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IN VITRO MICROPROPAGATION AND *EX VITRO* ACCLIMATION OF *BUPLEURUM KAOI* – AN ENDANGERED MEDICINAL PLANT NATIVE TO TAIWAN

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Summary

This study reports an improved protocol for *in vitro*-shoot multiplication and *ex vitro* acclimation of *Bupleurum kaoi*, an endangered medicinal herb. Nodal segments were cultured in half-strength Murashige and Skoog (MS) basal medium supplemented with different concentrations of benzyladenine (BA) and kinetin. The presence of 0.25 mg l^{-1} BA induced the highest number of shoots per explant after 8 wk of culture. Although BA was more effective than kinetin on shoot multiplication, it induced hyperhydric shoots at all concentrations tested. The use of dispense paper (DP) instead of aluminum foil (AF) for container closure was found to reduce hyperhydricity and improve *ex vitro* acclimation. The best survival rate (61%) was obtained when plantlets were grown in MS basal medium containing 0.5 mg l^{-1} indole-3-butyric acid and 0.1–0.2 mg l^{-1} α-naphthaleneacetic acid using DP as container closure. Leaves of the plant treated with AF6 (two layers of AF as container closure and 6 wk of incubation) lacked epicuticular wax and possessed larger stomata, higher stomata density, and fewer functional stomata compared to those of plants treated with AF2 + DP4 (two layers of AF for 2 wk, then replaced AF by three layers of DP for 4 wk) and *ex vitro*-acclimated plantlets.

Key words: acclimation; *Bupleurum kaoi*; container closure; hyperhydricity; proliferation; rooting; scanning electron microscope.

Introduction

In traditional Chinese medicines in Taiwan, roots of *Bupleurum kaoi*, *Bupleurum falcatum*, and *Bupleurum chinese* are used as substitutes of *Radix blpleuri*, commonly known as 'Chai-hu.' All these four species contain saikosaponins as main active ingredients. Among them, saikosaponin-a and saikosaponin-d have been recognized as pharmacologically active compounds known for anti-allergic, analgesic, and anti-inflammatory properties, and also for lowering fever (Kan, 1985; Hiraoka, 1989; Yen et al., 1991; Tang and Eisenbrand, 1992; Lin and Yen, 1999). The saikosaponin-d content in *B. kaoi* has been reported to be higher than that in *B. falcatum* and *B. chinese* by 2.4- and 19.2-fold, respectively (Yen et al., 1991).

A large number of medicinally important plant species have been micropropagated successfully by tissue-culture techniques (Tsay, 1992, 1999; Makunga et al., 2003; Nalawade et al., 2003). Micropropagation of *B. falcatum* through embryogenesis (Hiraoka, 1989) or shoot-tip culture (Hsu et al., 1993) has been reported. In *B. kaoi*, although shoots could be multiplied *in vitro* (Chen et al., 2004), the occurrence of a high percentage of hyperhydric shoots was problematic. Hyperhydricity is a physiological and morphological malformation commonly observed under tissue-culture conditions. This problem has resulted in poor *ex vitro* establishment and increased cost of production (Paek et al., 1991; Preece and Sutter, 1991; Ziv, 1991). In hyperhydric shoots, leaves usually manifested no epicuticular wax formation, poor cuticle development, and improper function of stomata. These features could result in excessive water loss and poor photosynthetic capacity in *ex vitro*-acclimated plants (Paek et al., 1991; Preece and Sutter, 1991; Ziv, 1991; George, 1993; Miguens et al., 1993; Jeong et al., 1995; Chen et al., 1998; Tsay, 2000). The presence of an unbalanced gaseous environment in the culture container has been reported as one of the causes of hyperhydricity in *in vitro*-grown shoots/plants, which can be improved by using better ventilating container closures (Kozai, 1991; Rossetto et al., 1992; Majada et al., 1998, 2000; Nauyen et al., 1999; Tsay, 2000; Zobayed et al., 2001; Lai et al., 2005).

The aim of the present study was to develop an improved protocol for *in vitro*-shoot multiplication, rooting, and *ex vitro* acclimation of *B. kaoi* by overcoming hyperhydricity.

