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SHORT COMMUNICATION

Somatic Embryogenesis and in vitro Flowering in *Saposhnikovia divaricata*

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Abstract Efficient somatic embryogenesis (SE) and in vitro flowering and fruiting were achieved in *Saposhnikovia divaricata* (Turcz.) Schischk. Friable embryogenic callus developed from the root, internode, and leaf explants on Murashige and Skoog medium (MS) with 2.26 μM 2,4-dichlorophenoxyacetic acid (2,4-D), and subsequently developed into somatic embryos on MS medium containing 4–5% sucrose, 1.74 μM naphthaleneacetic acid (NAA), 4.44 μM 6-benzylaminopurine (BA), and 1.90 μM abscisic acid (ABA). Then the mature embryos were separated and transferred onto MS with 3% sucrose and 0.6% agar for further development and conversion to plantlets. In vitro flowering and fruiting were obtained when the subcultures were carried out for over 15 months. Paclobutrazol (PP333) or ethephon (ETH) at low levels promoted flowering significantly. Also, abnormal rootless somatic embryos of *S. divaricata* could form flowers and fruits in vitro.

Keywords *Saposhnikovia divaricata* (Turcz.) Schischk. · Somatic embryogenesis · In vitro flowering · In vitro fruiting · Growth retardants · Abnormal somatic embryo

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Introduction

Saposhnikovia divaricata (Turcz.) Schischk. is a perennial herb belonging to the family Umbelliferae. As one of the most valuable medicinal plants in China, it is widely utilized in the treatment of skin allergies, arthritis, asthma, and tetanus (Wu 1992). However, the natural sources of *S. divaricata* have been seriously damaged because of environmental destruction and the destructive collection of the roots in recent decades. It is critical to establish an efficient regeneration system to sufficiently multiply the herb, and to this end a reproducible somatic embryogenesis (SE) system has been successfully achieved in our lab (Ma and others 2005).

After monthly subcultures over 15 months, we observed that some plantlets could flower and fruit in vitro at the height of 2–5 cm, whereas natural plants flower at the height of 30–80 cm after 3 years. Although in vitro flowering has been reported in many plant species, for example, *Bamboo* (Joshi and Nadgauda 1997), *Boronia megastigma* (Roberts and others 1993), *Arachis hypogeae* (Narasimhulu and Reddy 1984), and *Vigna mungo* (Ignacimuthu and others 1997), there has been no report so far on in vitro flowering in *S. divaricata* via somatic embryogenesis (SE). Furthermore, only 10% of in vitro flowering plantlets were obtained casually and the flowering ability could not be regained under the used conditions. In these experiments, the cultures were kept in closed jars wrapped with a plastic lid that had low gas permeability. Thus, in such a closed system ethylene gas could have accumulated (Buer and others 2003). The effect of ethylene on in vitro flowering was therefore tested in further experiments. In addition, the effects of the growth retardant paclobutrazol were also studied. Here we report on an optimal regeneration system based on our former studies (Ma and others 2005) and new findings on in vitro flowering in *S. divaricata*.

Materials and Methods

In vitro Germination of Seeds

Tissue cultures were started from seeds of *S. divaricata* obtained from China Shen-nong Seed Corporation in August 2004. After several washes in running tap water, the seeds were surface sterilized in 70% ethanol for 20 s, followed by 0.1% mercuric chloride for 6–8 min, and washed five times with sterile distilled water. Subsequently, they were transferred onto Murashige and Skoog (MS) medium with 3% sucrose and 0.6% agar. After 4 weeks, the seedlings (2–4 cm in length) were removed from the culture jars, and 0.5 cm segments of the root, internode, and leaf were used as explants for callus production.

