each treatment, 30 epidermal cells and ten stomata in four replicates were recorded.

Statistical analysis

Quantitative data were first analyzed by ANOVA (SAS® Inc., 2001). For those treatments where ANOVA showed significance, (P<0.05), the least significant difference (LSD) test was used to compare means.

RESULTS

Influence of ventilation closure on growth parameters of leaf and root

In vitro plants grown under ventilation closure treatments DP2 and DP3 had larger leaves than plants derived from AF and DP4 treatments. Plants in AF treatment developed the smallest leaves (Table 1).

In vitro shoots of *S. yoshimurae* cultured on MS basal medium without growth regulators induced 100% rooting in all ventilation closure treatments (data not shown). However, number and length of roots varied among the treatments. The maximum number of roots (2.93 per shoot) and the shortest root length (6.81 cm) were observed in AF treatment (Table 1). Among the three DP treatments, number and length of roots showed marginal differences.

Influence of ventilation closure on *ex vitro* acclimation rate

In vitro plants derived from different ventilation closure

Table 1. Influence of container closure type on leaf growth and *in vitro* rooting in *S. yoshimurae*[x].

Container closures	Layers	Leaf dimension (cm)[y]		Rooting[y]	
		length	width	Number	Length (cm)
Aluminum foil	2	1.52 ± 0.15[c]	0.92 ± 0.10[b]	2.93 ± 0.41[a]	6.81 ± 2.67[c]
Dispense paper	2	1.92 ± 0.09[a]	1.36 ± 0.07[a]	2.67 ± 0.18[ab]	9.46 ± 0.15[a]
Dispense paper	3	1.90 ± 0.17[a]	1.30 ± 0.10[a]	2.07 ± 0.47[c]	8.62 ± 0.02[b]
Dispense paper	4	1.78 ± 0.09[b]	1.10 ± 0.05[b]	2.47 ± 0.13[b]	8.84 ± 0.48[b]

Means ± standard error followed by the same letter/s are not significantly different at 5% level by LSD test.
[x]*In vitro* shoots cultured on MS basal medium with BA 1 (mg/l) + NAA (0.2 mg/l) for 8 weeks.
[y]Each value is the mean for 15 leaves or 15 roots.

treatments showed varying survival percentages at 4 and 8 weeks of observation (Figure 1). The highest survival rate (77.8%) was obtained with DP3 treatment after 4 weeks of acclimation in the growth chamber. This percentage decreased to 66.7% when plants were shifted to room conditions. AF resulted in the lowest survival (27.8%) which further decreased to < 7% on shifting plants to ambient conditions. Among the three DP treatments, DP4 showed the poorest survival rate, but it still exceeded AF (Figure 1).

Figure 2A shows the *in vitro* plantlets of *S. yoshimurae* under the DP3 treatment after 8 weeks of incubation. Plants derived from DP3 and DP4 treatments had better growth than AF and DP2 treated ones (Figure 2B). *In vitro* plants transferred to the PC box became established (Figure 2C) when PC box was kept inside a growth chamber for 4 weeks and on its subsequent transfer to 9-inch pot under outdoor conditions (Figure 2D).

