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Propagation of *Sedum spectabile* Boreau in Leaf Culture *in Vitro*

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Abstract

An efficient protocol was established for *Sedum spectabile* Boreau propagation. Various leaf parts were used as explants to regenerate plantlets, the stem segments of which were cultured for shoot proliferation and plantlet multiplication. The results showed that the leaf base was the optimal explant, as compared to both the middle and the top of leaves, for shoot formation. The highest shoot induction of 88.9% was observed on MS medium supplemented with 0.6 mg/l TDZ and 0.1 mg/l NAA. Hyperhydric leaves obtained in primary culture developed first into abnormal somatic embryos 10 days after subculture, and then into hyperhydric plantlets after an additional 10 days. The hyperhydric plantlets reversed to normal plantlets when plant growth regulators were removed from culture medium. Further, stem segments from reversed plantlets were used for shoot regeneration and root induction. Optimal shoot regeneration was obtained in MS medium containing 0.6 mg/l TDZ with 0.1 mg/l NAA. Root induction and root mean number were all higher on auxin-free medium than on medium containing auxins.

Keywords: hyperhydricity, leaf, reversion, tissue culture

Introduction

Sedum spectabile Boreau belongs to the *Crassulaceae* family and is widely distributed throughout the warm and temperate zones. It is an herbaceous perennial plant and has strong tolerance to drought (Rabas and Martin, 2003), low temperatures (Iles *et al*., 1993; Iles and Agnew, 1995), and shade (Jiang *et al*., 2007), all characteristics of wide adaptability and easy manageability. Cultivars of *Sedum spectabile* Boreau have showy white, pink, or red flowers, highlighting it as a fairly ornamental plant. It has also been reported to remedy polluted soil by Hg or Co (Wang *et al*., 2008).

Sedum spectabile Boreau is often propagated by either cutting or dividing its roots. These conventional methods, however, are slow and require a large number of parent propagules. It is thus important to study *Sedum spectabile* Boreau propagation to ultimately apply it to various uses, such as garden sets and medicinal cultivation. The *in vitro* culture system stands as a candidate for overcoming any prior deficiencies. Researchers have investigated *in vitro* cultures of *Sedum spectabile* Boreau using stem segments with terminal buds (Ren and Dong, 2006; Xing *et al*., 2010), intact leaves (Zhang and Cheng, 2007), and petals (Wojciechowicz, 2009) as explants. No further reports on morphogenesis and somatic embryo induction of leaf parts have been reported. In the study, different leaf parts were used as explants to investigate the morphogenesis process. Hyperhydric plantlets were found to reverse to normal, and the stems of these reversed plantlets were used to regenerate shoots and roots for propagation. The effects of varying concentrations of plant growth regulators (PGRs) on the differentiation of leaves, hyperhydric shoots, and stem segments were analyzed to ultimately establish an efficient protocol for the propagation of *Sedum spectabile* Boreau.

Materials and methods

Plant materials

Leaves of *Sedum spectabile* Boreau cultivar 'Pink' were used in this study. Leaves were first washed in running tap water for 30 minutes, sterilized by 70% ethanol for 10 seconds followed by 0.1% mercuric chloride for 8 minutes, and finally washed five times in sterilized distilled water. Leaves were cut into three parts: leaf top, leaf middle, and leaf base (Fig. 1 A, B), and cultured on primary culture media.

Primary culture

Treated leaves were inoculated on Murashige and Skoog (1962) (MS) medium supplemented with different concentrations of benzyladenine (BA; 0.5, 1.0, 2.0 mg/l) or Thidiazuron (TDZ; 0.2, 0.4, 0.6 mg/l) with 0.1 mg/l a-Naphthylacetic acid (NAA). MS medium without plant growth regulators (PGRs) was used as a control.

Subculture-induction of hyperhydric leaves and reversion of hyperhydric plantlets

Hyperhydric leaves obtained from primary culture were inoculated on MS medium supplemented with 1.0 mg/l BA or 0.4 mg/l TDZ with 0.1 mg/l NAA, and the effects of PGRs on their morphogenesis was discerned. MS medium without PGRs was used as a control.

Hyperhydric plantlets induced from hyperhydric leaves were cultured on PGR-free MS medium and MS medium containing 0.4 mg/l TDZ and 0.1 mg/l NAA to investigate factors affecting reversion.