Callus Induction from Different Explants and Maintenance

For induction of embryogenic callus, the explants were inoculated onto MS medium containing 3% sucrose, 0.6% agar, and different levels of auxin and cytokinin (Table1). Two weeks later, friable, light yellow, nodular calluses appeared. There were 30–40 replicates per treatment in each experiment. To optimize the media for regeneration, different maintenance media for long-term culture of the embryogenic calluses were tested, and MS medium containing 3% sucrose, 0.6% agar, and 2.69 µM naphthaleneacetic acid (NAA) was found to be the optimal maintenance medium to control the appearance of abnormal embryos (Ma and others 2005).

Somatic Embryo Initiation and Proliferation from Callus

Somatic embryos were induced from friable and nodular calluses (40–50 mg) on MS with 4–5% sucrose, 0.6% agar, and different concentrations of auxin, cytokinin, and abscsic acid (ABA) as the differentiation medium for the embryos (Table 2). There were 50–60 replicates per treatment in each experiment.

Maturation, Conversion, and Acclimatization of Somatic Embryos

When cotyledons appeared on the somatic embryos, they were separated and transferred onto MS with 3% sucrose and 0.6% agar for maturation and conversion. Converted plantlets were removed from the culture jars and transferred to pots containing soil and sand (1:1) for 2–3 weeks at room temperature. Finally they were planted in soil outdoors.

Long-term Subculture and Promotion of Flowering

Calluses were subcultured on maintenance medium every 4-week period; the subcultured calluses could develop into somatic embryos at any time according to production needs. After subculturing for 15 months, 10% in vitro flowering plantlets were obtained and some of them could develop into fruit.

To improve in vitro flowering, paclobutrazol (PP333) or ethephon (ETH) in different concentrations was added to the maturation medium after sterilization by filtration through membrane filters (0.2 µm). The capless culture jars were placed in a sterile chamber as an open system (Southeast PGX-260D) to investigate the effect of ETH, and the calluses were transferred onto the same medium every 2–3-week period to prevent drying of the medium in the open jars. The control was MS medium containing 3% sucrose and 0.6% agar. There were 50–60 replicates per treatment in each experiment.

Culture Conditions, Observations, and Statistical Analysis

In all cases the pH of the medium was adjusted to 5.8 before addition of agar and sterilization at 121°C for 18–20 min. All the explants were cultured on cooled nutrient

Table 1 Effects of different PGRs on the induction of embryonic callus from root, leaf, and internode

PGRs treatments (µM)	Callus induction from root (%)	Callus induction from internode (%)	Callus induction from leaf (%)	Callus appearance
2.69 NAA + 4.44 BA	100 a	91 b	97 a	Pale green, friable
2.26 2,4-D	100 a	100 a	100 a	Light yellow, friable
4.52 2,4-D	90 b	90 b	91 b	Yellow, friable
9.05 2,4-D	67 c	70 c	73 c	Brown, friable
4.44 BA	0 d	0 d	0 d	No callus
2.69 NAA	84 b	88 b	86 b	Light yellow, friable

Basal medium is MS medium. Each value represents the mean of 30–40 replications. Different letters within columns indicate significant differences ($P < 0.05$, LSD)

Table 2 Effects of different PGRs on the induction of somatic embryos, frequency of abnormal somatic embryos, growth rate, and development capacity

PGRs treatments (μM)	No. of somatic embryos per 100 mg callus	Frequency of abnormal somatic embryo (%)	Growth rate[a]	Development capacity
2.26 2,4-D	28 c	49.6 e	+	Easy
2.69 NAA	25 c	26.3 d	+	Easy
4.44 BA	10 e	49.9 e	+	Uneasy
4.65 KIN	23 c	47.6 e	+ +	Uneasy
2.26 2,4-D + 4.44 BA	25 c	52.1 e	+ +	Uneasy
2.69 NAA + 4.44 BA	29 c	29.8 d	+ +	Uneasy
2.69 NAA + 8.88 BA	18 d	69.2 f	+ +	Uneasy
1.74 NAA + 4.44 BA	54 a	19.6 c	+ +	Uneasy
1.74 NAA + 2.22 BA	31 c	32.1 d	+ + +	Easy
1.74 NAA + 4.65 KIN	38 b	13.4 b	+ +	Easy
1.74 NAA + 4.44 BA + 0.76 ABA	50 a	18.7 c	+ + +	Easy
1.74 NAA + 4.44 BA + 1.90 ABA	48 a	10.1 a	+ + +	Easy
1.74 NAA + 4.44 BA + 3.80 ABA	25 c	18.3 c	+ +	Easy
1.74 NAA + 4.65 KIN + 1.90 ABA	34 b	9.8 a	+	Easy