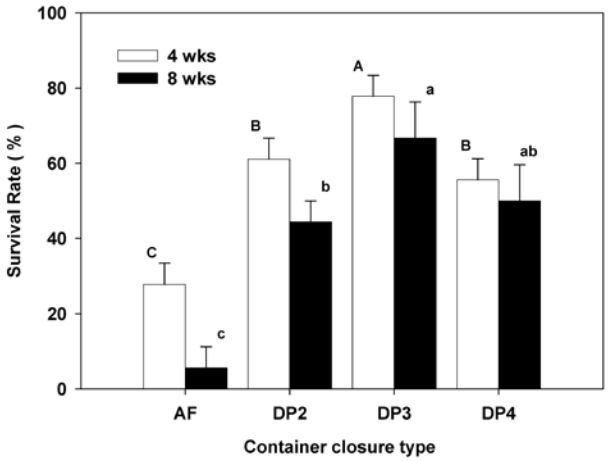

Figure 1. Influence of container closure type on survival rate of *ex vitro* acclimated plants of *S. yoshimurae*. *In vitro* rooted plantlets of *S. yoshimurae* derived from various closure treatments were kept in growth chamber for 4 weeks (□) and there after under room condition for another 4 weeks i.e total 8 weeks (■). The same letter/s above columns with same symbol is not significantly different from each other at the 5% level by LSD test. Each treatment consisted of 18 plants.

Scanning Electron Microscopy (SEM) study of leaf surfaces of *in vitro* plants and *ex vitro* acclimated plants

AF as ventilation closure affected the maximum density of epidermal cells (15094 cells/mm^2) on the adaxial surface of leaves (Table 2). However, cell dimensions in AF were the lowest of all the DP treatments. We observed a direct correlation between ventilation closure and epidermal cell density. Higher ventilation resulted in lower density. Among the four ventilation closure treatments, the minimum cell density (640 cells/mm^2) was observed with DP2. However, a reverse trend was observed with respect to cell dimensions. Among all the ventilation closure treatments, the maximum cell dimensions were obtained with DP2 (Table 2).

As with epidermal cells, a more or less identical trend was observed for stomata density and dimensions (Table 2). All four ventilation closure treatments and *ex vitro* acclimated plants showed a marked difference in the number of open and closed stomata. The maximum amount of open stomata (33/mm^2) was recorded under AF treatment. Again, the reverse was true for the number of closed stomata. The highest density of closed stomata (21/mm^2) was observed in leaves of *ex vitro* acclimated plants. The sizes of guard cells and stomatal apertures were also influenced by the type of ventilation closure treatment in leaves of *S. yoshimurae*. The largest guard cells (0.27 μm) and maximum aperture (0.08 μm) were recorded in the AF treatment (Table 2). The SEM examination of leaf surface of *S. yoshimurae* plants derived from different ventilation closure treatments is shown in Figure 3A-F. Abaxial surface of leaves derived from DP as ventilation closure showed both open and closed stomata (Figure 3C-3E) while all the stomata were open on both the adaxial (Figure 3A) and abaxial (Figure 3B) surfaces of leaves under AF treatment. In leaves from *ex vitro* acclimated plants, all the stomata were found closed (Figure 3F).