Shoot development and root induction of stem segments from recovered plantlets

Stems of recovered plantlets were cut into 2-3 cm segments with three nodes and inoculated on MS medium supplemented with different concentrations of TDZ (0.2, 0.4, 0.6 mg/l) with 0.1 mg/l NAA to regenerate shoots. The same stem segments were cultured on MS medium supplemented with NAA (0.1, 0.5, 2.5 mg/l) or indolebutyric acid (IBA; 0.1, 0.5, 2.5 mg/l) to induce roots. MS medium without PGRs was used as a control.

Acclimatization

The rooted plantlets were potted in a mixture of humus and perlite (2:1, v:v) and placed in a greenhouse exposed to a natural light/dark circle of sunlight at 24±2°C in 60% relative humidity.

Culture condition

The above culture media contained 30.0 g/l sugar, 6.0 g/l agar, and was adjusted to pH 5.8 before autoclaving at a pressure of 105 kPa for 20 minutes at 121°C. All cultures were incubated under a 16 h photoperiod at 40 μmol m^{-2} s^{-1} at 23±2°C in 70-80% relative humidity.

Statistical analysis

Each treatment consisted of 42 inoculated explants and was performed in triplicate when determining hyperhydric plantlet reversion. Five replicates were used per treatment with the 15 explants in the other experiments. All data was recorded after 4 weeks of culture and was analyzed for variance. Additionally, treatment means were compared using Duncan's multiple range test (Duncan, 1995) at 5% significance.

Results and discussion

The morphogenesis of leaves in the primary culture

All leaves expanded a week after inoculation. Three types of organogenesis after swollenness were observed, Type I extended at twice to three times over original volume and showed hyperhydricity (Fig. 1 CI). Moreover, abnormal somatic embryo regenerated from these leaves and developed into hyperhydric plantlets (Fig. 1 E, F, G). Comparatively, type II samples were less swollen and normal shoots were initiated from the cut surface of the lower side of these leaves (Fig. 1 CII, D). Although type III sam-

Tab. 1. Leaf Morphogenesis 4 weeks after inoculation

Explant type	BA concentration (mg/l)	TDZ concentration (mg/l)	Embryo induction rate (%)	Shoot induction rate (%)	Mean shoot number
Leaf top	0	0	0 f	0 j	0 g
	0.5	0	0 f	0 j	0 g
	1.0	0	0 f	0 j	0 g
	2.0	0	14.3 b	14.3 h	0.1 f
	0	0.2	0 f	33.3 e	0.7 c
	0	0.4	0 f	50.0 c	0.3 e
	0	0.6	8.3 e	25.0 f	0.6 cd
Leaf middle	0	0	0 f	0 j	0 g
	0.5	0	11.1 d	0 j	0 g
	1.0	0	50.0 a	0 j	0 g
	2.0	0	12.5 c	0 j	0 g
	0	0.2	0 f	60.0 b	2.4 a
	0	0.4	8.3 e	25.0 f	0.4 de
	0	0.6	0 f	50.0 c	0.5 cde
Leaf base	0	0	0 f	0 j	0 g
	0.5	0	0 f	0 j	0 g
	1.0	0	11.1 d	11.1 i	0.1 f
	2.0	0	0 f	0 j	0 g
	0	0.2	0 f	44.4 d	0.6 cd
	0	0.4	11.1 d	22.2 g	0.7 c
	0	0.6	11.1 d	88.9 a	1.7 b

Note: Different letters in the same line denote significant differences (Duncan's test, $p<0.05$)

ples were also less swollen, there existed no visible changes after a month (Fig. 1 CIII).