Materials and Methods

In vitro *shoot culture initiation and shoot proliferation.* Nodal segments of *B. kaoi* were obtained from plants growing in the field of the Agricultural Research Institute in Taiwan (Fig. 1*A*). Explants were disinfected by dipping in a 70% alcohol for 10 s, 10 min in 0.5% NaOCl solution, and rinsed three times with sterile-distilled water. For culture initiation, nodal segments (*c.* 1 cm long) were cultured on the medium containing half-strength MS (Murashige and Skoog, 1962) salts and vitamins, 30 g l^{-1} sucrose, 9.5 g l^{-1} Bacto-agar (Difco, USA), 1 mg l^{-1} BA and 0.1 mg l^{-1} NAA. For shoot

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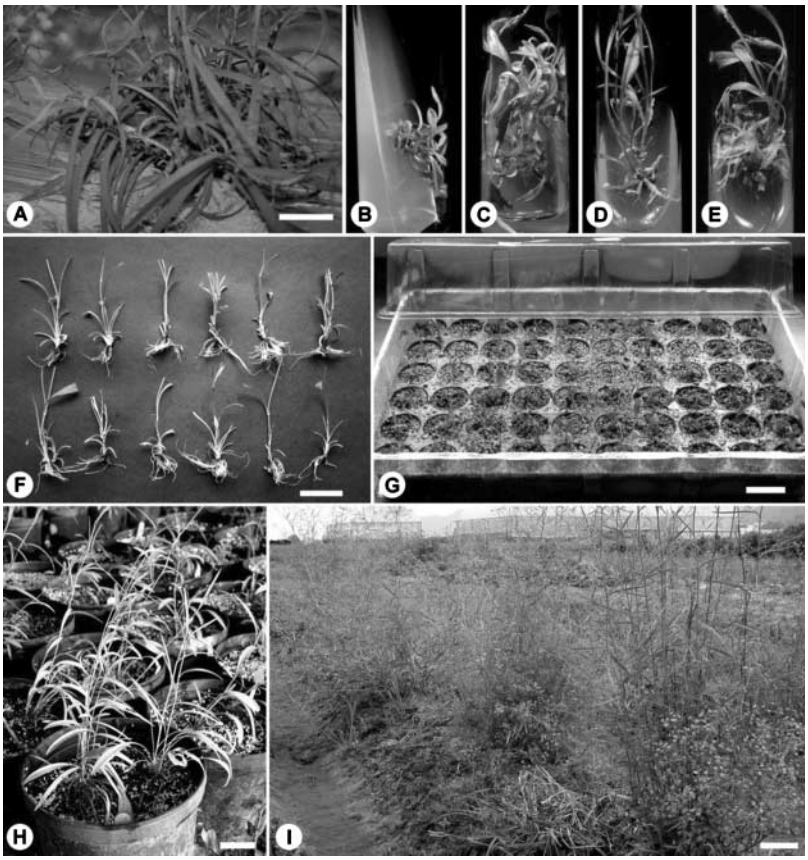

FIG. 1. *A*, Growth of new shoots in 1-yr-old *B. kaoi* plant (*bar* = 5 cm); *B*, initial aseptic shoots in nodal segment; *C*, *in vitro* shoot proliferation on the same medium after 6 wk of culture; *D*, rooting of shoots in the medium with 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} NAA; *E*, rooting of shoots in the medium with 0.5 mg l^{-1} IBA and 0.8 mg l^{-1} NAA; *F*, rooted shoots derived from medium as in *D* but treated with AF2 + DP4 as container closure (*bar* = 2 cm); *G*, plantlets after 4 wk of transfer to transparent PC plastic box with cover, kept inside a growth chamber (*bar* = 5 cm); *H*, plants grown in pots kept outdoors for 4 mo. (*bar* = 10 cm); *I*, tissue culture (TC) plants grown for more than 1 yr in the field (*bar* = 20 cm).

proliferation, different concentrations of BA (0.25–2.0 mg l^{-1}) and kinetin (0.1–0.8 mg l^{-1}) were tested. Cultures were evaluated in terms of number of shoots (< 2 cm long) per explant after 6 and 8 wk of culture.

