

## Short Communication

## Adventitious shoot regeneration and micropropagation of the Japanese endangered *Hylotelephium sieboldii* (Sweet ex Hook.) *H. Ohba* and *H. sieboldii* var. *ettyuense* (Tomida) *H. Ohba*

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**Abstract** Due to indiscriminate collection, the natural habitat of *Hylotelephium sieboldii* and *H. sieboldii* var. *ettyuense* have been significantly reduced. For *ex situ* conservation and efficient vegetative propagation, a micropropagation system based on adventitious shoot regeneration was developed for these two endangered species. Leaves, stems and roots of *in vitro*-grown plantlets, and flower buds of greenhouse-grown plants were used as explants. For *H. sieboldii*, adventitious shoots were most efficiently regenerated from flower bud explants on a medium containing 1 mg l<sup>-1</sup> each of NAA and BA. Adventitious shoot regeneration from flower bud explants under this condition was also obtained in *H. sieboldii* var. *ettyuense*, but with lower efficiencies. Adventitious shoots of both species rooted and developed into plantlets on a medium containing 0.1 mg l<sup>-1</sup> IBA. Almost all of these plantlets were successfully transplanted to the greenhouse. At least at early stage of growth, they showed no apparent morphological alterations.

**Key words:** Adventitious shoots, *ex situ* conservation, *Hylotelephium*, micropropagation, ornamental plants.

The potential use of micropropagation in *ex situ* conservation of threatened plants has already been demonstrated for a number of species (Fay 1992; Malda et al. 1999; Nakano et al. 2004). Micropropagation allow the establishment of a large stock of plants within a short period of time from a minimum of original plant materials, thus imposing a minimum impact on the endangered native populations (Fay 1992).

The Crassulaceous perennial plants *Hylotelephium sieboldii* (Sweet ex Hook.) *H. Ohba*, generally called 'misebaya', and *H. sieboldii* var. *ettyuense* (Tomida) *H. Ohba*, generally called 'ettyu-misebaya', are found only in Shodoshima Isl., Kagawa Pref. (Yuasa 1969) and Toyama Pref. (Ohba 1981, 1992), respectively, Japan. They have beautiful foliage and flowers, and therefore, have been used widely for ornamental purposes in Japan. Due to indiscriminate collection with subsequent eradication of the natural populations, the natural habitat of these species has been increasingly reduced. *H. sieboldii* and *H. sieboldii* var. *ettyuense* are listed as endangered and critically endangered, respectively, in the Red Data list of Japan (Environment Agency of Japan 2000). In addition, although these species are generally propagated by division or cutting, propagation efficiency

by these methods is relatively low. Micropropagation techniques may be of advantage in *ex situ* conservation and efficient vegetative propagation of these species. To date, tissue culture studies have been reported for several Crassulaceous species such as *Bryophyllum calycinum* (Karpoff 1982), *Crassula ovata* (Frello et al. 2002), *Echeveria derenbergii* (Frello et al. 2002), *Kalanchoe* spp. (Smith and Nightingale 1979; Frello et al. 2002), *Sedum* spp. (Brandão and Salema 1977; Frello et al. 2002) and *Sempervivum tectorum* (Dobos et al. 1994). However, no reports on micropropagation of *H. sieboldii* and *H. sieboldii* var. *ettyuense* have yet been published. In the present study, we describe a multiplication protocol based on adventitious shoot regeneration of these two species.

Potted plants of one *Hylotelephium sieboldii* strain, 15061, and four *H. sieboldii* var. *ettyuense* strains, 31656, 31657, 31702 and 31703, were used in the present study. All the potted plants were initiated from wild materials by cuttings. They were cultivated in the greenhouse without heating and shading.

In preliminary experiments on these species, we attempted to induce adventitious shoot regeneration from leaves, stems and nodes of potted plants. However, these

Abbreviations: BA, 6-benzyladenine; IBA, indolebutylic acid; NAA,  $\alpha$ -naphthaleneacetic acid.

Table 1. Effects of NAA, BA and explant type on adventitious shoot regeneration in *Hylotelephium sieboldii* strain 15061.