Small structures emerged from the edges of hyperhydric leaves (Type I) 3 weeks after inoculation. These structures became scattered and independent after 4 weeks and finally developed into abnormal, hyperhydric somatic embryos (Fig. 1 E). Embryo induction of the leaf middle reached the highest rate of 50.0% on medium containing 1.0 mg/l BA. The highest embryo induction rates of leaf top and leaf base were 14.3% and 11.1%, respectively (Tab. 1). Enlarged leaves and induced somatic embryos, however, were all hyperhydric (Fig. 1 E, F). Hyperhydricity is also known as 'vitrification' in fact, Franck *et al*. (2004) characterized hyperhydric materials by their translucent aspect from chlorophyll deficiency, undeveloped cell wall, and high water content. Moreover, Rojas-Martínez *et al*. (2010) defined the hyperhydricity phenomenon as a physiological disorder in tissue-cultured plant material, resulting from waterlogging and apoplast stresses. A reason for hyperhydricity may be due to the different physiological explant statuses. The physiological states of leaves were different at various attachment regions on the same plantlet. Leaves from differing nodes showed varied morphology in identical medium, with only some being hyperhydric. Another potential reason is a high PGR concentration. Kadota and Niimi (2003) reported that TDZ and CPPU caused greater hyperhydricity than BA when culturing pear cultivar *in vitro*, but BA caused higher leaf hyperhydricity than TDZ in the present study.

Shoots were induced on type II leaves. Medium containing 0.2-0.6 mg/l TDZ and 0.1 mg/l NAA was more effective in multiple shoot inductions than that containing 0.5-2 mg/l BA and 0.1 mg/l NAA. The highest rates of shoot induction from leaf top, leaf middle, and leaf base