Basal medium is MS medium. Each value represents the mean of 50–60 replications. Different letters within columns indicate significant differences ($P < 0.05$, LSD)

[a] + indicates the status of growth rate; more than one + indicates a better growth rate

media under complete aseptic conditions in the sterilized laminar airflow hood. The culture jars were placed in an air-conditioned culture room at 25 ± 2°C with a 14-h/10-h photoperiod with cool-white fluorescent lamps providing 80 μmol m^{-2} s^{-1} fluorescent light.

All procedures were observed and recorded every 2 days but the observations of the flowers and fruits were recorded daily. Photos were taken with a Sony F717 camera. All experimental data were statistically analyzed by one-way analysis of variance (ANOVA) using the protected least-significant-difference (LSD) test ($P < 0.05$) to compare treatment means followed by the LSD multiple-comparison test.

Results and Discussion

Somatic Embryogenesis (SE)

An optimized regeneration system for long-term subculture and continuous high frequency of SE and plantlet production in *S. divaricata* was developed. On the induction medium containing 2.26 μM 2,4-D, embryogenic callus was successfully induced from the root, internode, and leaf explants (Table 1). The texture and color of the callus depended on the concentrations of auxin. A low concentration of auxin (range = 2.26–4.52 μM) made the calluses friable, light yellow, and nodular, and they could develop into somatic embryos (Fig. 1a). On the medium with 2.69 μM NAA and 4.44 μM BA, the callus turned green, was fast-growing, and harder; such calluses always developed into buds. When the embryogenic callus was transferred to MS supplemented with 2.69 μM NAA for subculture, the callus proliferated normally to form a yellow-white friable callus. It is obvious that 2.69 μM NAA could be the ideal substitution for 2,4-D in the long-term subculture to decrease the frequency of abnormal embryos (Ma and others 2005). In conclusion, a low concentration of auxin was required in the induction (2.26 μM 2,4-D) and maintenance (2.69 μM NAA) of embryogenic callus.

When embryonic calluses were cultured on the differentiation medium for 3 weeks, somatic embryos appeared (Fig. 1b). The optimal differentiation medium was selected by comparing the mean number of somatic embryos per explants, mean frequency of abnormal somatic embryos, growth rate, and development capacity. Table 2 shows that MS containing 1.74 μM NAA, 4.44 μM BA, and 1.90 μM ABA was an optimal differentiation medium according to these criteria. ABA and 4–5% sucrose were effective in decreasing the frequency of abnormal embryos (Ma and others 2005). With these combinations of plant growth regulators (PGRs) in maintenance and differentiation media, we have kept the regeneration system in our lab going for over 3 years.

The matured somatic embryos were separated (Fig. 1c) and converted into plantlets within 5 weeks (Fig. 1d). When the plantlets with well-developed roots and shoots were transferred to the field, there was about an 81% survival rate. They were suitable for field planting after 1 month (Fig. 1e).