The pH of all media was adjusted to 5.7 ± 0.1 with 0.1 N NaOH and 0.1 N HCl before autoclaving at 1.05 kg cm^{-2} and 121°C for 20 min. Shoot initiation and multiplication experiments were carried out using glass test tubes (25 × 120 mm, Pyrex, Japan), each with 10 ml medium. For rooting and container closure experiments, 125 ml Erlenmeyer flasks (Pyrex, Japan), each with 25 ml medium, were used. Explants were incubated at 25 ± 2°C under a 14-h photoperiod provided by white fluorescent lamps with a photosynthetic photon flux density of 38 μmol m^{-2} s^{-1}.

Influence of container closure on in vitro *rooting and* ex vitro *acclimation*. Culture containers were first sealed with two layers of aluminum foil (AF) and incubated for 4 (AF4), 3 (AF3), 2 (AF2), or 1 wk (AF1), then replaced by three layers of dispense paper (DP) [95 × 95 × 0.46 mm, gas flow 0.5 ml s^{-1}, made from soft- and hard-fiber (50:50), Cheng Long, Taiwan], followed by incubation for 2 (DP2), 3 (DP3), 4 (DP4), and 5 wk (DP5), respectively.

For root induction, regenerated shoots were cultured on MS basal medium containing 0.5 mg l^{-1} indole-3-butyric acid (IBA). Rooting response was evaluated after 6 wk of culture. For *ex vitro* acclimation, the rooted shoots (3–4 cm long) from different container closure treatments were immersed in 1000 × dilute Benlate (Dupon, USA) solution for 1 h before transferring to a pot (5 cm diameter × 4 cm depth) containing autoclaved mixture of BioMix, vermiculite and perlite (1:1:1). The pot was kept inside a transparent polycarbonate (PC) plastic box (55 × 34 × 9 cm) to maintain high relative humidity. The box was kept in a growth chamber (Hotech, Model 624 HD, Taiwan) with 14-h photoperiod and light intensity of 100 μmol m^{-2} s^{-1} at day/night temperature of 22/18°C. After 4 wk, the box was transferred to the room temperature for another 4 wk.

SEM study of leaf surfaces of in vitro- *and* ex vitro-*acclimated plants*. *In vitro* leaves were taken from 6-wk-old plantlets treated with AF6 and AF2 + DP4, while *ex vitro* leaves were excised from plants grown for 4 wk in a growth chamber, followed by 2 wk under the ambient room conditions. Both adaxial and abaxial leaf surfaces were examined by a scanning electron microscope (SEM, LEO 1430, Japan). Leaf blade (*c*. 25 × 25 mm), taken from the third or fourth leaf from the shoot tip, was fixed with liquid nitrogen for 30 s, followed by gold coating for 180 s using the SEM cryo preparation system (PolarPrep 2000, Polaron, UK). Leaves collected were either fixed immediately after sampling or after 30 min at ambient room conditions. Data on epidermal cell and stomata density were recorded.

Experimental design and data analysis. A completely randomized design was used for all experiments. In multiple shoot experiments, each treatment consisted of four replicates, each with five nodal segments. For rooting and container closure experiments, each treatment consisted of nine *in vitro* shoots with three replicates. In root length and diameter, 15 *in vitro*-rooted shoots in three replicates were recorded, while each treatment in *ex vitro* acclimation experiments consisted of six rooted shoots with three replicates. To study the epidermal cells and stomata using SEM, 20 epidermal cells and 10 stomata for each leaf sample with three replicates for each treatment were examined. Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using SAS 8.2 statistical software (SAS Institute Inc., 2001).

Results and Discussion

Establishment of shoot culture and influence of BA and kinetin on shoot proliferation. *In vitro* shoots were initiated from nodal segments of *B. kaoi* grown on MS basal medium supplemented with 1.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Fig. 1*B*). Multiple shoots were formed during subculture on the same medium (Fig. 1*C*). Different concentrations of BA and kinetin resulted in varying shoot proliferation rates (Table 1). The maximum number of shoots (7.3) per explant was obtained in the presence of 0.25 mg l^{-1} BA. Further increase in shoot number did not occur by increasing the BA concentrations higher than 0.25 mg l^{-1}. Explants grown on different concentrations of kinetin (0.1–0.8 mg l^{-1}) formed fewer shoots compared to BA. Although BA resulted in higher shoot proliferation rates, shoots were hyperhydric in appearance. On the other hand, shoots grown on media supplemented with kinetin or devoid of growth regulators were normal and non-hyperhydric. Similar results have also been observed in carnation (*Dianthus caryophyllus*), where higher concentrations of BA increased the production of hyperhydric shoots (Chen et al., 1998).