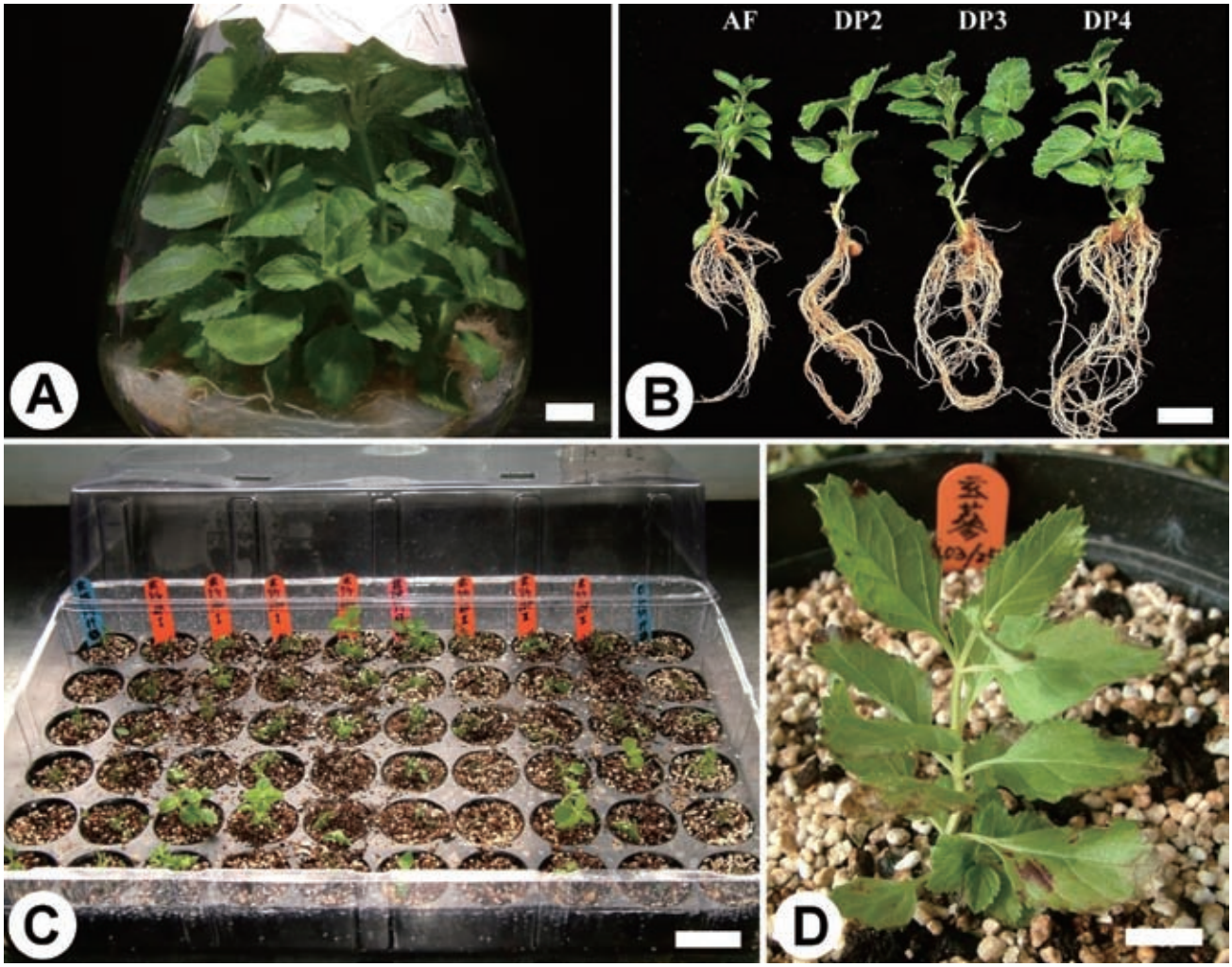

Figure 2. A: In vitro rooted shoots of *S. yoshimurae* under DP2 treatment (Bar=1 cm); B: rooted plantlets under different container closure treatments (Bar=2 cm); C: plantlets inside transparent PC plastic box with cover, kept inside a growth chamber for 4 weeks followed by exposure to room conditions for 4 weeks (Bar=5 cm); D: Plant established in pot kept in outdoor conditions for 2 months. (Bar=2 cm).

DISCUSSION

Growth rate and many other physiological and morphological characteristics of plants developed under *in vitro* conditions have been reported to be influenced by the physical and chemical micro-environment of culture vessels (Walker et al., 1988). In the present study, *in vitro* plants grown under higher ventilation closure treatments had larger leaves than plants derived from lower ventilation closures. These observations are consistent with earlier reports on different plant species (Sallanon and Maziere, 1992; Lai et al., 1998; Zobayed et al., 1999a, 1999b, 2001), which showed that plants under a diffused ventilation conditions had larger leaf area.

Though different ventilation closure treatments had no effect on the rooting response of *in vitro* shoots of *S. yoshimurae* cultured on MS basal medium without growth regulators, growth parameters in terms of number and length of roots varied among the treatments. This difference was marginal among the DP treatments. We have observed similar results with carnation (Chen et al., 1998) and *B. kaoi* (Chen et al., 2004a; 2006).