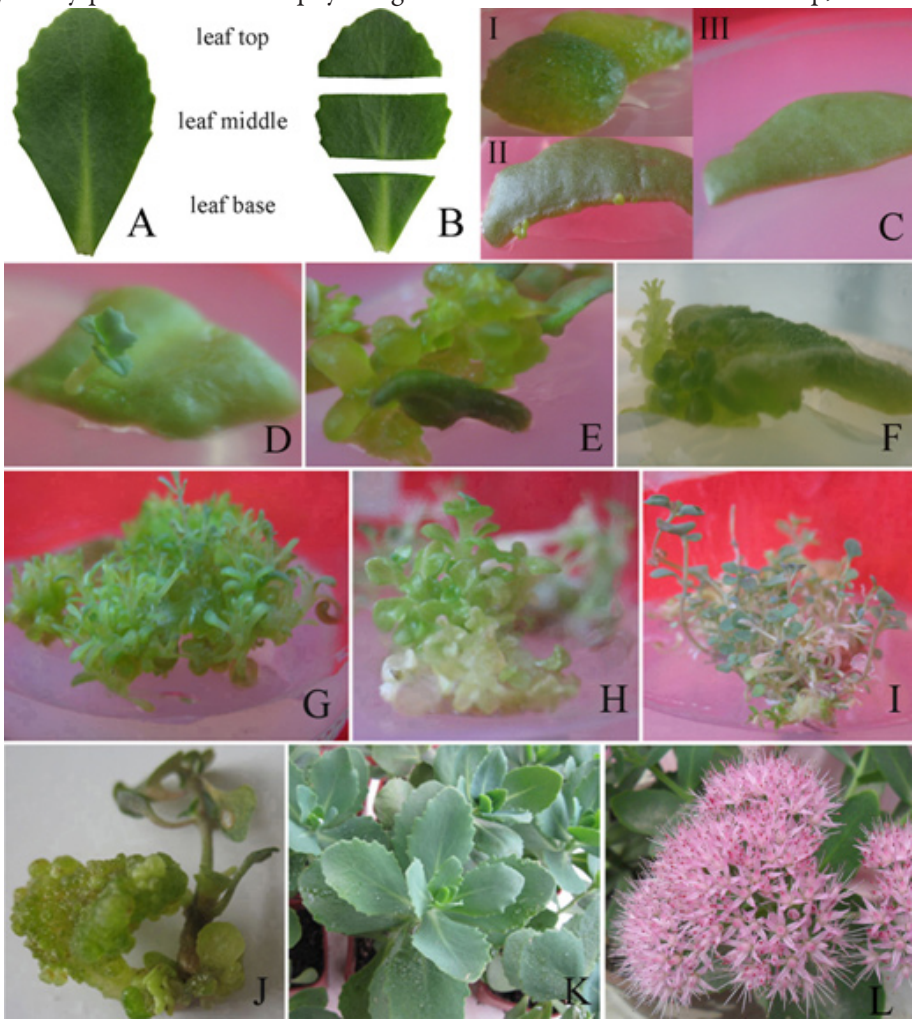

Fig. 1. Leaf explant preparation, regeneration, and micropropagation of *Sedum spectabile* Boreau. (A) and (B) Leaf explants preparation; (C) I, II, III. Three types of organogenesis of one leaf: type I (leaf base), type II (leaf top) and type III (leaf top), respectively; (D) Shoot regeneration of leaf base explants in culture after 3 weeks; (E) Abnormal somatic embryos from leaf explants after 4 weeks in culture; (F) Organogenesis of hyperhydric leaves after 2 weeks of subculture; (G) Hyperhydric plantlets from hyperhydric somatic embryos; (H) Intermediate type of hyperhydric and normal plantlets; (I) Normal plantlets from hyperhydric plantlets after 4 weeks of culture on PGR-free MS medium; (J) Organogenesis of stem segments from reversed plantlets on MS medium supplemented with 0.6 mg/l TDZ and 0.1 mg/l NAA; (K) Acclimatization of leaf-derived plantlets; (L) Flowering of leaf-derived plantlets

were detected on medium containing 0.4 mg/l, 0.2 mg/l, and 0.6 mg/l TDZ, respectively. Leaf base had the highest induction rate of 88.9% compared to the other leaf parts. Additionally, it was observed that all shoots grew from the morphologic leaf bottoms (Fig. 1 CII, D), indicating the existence of leaf polarity under certain induction conditions.

Effects of PGRs on the morphogenesis of hyperhydric leaves and plantlets

Since the embryo induction rate of leaf middle and leaf base on media containing 1.0 mg/l BA or 0.4 mg/l TDZ with 0.1 mg/l NAA was high in primary culture, this culture medium was selected to subculture hyperhydric leaves. The first abnormal somatic embryogenesis was observed 10 days after subculture in medium containing 1.0 mg/l BA, which then began to differentiate after 20 days. No significant difference between embryo induction and differentiation rate was observed in medium containing 0.4 mg/l TDZ and 1.0 mg/l BA, whereas significant difference was observed when this was compared to the control for induction (Tab. 2). Although abnormal somatic embryos developed into plantlets after 20 days of subculture, plantlets showed a lower differentiation level without roots and were hyperhydric (Fig. 1 G). These monstrous plantlets should be reversed into normal plantlets before being transplanted for garden sets.

After inoculation on culture medium with PGRs for 4 weeks, plantlets still remained in a state of hyperhydricity, not producing roots. Hyperhydric plantlets, however, began to reverse, and slender leaves turned normal and obvated, and started to root on PGR-free medium after 3 weeks (Fig. 1 H, I). The reversion rate of hyperhydricity was 89.2%, and rooting rate achieved was 82.3%. PGRs were the main factors affecting hyperhydricity of *Sedum spectabile* Boreau, possibly through the concentration of cytokinins. Kevers *et al*. (1984) reported that excess of either cytokinins or NH_4^+ ions indirectly led to cellulose and lignin deficiencies, allowing increased water uptake from reduced wall pressure, bringing about hyperhydric malformations. Recently, Yu *et al*. (2011) argued that zeatin was the most important factor for hyperhydricity, followed by sucrose concentration, $AgNO_3$, and indoleacetic acid. Additionally, other studies also showed that cytokinins caused hyperhydricity in vitro (Debergh *et al.,* 1981; Debergh and Maene 1984; Pagues *et al*., 1987). In the pre study, the hyperhydric phenomenon occurred in almost all culture media containing high concentrations of cytokinins.

In addition to PGRs, agar concentration (6, 7 and 8 g/l) and pH value (5.8, 6.4 and 7.0) were investigated for hyperhydric plantlet reversion (data not shown). These two factors affected water content, chlorophyll, and anthocyanin synthesis, and ultimately, could not induce normal plantlets from hyperhydric ones. Previous studies indicated that increased agarose concentration could reduce hyperhydricity rate (Borman and Vogelmann, 1984; Debergh, 1983), yet this result could not be repeated in the present study, most likely due to differences in plant materials.