Influence of container closure on in vitro *rooting and* ex vitro *survival of plantlets.* The highest frequency (77.8%) of shoots forming roots and the lowest plant survival rate (33.3%) were obtained in the treatment of using aluminum foil as a container closure for 6 wk (AF6). The lowest rooting frequency (58.9%) was observed with the treatment AF1 + DP5 (Table 2). On the other hand, the plantlet survival rate improved markedly with the use of DP as container closure, with the highest survival rate (77.8%) observed in AF2 + DP4.

Effect of auxin on in vitro *rooting and* ex vitro *acclimation of plantlets.* Shoots grown on MS basal medium supplemented with 0.5 mg l^{-1} IBA and 0.2 mg l^{-1} NAA formed roots at the highest frequency (94.4%), with the maximum number of roots (15.4). The level of NAA higher than 0.2 mg l^{-1} resulted in thicker roots and callus formation at the shoot base (Fig. 1*D*, *E*). These rooted plants showed the maximum survival rate (91.7%) after 4 wk of acclimation (Table 3). As *B. kaoi* in this study, IBA has been reported to be a better auxin for rooting in several herbaceous species compared to IAA and NAA (Lewandowski, 1991; Rani and Grover, 1999; Huang et al., 2000; Martin, 2002). Similarly, induction of better rooting response using the combination of two auxins has also been reported (Lewandowski, 1991; Rani and Grover, 1999; Huang et al., 2000). Healthy plants derived from *in vitro* container closure treatments after acclimation grew well in the field for more than 1 yr without noticeable morphological abnormalities (Fig. 1*I*).

TABLE 1

INFLUENCE OF CYTOKININ ON SHOOT MULTIPLICATION AND HYPERHYDRICITY IN *BUPLEURUM KAOI* AFTER 6 AND 8 WK OF CULTURE

Cytokinin (mg l^{-1})		No. of shoots per explant[z]		Percentage of hyperhydric shoots[z]	
Kinetin	BA	6 wk	8 wk	6 wk	8 wk
0	0	1.3 g[y]	1.9 e[y]	0 c	0 c
0.1	0	2.5 f	2.6 de	0 c	0 c
0.2	0	2.6 ef	3.0 d	0 c	0 c
0.4	0	2.9 de	4.1 c	0 c	0 c
0.8	0	2.7 def	2.7 d	0 c	0 c
0	0.25	6.0 a	7.3 a	10.0 b	16.4 b
0	0.5	5.0 b	6.2 b	22.0 a	46.8 a
0	1.0	4.1 c	6.0 b	26.8 a	46.7 a
0	2.0	3.0 d	3.9 c	33.3 a	41.0 a

[z] Shoots <2 cm in length were not recorded.

[y] Each treatment consisted of four replicates, each with five *in vitro* shoots. Means with the same *letter(s)* within the same *column* are not significantly different at the 5% level.

Leaf surfaces of in vitro- *and* ex vitro-*acclimated plants.* Epidermal cell densities on adaxial and abaxial surfaces of leaves derived from different container closure treatments varied greatly. The highest epidermal cell density (2720 mm^{-2}) was recorded on the abaxial surface, followed by 1832 mm^{-2} on the adaxial surface of leaves derived from AF6 container closure treatment (Table 4). In general, cell density was higher on abaxial compared to adaxial surfaces. *Ex vitro*-acclimated plants showed the minimum density on both leaf surfaces. Also, the cell density in both leaf surfaces was lower in AF2 + DP4 compared to AF6 (Table 4). We observed an identical trend with respect to stomata densities. Ventilation closure treatment of AF6 recorded the higher stomatal density than AF2 + DP4 on both the leaf surfaces. The minimum stomata density (133 mm^{-2}) was again observed on the abaxial leaf surface of *ex vitro*-acclimated plants (Table 4). Our findings are in line with the results of Zobayed et al. (2001), who observed a higher stomatal density in tobacco and cauliflower leaves derived from airtight or diffusive culture vessels compared to that of *ex vitro*-grown or forced ventilated plants. However, in *Datura insignis*, there was no significant difference in stomatal densities between the *in vitro*- and *ex vitro*-grown plants, although the ratio of normal and abnormal stomata differed (Miguens et al., 1993). The size of stomatal aperture of AF6 leaf was significantly larger than AF2 + DP4 leaf and the adaxial and abaxial leaf surfaces of *ex vitro*-acclimated plants.