Our results on survival percentages of plants demonstrate that ventilation closure has a direct bearing on success in the hardening process and further acclimation of *in vitro* plants of *S. yoshimurae*. In general, DP as a container closure improved *ex vitro* acclimation of plants. Similar observations in other crops have been reported earlier (Whish et al., 1992; Majada et al., 1998; Chen et al., 2004b; 2005). In our previous research on carnation, DP as a container closure resulted in better plant growth and non-hyperhydric shoots (Chen et al., 1998), *B. kaoi* (Chen et al., 2004a, 2006) and *S. miltiorriza* (Chen et al., 2005).

SEM studies demonstrate that the type of ventilation closure affected the anatomical features of leaf surfaces,

Table 2. Influence of container closure type on anatomical structure of surfaces of leaves derived from in vitro acclimated plantlets of *Scrophularia yoshimurae*[x].

Ventilation closure	Layers	Epidermal cell[y]			Stomatal density (mm^2)			Guard cell (μm)[z]	Stomatal aperture (μm)[z]
		Total number/mm^2	length (μm)	width (μm)	Total number	open	closed	length	width
Aluminum foil	2	15094 ± 33.3[a]	0.43 ± 0.06[c]	0.21 ± 0.04[c]	38[a]	33[a]	5[c]	0.27 ± 0.04[a]	0.08 ± 0.02[a]
Dispense paper	2	640 ± 32.0[c]	0.63 ± 0.10[ab]	0.39 ± 0.07[ab]	17[c]	6[c]	11[b]	0.19 ± 0.10[b]	0.05 ± 0.01[b]
Dispense paper	3	928 ± 42.3[b]	0.55 ± 0.08[b]	0.32 ± 0.06[b]	17[c]	6[c]	11[b]	0.17 ± 0.10[b]	0.04 ± 0.02[b]
Dispense paper	4	1163 ± 33.3[b]	0.51 ± 0.09[b]	0.25 ± 0.04[c]	25[ab]	14[b]	11[b]	0.22 ± 0.04[ab]	0.05 ± 0.02[b]
Ex vitro acclimated plants	---	480 ± 42.3[c]	0.78 ± 0.13[a]	0.48 ± 0.11[a]	21[b]	0[d]	21[a]	0.21 ± 0.03[ab]	---[#]

Means ± standard error followed by the same letter(s) are not significantly different at 5% level by LSD test.
[x]Ex vitro acclimated plants constituted *in vitro* plants obtained from GR free MS medium under different container closure treatments and acclimated in growth chamber for 8 weeks.
[y]Each value is the mean of 30 cells.
[z]Each value is the mean of 10 stomata.
[#]All stomata were closed.

which in turn influenced the survival rate of *in vitro* raised plants. AF or an increased number of DP layers reduced gaseous exchange between the culture vessel and the outside environment. Similar observations in *B. kaoi* (Chen et al., 2006), cauliflower and tobacco (Zobayed et al., 2001), *D. caryophyllus* (Majada et al., 2000), *Delphinium* spp. (Santamaria et al., 1993), and rose (Sallanon et al., 1993) have been reported. Higher ventilation was shown to decrease stomatal density, resulting in more functional stomata. The hyperhydric and/or *in vitro* plants derived from airtight culture vessels having a higher number of improperly functioning stomata have been reported. These disorders result in excessive water loss and poor photosynthesis during *ex vitro* acclimation, which lead to a lower survival rate (Preece and Sutter, 1991; Ziv, 1991; Sallanon et al., 1993; Jeong et al., 1995; Majada et al., 1998; Zobayed et al., 1999a, 1999b, 2001; Chen et al., 2006). Our results confirm earlier findings that normal functioning of stomata in *in vitro* plants grown under high relative humidity conditions (AF as ventilation closure in the present study) is disrupted (Sallanon et al., 1993; Santamaria et al., 1993; Cassells and Walsh, 1994; Majada et al., 1998; 2000) and adversely affects survival rate.