NAA (mg l <sup>-1</sup> )	BA (mg l <sup>-1</sup> )	% of explants producing adventitious shoots				No. of adventitious shoots per explant which produced adventitious shoots			
		Leaf	Stem	Root	Flower bud	Leaf	Stem	Root	Flower bud
0	0	3.7 a	3.5 a	0 a	0 a	1.2 b	1.0 a	0 a	0 a
0	0.1	14.3 b	17.8 b	5.3 ab	0 a	1.6 b	1.1 a	1.2 b	1.2 b
0	1	31.3 cd	34.0 c	11.3 b	1.4 a	1.8 b	1.6 a	1.0 b	2.2 c
0.1	0	1.7 a	2.2 a	0 a	0.8 a	1.1 b	1.1 a	0 a	1.0 b
0.1	0.1	29.5 c	23.7 b	24.9 c	38.6 c	3.5 c	2.4 b	2.9 c	3.9 de
0.1	1	38.3 d	38.5 c	26.5 c	39.3 c	3.8 c	3.1 b	3.6 cd	4.4 e
1	0	0 a	0.5 a	0 a	4.0 a	0 a	1.0 a	0 a	1.2 b
1	0.1	15.8 b	22.3 b	12.6 b	25.9 b	1.7 b	2.5 b	1.2 b	3.5 d
1	1	34.2 cd	38.6 c	33.4 d	43.7 c	5.1 d	4.2 c	4.2 d	6.4 f

Leaf, stem and root explants were prepared from *in vitro*-grown plantlets 2 months after subculture, whereas flower bud explants were prepared from potted plants grown in the greenhouse. Data were recorded 3 months after culture initiation. Values represent the mean of 3 independent experiments each of which consisted of 10–15 explants. Means in the same column followed by the same letter are not significantly different at  $p=0.05$  with LSD test.

organs were severely damaged by surface-disinfecting treatments, and almost all of them were bleached and died even after treatment with 0.5% NaOCl for 3 min (data not shown). When lower concentrations of NaOCl and shorter treatment times were applied, microbial contamination could not be eliminated completely. Thus, for *H. sieboldii* and *H. sieboldii* var. *ettyuense*, leaves, stems and nodes of potted plants are unsuitable as explants. On the other hand, about 40% of apical segments and over 95% of flower buds dissected from potted plants survived after surface-disinfection with 0.5% NaOCl for 5 min. Therefore, leaves, stems and roots of *in vitro*-grown plantlets derived from apical segments of potted plants, and flower buds of potted plants were used as explants for adventitious shoot induction in the present study.

*In vitro*-grown plantlets derived from apical segments of potted plants were established and maintained as follows. Branches (2–5 cm in length) were harvested from potted plants and leaves were removed. Branches were then surface-disinfected with a 0.5% NaOCl solution containing 0.1% Tween20 for 5 min followed by 3 rinses with sterilized, distilled water. Apical segments (ca. 0.5 cm in length) were dissected from the branches and placed on half-strength MS medium (Murashige and Skoog 1962) containing 0.1 mg l<sup>-1</sup> IBA. All media used in the present study contained 30 g l<sup>-1</sup> sucrose and 2 g l<sup>-1</sup> gellan gum, and adjusted to pH 5.6–5.8 prior to autoclaving at 121°C for 15 min. Culture flasks (100 ml) were filled with 50 ml of the medium and 3 apical segments were cultured per flask. All cultures throughout this study were kept at 25°C under continuous illumination at 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with white fluorescent lamps. Apical segment-derived plantlets of 3–5 cm in height were established after 2 months and they could be maintained by transferring apical or lateral cuttings to fresh medium of the same composition every 3 months.

Explants for adventitious shoot induction were prepared as follows. Leaves (1–1.5 cm in length) were harvested from *in vitro*-grown plantlets 2 months after subculture and cut into pieces of ca. 0.5×0.5 cm. Stems and roots were also harvested from *in vitro*-grown plantlets and cut into segments of ca. 0.5 cm in length. Flower buds, 2–3 weeks before anthesis and 5–8 mm in length, were harvested with the pedicel from potted plants and surface-disinfected as described above for branches, and pedicels were removed from the flower buds. Leaf, stem, root and flower bud explants were placed on half-strength MS media without plant growth regulators, or containing NAA in combination with BA (Table 1). Plastic Petri dishes were filled with 30 ml of each medium and 10–15 explants were cultured per dish. Three months after culture initiation, the percentage of explants producing adventitious shoots and the number of adventitious shoots per explant which produced adventitious shoots were recorded. Experiments were replicated 3 times, and statistical analysis was carried out by analysis of variance using LSD test ( $p=0.05$ ).

Effects of NAA, BA and explant type on adventitious shoot regeneration were examined using *H. sieboldii* strain 15061 (Table 1). Within 2 weeks after culture initiation green and compact calli started to be produced mainly from the cut surface of explants, and adventitious shoots were produced from the calli 3–5 weeks later (Figure 1A). Although adventitious shoot regeneration was observed in all types of explants, both the percentage of explants producing adventitious shoots and the number of adventitious shoots per explant which produced shoots were higher in flower bud explants. For all types of explants, inclusion of both NAA and BA in regeneration media allowed efficient regeneration of adventitious shoots. A medium containing 1 mg l<sup>-1</sup> each of NAA and BA was most favorable for adventitious shoot regeneration of *H. sieboldii* strain 15061, on which 43.7% of flower bud explants produced shoots and 6.7