Results of the SEM study revealed a noticeable difference in the leaf surface of plants grown under different conditions. This is evidenced from AF6 shoots that appeared to be hyperhydric (Fig. 2*A*), while AF2 + DP4 shoots are normal (Fig. 2*B*). In addition, the leaf surface of AF6 plants (Fig. 2*C*) showed a lack of epicuticular wax formation compared to leaves derived from AF2 + DP4 (Fig. 2*E*, *F*) or *ex vitro*-acclimated plants (Fig. 2*D*). The stomata in the AF2 + DP4 leaf (Fig. 2*E*, *F*) were similar to those of *ex vitro*-acclimated plants. However, the AF6 leaf showed various abnormal stomatal characteristics, including larger size and circular or protruding appearance (Fig. 2*C*). On the other hand, leaves of the *ex vitro*-acclimated plants possessed sunken stomata (Fig. 2*D*), while both sunken (Fig. 2*F*) and slightly protruding stomata (Fig. 2*E*) were observed in AF2 + DP4 leaves. Furthermore, we observed widely open stomata when AF6 and AF2 + DP4 leaves were fixed with liquid nitrogen (Fig. 2*C*, *E*), but stomata were closed in fixed leaves of *ex vitro*-acclimated plants. Similarly, closed stomata were also observed in AF2 + DP4 leaves, which were fixed 30 min after sampling (Fig. 2*D*). Similar abnormalities in size and shape of stomatal aperture as well as guard cells have also been reported in leaves of hyperhydric plants of *D. insignis* and *D. caryophyllus* (Miguens et al., 1993).

Results from several lines of study show that stomata in hyperhydric leaves or sometimes *in vitro* leaves do not close in response to darkness, relative humidity, CO_2, ABA, or Ca^{2+}, which affect the function of guard cells (Ziv, 1991; Zobayed et al., 2001). In this study, stomata from AF6 leaves remained open even after

TABLE 2

INFLUENCE OF CONTAINER CLOSURE TYPE AND INCUBATION PERIOD ON *IN VITRO* ROOTING AND *EX VITRO* ACCLIMATION OF *B. KAOI* PLANTLETS

Container closure[z]	Rooting (%)	No. of roots per shoot	Mean root length (cm)	Survival rate (%)	
				4 wk	8 wk
AF6	77.8 ± 5.89 a[y]	7.0 ± 0.53 a[y]	1.69 ± 0.09 a[y]	33.3 ± 6.42 c[y]	11.1 ± 6.42 b[y]
AF4 + DP2	73.3 ± 8.67 b	6.6 ± 0.78 a	1.59 ± 0.10 a	74.1 ± 7.41 a	37.0 ± 3.70 a
AF3 + DP3	71.7 ± 12.1 b	6.4 ± 1.09 a	1.51 ± 0.10 a	74.1 ± 3.70 a	44.4 ± 0.00 a
AF2 + DP4	72.2 ± 5.44 b	6.5 ± 0.49 a	1.59 ± 0.09 a	77.8 ± 6.42 a	48.1 ± 7.41 a
AF1 + DP5	58.9 ± 1.78 c	5.3 ± 0.16 b	1.19 ± 0.10 b	55.6 ± 6.42 b	44.4 ± 6.42 a

[z] AF6, two layers of aluminum foil as container closure for 6 wk of incubation; AF4 + DP2, AF3 + DP3, AF2 + DP4, and AF1 + DP5, two layers of aluminum foil as container closure for 4, 3, 2, and 1 wk of incubation, followed by AF exchanged with three layers of dispense paper for the next 2, 3, 4, and 5 wk of incubation, respectively.