Culture vessels can be considered tiny greenhouses. Micro-environmental conditions in culture vessels can be controlled by techniques similar to greenhouse control such as enhancing natural/forced ventilation. Controlled micropropagation systems, especially under forced ventilation have been shown to produce morphologically superior as well as physiologically normal plants (Zobayed et al., 1999a, 1999b, 2001). The head space of vessels with low ventilation accumulates components including ethylene, CO_2, acetaldehyde, and ethanol (Zobayed et al., 1999a, 1999b, 2001). These micro-environment changes in the vessels are a result of altered anatomical features in leaves as evident from the present study. Under a low ventilation closure like AF in our previous study and under the higher number of DP in the present study, an accumulation of ethylene and CO_2 was demonstrated (Lai et al., 2005). The poor growth and low survival percentage of plants under AF can be attributed to the inhibitory effect of ethylene as well as to the non-utilization of CO_2 due to low density and the high number of non-functioning stomata (Lai et al., 2005). Further, it could be understood that improved growth parameters under high ventilation closures are a function of a balanced CO_2 supply enabling the plants to benefit from net photosynthetic assimilate production (Zobayed et al., 1999a, 1999b).

CONCLUSION

Results obtained in the present study clearly demonstrate that ventilation closure of culture vessels has a definite influence on plant growth parameters, anatomical features of leaf and in turn survival rate of plants raised under *in vitro* culture conditions. Use of dispense papers as ventilation closure instead of