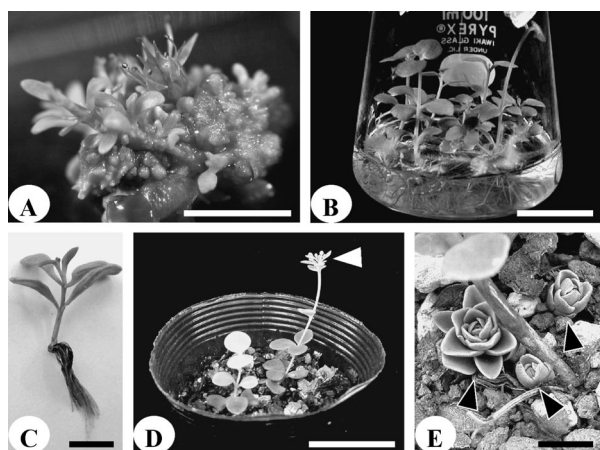


Figure 1. Adventitious shoot regeneration and micropropagation of *Hylotelephium sieboldii* strain 15061. (A) Adventitious shoots developed from a flower bud explant 2 months after culture initiation on a medium containing  $1 \text{ mg l}^{-1}$  each of NAA and BA. Bar=5 mm. (B) Plantlets obtained 2 months after transfer of flower bud-derived adventitious shoots to a medium containing  $0.1 \text{ mg l}^{-1}$  IBA. Bar=2 cm. (C) A plantlet with a well-established root system. Bar=1 cm. (D) A flower bud-derived small plant with flowers (arrowhead). Bar=3 cm. (E) Development of winter buds (arrowheads) at the base of a small plant. Bar=5 mm.

shoots were obtained per flower bud explant which produced shoots.

Application of flower bud culture using a medium containing  $1 \text{ mg l}^{-1}$  each of NAA and BA to *H. sieboldii* var. *ettyuense* resulted in adventitious shoot regeneration but with lower regeneration frequencies (18.4–27.9%) and numbers of shoots per flower bud explant which produced shoots (3.6–4.1) than the *H. sieboldii* (Table 2). Further studies should be directed to improve the regeneration efficiency in *H. sieboldii* var. *ettyuense*. These were no significant differences in the regeneration frequency and the numbers of shoots among 4 strains of *H. sieboldii* var. *ettyuense* collected from different places (Table 2), indicating that there is no significant variability in the ability to regenerate adventitious shoots among populations of *H. sieboldii* var. *ettyuense*.

In some experiments, adventitious shoot masses obtained after 3 months on a medium containing  $1 \text{ mg l}^{-1}$  each of NAA and BA were isolated from the explants, divided into 2 pieces after excising elongated shoots of over 10 mm in length, and transferred onto fresh medium of the same composition for shoot multiplication. Irrespective of the initial explant source and plant genotype, 2 to 3-fold increases in the number of shoots were obtained after 3 months. Although shoot masses could be subcultured every 3 months, the shoot multiplication rate decreased and the number of small shoots of below 5 mm in length increased after the third subculture (data not shown). In addition, repetition of *in vitro* multiplication cycle has generally been demonstrated to decrease the shoot quality and increase

Table 2. Comparison of adventitious shoot regeneration from flower bud explants among *Hylotelephium sieboldii* and *H. sieboldii* var. *ettyuense* strains.

Species and strains	% of explants producing adventitious shoots	No. of adventitious shoots*
<i>H. sieboldii</i>		
15061	43.7 b	6.4 b
<i>H. sieboldii</i> var. <i>ettyuense</i>		
31656	18.4 a	3.6 a
31657	20.5 a	3.8 a
31702	27.9 a	4.1 a
31703	23.8 a	3.8 a

Flower bud explants were prepared from potted plants grown in the greenhouse. Data were recorded 3 months after culture initiation on a medium containing  $1 \text{ mg l}^{-1}$  each of NAA and BA. Values represent the mean of 3 independent experiments each of which consisted of 10–15 explants. Means in the same column followed by the same letter are not significantly different at  $p=0.05$  with LSD test.

\*Number of adventitious shoots per explant which produced adventitious shoots.

phenotypic variation in regenerants (Westerhof et al. 1984; Nakano et al. 1999).