[y] Means with the same letter(s) within the same column are not significantly different at the 5% level by LSD test.

TABLE 3

INFLUENCE OF IBA AND NAA ON ROOTING AND *EX VITRO* ACCLIMATION OF *B. KAOI* PLANTLETS

Auxin ($mg\,l^{-1}$)							Survival rate (%)	
IBA	NAA	Rooting (%)	No. of roots per shoot	Mean root length (cm)	Mean root diameter (mm)	Basal callusing (%)	4 wk	8 wk
0.5	0.1	92.2 ± 5.3 a[z]	8.6 ± 1.42 c[z]	1.54 ± 0.226 b[z]	0.62 ± 0.030 c[z]	16.7 ± 3.2 c[z]	91.7 ± 8.3 a[z]	61.1 ± 12.1 a[z]
0.5	0.2	94.4 ± 3.2 a	15.4 ± 0.38 a	1.78 ± 0.050 a	0.65 ± 0.044 c	19.4 ± 5.3 c	91.7 ± 0.0 a	55.6 ± 2.8 a
0.5	0.4	72.2 ± 10.7 b	10.6 ± 2.16 b	1.66 ± 0.138 ab	0.77 ± 0.046 b	52.8 ± 2.8 b	83.3 ± 4.8 b	58.3 ± 4.8 a
0.5	0.8	66.7 ± 10.1 b	11.2 ± 2.61 b	1.27 ± 0.067 c	1.13 ± 0.087 a	75.0 ± 5.3 a	66.7 ± 17.4 c	38.9 ± 20.0 b

[z] Means with the same letter(s) within the same column are not significantly different at the 5% level by LSD test.

30 min of sampling under ambient room conditions, as compared to closed stomata in leaves of AF2 + DP4 and *ex vitro*-acclimated plants. In general, leaves once excised from the plant are capable of triggering the ABA signal leading to stomata closure in order to reduce water loss from the leaf surface by evaporation (Taiz and Zeiger, 1998). In view of this, stomata in AF6 leaves appear to be less functional than those of AF2 + DP4 and *ex vitro*-acclimated leaves. Similar observations have also been observed in *in vitro* leaves of *D. insignis* and *D. caryophyllus* grown in airtight culture containers (Miguens et al., 1993). We speculate that the lower

TABLE 4

INFLUENCE OF CONTAINER CLOSURE TYPE ON CHARACTERISTICS OF LEAF SURFACES OF *IN VITRO*- AND *EX VITRO*-ACCLIMATED PLANTLETS OF *B. KAOI* AS REVEALED BY SCANNING ELECTRON MICROSCOPY

Characteristics of leaf surface[z]	Adaxial surface			Abaxial surface		
	Number mm^{-2}	Length (μm)	Width (μm)	Number mm^{-2}	Length (μm)	Width (μm)
Epidermal cells						
Ex vitro-acclimated plant	642 ± 30.0 c[y]	54.7 ± 0.74 a[y]	36.6 ± 1.44 a[y]	642 ± 87.0 c[y]	58.7 ± 2.12 a[y]	35.3 ± 1.64 a[y]
AF2 + DP4	950 ± 104.1 b	54.7 ± 0.43 a	24.8 ± 1.37 b	1783 ± 16.7 b	63.8 ± 1.60 a	34.4 ± 2.30 a
AF6	1832 ± 153.7 a	45.8 ± 0.47 b	26.9 ± 0.59 b	2720 ± 133.2 a	40.5 ± 1.43 b	19.6 ± 1.23 b
Stomatal aperture						
Ex vitro-acclimated plant	–[x]	–	–	133 ± 22.0 c	6.81 ± 0.77 b	1.68 ± 0.06 b
AF2 + DP4	125 ± 25.0 b	6.35 ± 0.05 b	1.45 ± 0.06 b	275 ± 38.2 b	7.63 ± 0.09 b	1.96 ± 0.14 b
AF6	253 ± 22.8 a	9.05 ± 0.25 a	2.91 ± 0.10 a	576 ± 64.0 a	9.05 ± 0.34 a	3.94 ± 0.25 a

[z] Leaf-blade about 25 mm^2 taken from third or fourth leaf from shoot tip in 6-wk-old *in vitro* AF6 and AF2 + DP4-treated plantlets and *ex vitro*-acclimated plants.