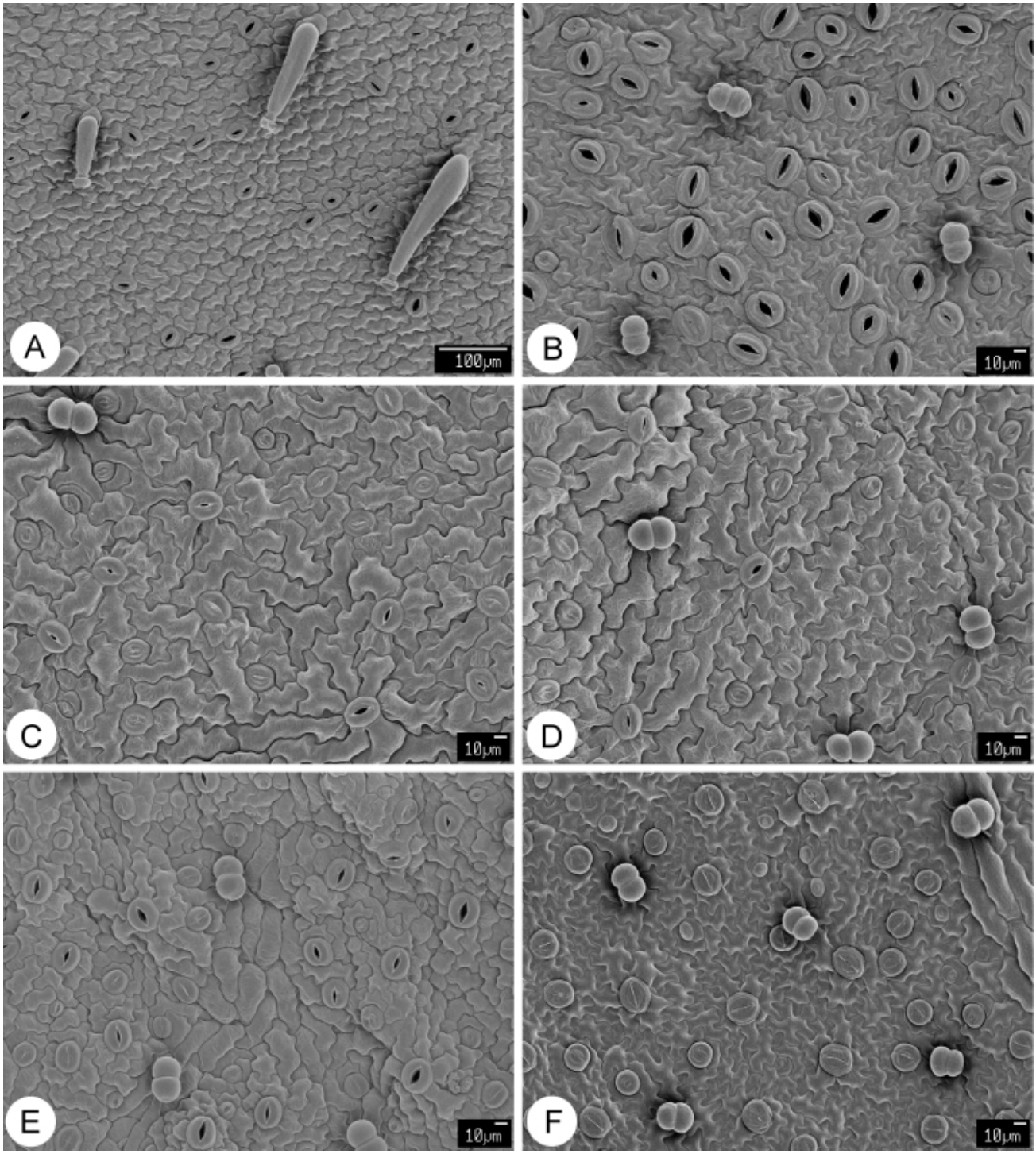

Figure 3. SEM examination of adaxial (A) and abaxial (B-F) surfaces of leaves of *S. yoshimurae*. A, B: *In vitro* leaves derived from containers having two layers of aluminum foil (AF); C: two layers of dispense paper (DP); D: three layers of DP; E: four layers of DP; F: leaf derived from *ex vitro* acclimated plant. (Bar, A=100 μm; B-F=10 μm).

commonly used aluminum foil improved not only plant growth parameters but also the morphological characteristics of leaves, *ex vitro* acclimation and survival of plants.

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透氣性培養容器封口對臺灣本土藥用植物—臺灣雙鋸齒葉玄參組培苗生長、馴化及葉表構造之影響

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臺灣雙鋸齒葉玄參（*Scrophularia yoshimurae*）為重要藥用植物，其組織培養大量繁殖瓶苗過程中，利用雙層鋁箔紙（aluminum foil, AF）或不同層數藥包紙（dispense paper, DP）之培養容器封口形成透氣率的差異，對於組織培養苗發根、馴化與葉表構造均可造成極大的影響。研究結果顯示，組培苗葉片之生長效率以鋁箔紙處理最差，其出瓶馴化成活率甚至低於 7%。利用掃描式電子顯微鏡（scanning electron microscopy, SEM）觀察不同透氣率處理與馴化成活植株葉表之結果顯示，包括表皮細胞、保衛細胞與氣孔之大小與密度等葉片特性，均受到培養容器封口透氣率的影響，進而導致出瓶組培苗的馴化存活率的差異。透氣率較低之鋁箔紙處理相較於透氣率較高之藥包紙處理，其組培苗葉片顯示具有最大表皮細胞密度（15094 cells/mm^2）與氣孔密度（38 stoma/mm^2）。利用透氣率較高之藥包紙作為培養容器封口，不僅可改善其組培苗葉片形態，更進一步促進組培苗之出瓶移植存活率達 66.7%。本研究結果顯示，藉由改善培養容器封口透氣率，不僅提供有效方法以促進組培苗之出瓶馴化成活率，更進一步利用掃描式電子顯微鏡的觀察證實，出瓶馴化成活率的差異主要是由於培養容器封口透氣率的不同。

關鍵詞：玻璃質化；組織培養；掃描式電子顯微鏡；臺灣雙鋸齒葉玄參；氣孔；透氣性培養容器封口。