Adventitious shoots of over 10 mm in length were transferred for rooting onto half-strength MS medium containing  $0.1 \text{ mg l}^{-1}$  IBA. In our preliminary experiments, this medium was favorable for root induction of adventitious shoots of *H. sieboldii* strain 15061 (data not shown). Culture flasks (100 ml) were filled with 50 ml of the medium and 3–5 shoots were cultured per flask. Irrespective of initial explant source and plant genotype, almost all of the shoots started to develop roots within one month and developed into plantlets, 3–5 cm in height and with a well-established root system, one month later (Figure 1B, C). Shoots from shoot masses after successive subculture did not elongate and develop roots very well. Plantlets with a well-established root system were then washed carefully with tap water to remove gellan gum, transferred to pots containing vermiculite, and acclimatized in a transparent plastic cabinet covered with a polyethylene sheet. Over 95% of the plantlets successfully acclimatized within 2 weeks. Acclimatized plants were then transplanted to pots containing soil and cultivated in the greenhouse under the same conditions as the mother plants. Initial growth of adventitious shoot-derived plants was very slow as plants vegetatively propagated by cutting. When plantlets were acclimatized before August, small plants produced flowers in October (Figure 1D). These plants also produced winter buds in December (Figure 1E) and survived the winter. Although no apparent morphological alterations were observed in these small plants, a detailed characterization is necessary when they grow into large plants (2–3 years later).

In the present study, a micropropagation system based on adventitious shoot regeneration was developed for the Japanese endangered *H. sieboldii* and *H. sieboldii*

var. *ettyuense*. This system consists of the following steps: (1) flower bud explants prepared from potted plants, or leaf, stem or root explants prepared from *in vitro*-grown plantlets were cultured on half-strength MS medium containing  $1 \text{ mg l}^{-1}$  each of NAA and BA for 2–3 months; (2) for shoot multiplication, adventitious shoot masses were subcultured onto fresh medium of the same composition and cultured for 2–3 months; (3) adventitious shoots elongating over 10 mm in length were transferred for rooting onto half-strength MS medium containing  $0.1 \text{ mg l}^{-1}$  IBA and cultured for 2 months; and (4) plantlets thus obtained were subjected to acclimatization for 2 weeks and transplanted to the greenhouse. Among 4 types of explants, flower buds is most appropriate since they had higher potential to produce adventitious shoots in *H. sieboldii*, which was also confirmed in *H. sieboldii* var. *ettyuense* (data not shown), and in addition, flower bud explants can be prepared after horticultural evaluation of the mother plants with minimal damage as suggested by Nakano et al. (2004). However, use of flower bud explants is restricted in the flowering season (October). On the other hand, leaves, stems and roots of *in vitro*-grown plantlets can be used as explants throughout the year once *in vitro*-grown plantlets are established, although efficiencies of adventitious shoot regeneration of these explants were slightly lower than flower bud explants.

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### References

- Brandão I, Salema R (1977) Callus and plantlets development from cultured leaf explants of *Sedum telephium* L. *Z. Pflanzenphysiol* 85: 1–8
- Dobos E, Dános B, László-Bencsik Á (1994) Callus induction and shoot regeneration in *Sempervivum tectorum*. *Plant Cell Tiss Org Cult* 36: 141–143
- Environment Agency of Japan (2000) Threatened Wildlife of Japan—Red Data Book 2nd ed., Volume 8, Vascular Plants, Japan Wildlife Research Center, Japan
- Fay MF (1992) Conservation of rare and endangered plants using *in vitro* methods. *In Vitro Cell Dev Biol-Plant* 28: 1–4
- Frello S, Venerus E, Serek M (2002) Regeneration of various species of *Crassulaceae*, with special reference to *Kalanchoe*. *J Hort Sci Biotechnol* 77: 204–208
- Karpoff AJ (1982) Hormones and early *in vitro* development of epiphyllous propagules on *Bryophyllum calycinum*. *Am J Bot* 69: 348–355
- Malda G, Suzán H, Backhaus R (1999) *In vitro* culture as a potential method for the conservation of endangered plants possessing crassulacean acid metabolism. *Sci Hort* 81: 71–87
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Nakano M, Mizunashi K, Tanaka S, Godo T, Nakata M, Saito H (2004) Somatic embryogenesis and plant regeneration from callus cultures of several species in the genus *Tricyrtis*. *In Vitro Cell Dev Biol-Plant* 40: 274–278
- Nakano M, Niimi Y, Kobayashi D, Watanabe A (1999) Adventitious shoot regeneration and micropropagation of hybrid tuberous begonia (*Begonia* × *tuberhybrida* Voss). *Sci Hort* 79: 245–251
- Ohba H (1981) Nomenclatural changes and notes on Japanese Sedoideae. *J Jpn Bot* 56: 181–187
- Ohba H (1992) Notulae Crassulacearum Asiae Orientalis (1). *J Jpn Bot* 67: 194–200
- Smith RH, Nightingale AE (1979) *In vitro* propagation of *Kalanchoe*. *Hort Sci* 14: 20
- Yuasa H (1969) *Sedum sieboldii* and its relative species (4). The native locality—a visit to Shodoshima Island. *News to Plant Collecting* 44: 59–60 (in Japanese)
- Westerhof J, Hakkaart FA, Versluijs JMA (1984) Variation in two *Begonia* × *hiemalis* clones after *in vitro* propagation. *Sci Hort* 24: 67–74