[y] Means with the same letter(s) are not significantly different at the 5% level by LSD test. Mean of 20 epidermal cells or 10 stomata with three replicates.

[x] Data were not collected.

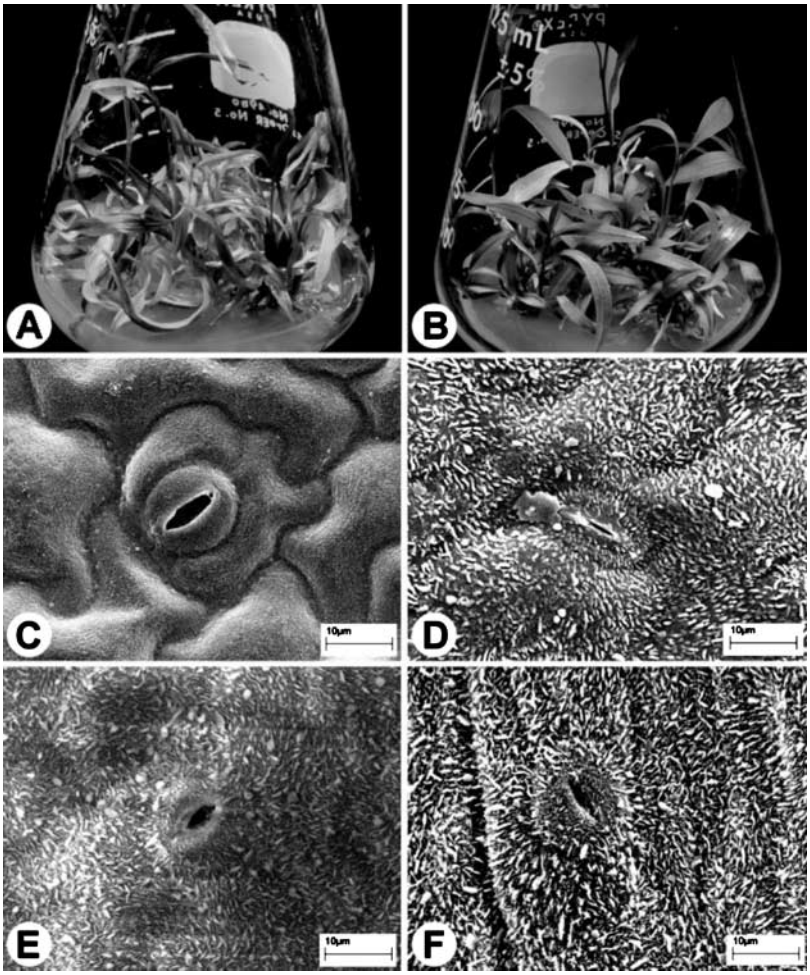

FIG. 2. *A*, *In vitro* plantlets with AF6 as ventilation closure; *B*, *in vitro* plantlets with AF2 + DP4 as ventilation closure; *C*, stomata in leaves under AF6 treatment; *D*, stomata in *ex vitro* acclimated leaves; *E*, *F*, *in vitro* AF2 + DP4 treated leaves (*bar* for $C-F = 10\,\mu m$). In *C–E*, samples were fixed in liquid nitrogen immediately after sampling; in *F*, after 30 min of sampling.

survival rate of AF6 plantlets in this study may be due to the lack of epicuticular wax, higher stomata density, larger size, and the presence of non-functional stomata in the leaves.

In conclusion, we report an improved procedure for *in vitro* multiplication and *ex vitro* acclimation *B. kaoi*. We found that a combination of dispense paper with aluminum foil as container closure improved the survival rate of *in vitro* as well as *ex vitro*-acclimated plants. SEM study revealed structural variation in leaf surfaces of *in vitro*- and *ex vitro*-acclimated plants and their relationship with hyperhydricity. No noticeable morphological abnormalities in tissue culture plants were observed even after 1 yr of growth under field conditions. Results of this study provide a practical method for improving the quality of *B. kaoi* micropropagation and may enhance commercial production and germplasm conservation of this endangered and medicinally important plant species.

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