Humidity (Curtis and Shetty, 1996), subculture times (Tsay, 1998), and soft culture media all (Kevers *et al*., 2004) could influence hyperhydricity. Saher *et al*. (2005a, 2005b) studied a mechanism of hyperhydricity, discovering that different pectin methyl esterase (PME) activities influence structural changes related to hyperhydricity in micropropagated carnation plants. Hyperhydric leaves of carnations adapted to hypoxia stress conditions through both oxidative pentose phosphate and fermentative pathway induction. It remains difficult to clarify physiological causes of hyperhydricity, mainly due to the multiplicity of factors involved in this process.

Regeneration of stem segments from plantlets

Shoot regeneration rate on culture media with PGRs was higher than on media without PGRs (Tab. 3). Moreover, the average number of shoots increased with higher TDZ concentration. The optimal effect of shoot induc-

Tab. 2. Subculture induction of hyperhydric leaves

Concentration combination of cytokinin and auxin (mg/l)	Embryo induction rate (%)	Mean differentiation rate (%)	Growth state
0	0 b	0 b	Hyperhydric leaves began to lose green, and showed yellow, brown; died after inoculation.
BA 1.0 + NAA 0.1	70.0 a	66.7 a	Some remained green and exhibited no change; others produced abnormal somatic embryos that soon differentiated into hyperhydric plantlets with slender and fleshy leaves, thick and fleshy stems, and no roots.
TDZ 0.4 + NAA 0.1	69.2 a	65.5 a	Some remained green and exhibited no change; others produced abnormal somatic embryos that soon differentiated into hyperhydric plantlets with slender and fleshy leaves, thick and fleshy stems, and no roots.

Note: Different letters in the same line denote significant differences (Duncan's test, $p<0.05$)

Tab. 3. Effects of different PGRs on induction of shoots

TDZ concentrations (mg/l)	NAA concentrations (mg/l)	Shoot regeneration rate (%)	Mean shoot number	Callus induction rate (%)
0	0	61.1 c	1.9 d	0 d
0.2	0.1	100.0 a	2.5 c	27.8 b
0.4	0.1	91.7 b	3.3 b	58.3 a
0.6	0.1	100.0 a	3.8 a	22.2 c

Note: Different letters in the same line denote significant differences (Duncan's test, $p<0.05$)

tion was obtained on culture medium containing 0.6 mg/l TDZ with 0.1 mg/l NAA. Most induced shoots were axillary (Fig. 1 J). Embryonic callus was induced 3 weeks after inoculation from the base of stem segments on culture media containing PGRs (Fig. 1 J). The highest callus induction rate was detected on culture medium containing 0.4 mg/l TDZ with 0.1 mg/l NAA. The obtained callus had strong regeneration ability (data not shown) and is a valid propagation material. Ren and Dong (2006) reported that a single bud developed into axillary buds on MS medium with 0.1 mg/l BA and 0.1 mg/l NAA, and finally into plexus buds. The callus was first shaped from the stem base, and then differentiated into shoots, similar to the present results.

The rooting results show that root induction rate and mean number were higher on auxin-free medium than on medium containing auxins. IBA was superior to NAA for rooting among treatments supplemented with auxins (Tab. 4). Rooted plantlets hardened after 4 weeks with a 100% survival rate. Healthy plants were obtained with normal flowers into the next year (Fig. 1 K, L).

Tab. 4. Effects of different PGRs on root induction

NAA concentrations (mg/l)	IBA concentrations mg/l)	Root induction rate %)	Mean root number
0	0	100.0 a	18.5 a
0.1	0	55.6 c	9.3 c
0.5	0	94.4 b	9.0 c
2.5	0	94.4 b	13.5 c
0	0.1	100.0 a	17.0 a
0	0.5	100.0 a	13.3 b
0	2.5	100.0 a	11.9 b

Note: Different letters in the same line denote significant differences (Duncan's test, $p<0.05$)

Conclusions

Hyperhydric leaves obtained in primary culture developed into somatic embryos, and then formed hyperhydric plantlets after subculture. Hyperhydric plantlets reversed and turned to normal plantlets by removing PGRs from culture media. The study showed that plant growth regulators potently affected hyperhydricity of *Sedum spectabile* Boreau. Additionally, the number of normal plantlets obtained from hyperhydric leaves was much more than the amount of shoots induced directly from normal leaves. Recovered plantlets flowered normally. With these results, a successful and effective pathway for *Sedum spectabile* Boreau propogation was concluded.

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