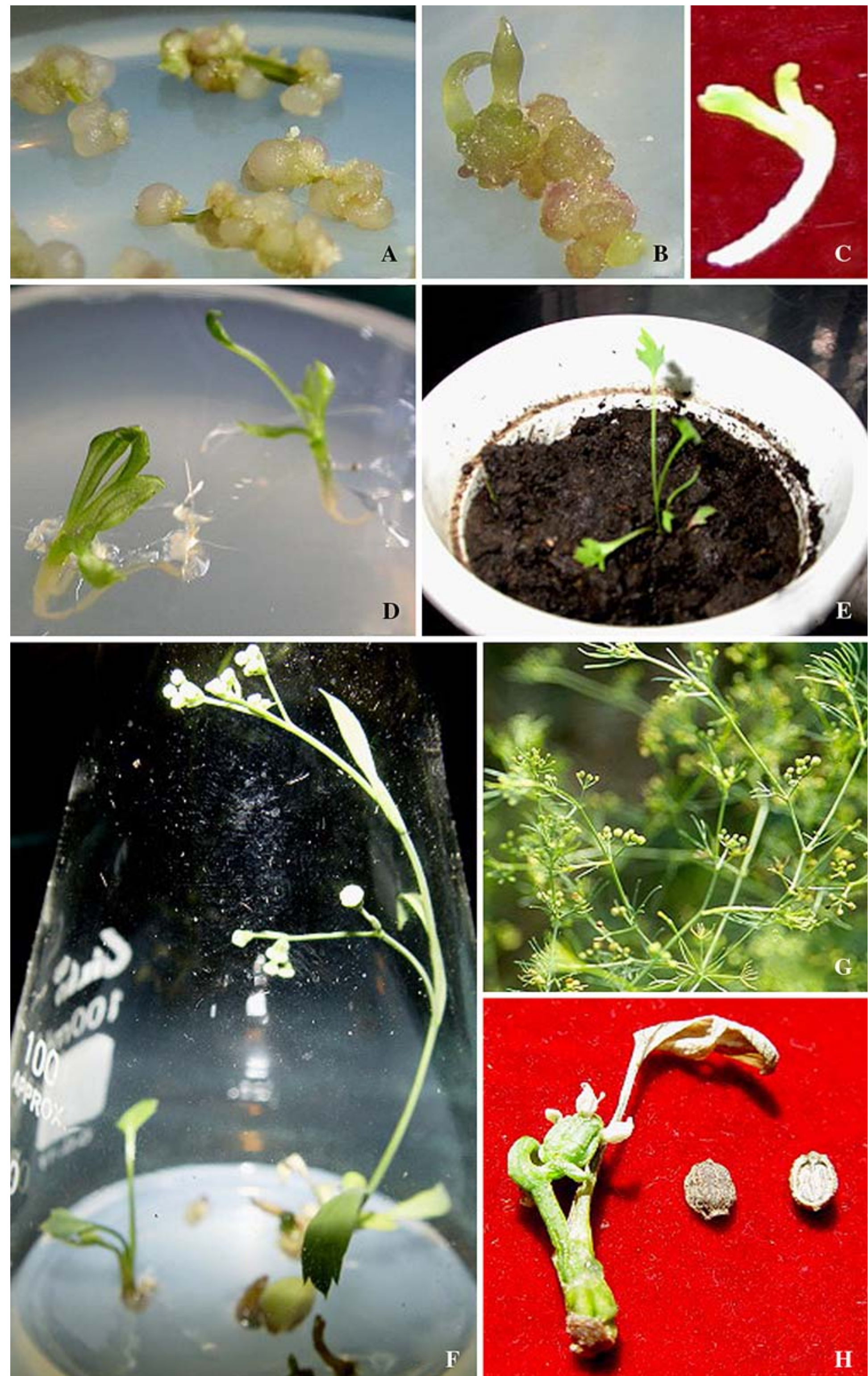

Fig. 1 Stages of somatic embryos and in vitro inflorescence development in *Saposhnikovia divaricata*. (**a**) The embryonic callus from internodes. (**b**) Somatic embryos were induced from the callus. (**c**) Converted seedling from matured embryo. (**d**) Regenerated plantlet. (**e**) Healthy transplanted plantlet in soil. (**f**) In vitro flowering from normal embryo. (**g**) Ex vitro flowering. (**h**) In vitro fruiting from abnormal embryo

In vitro Flowering

When the embryonic callus had been subcultured every 4 weeks for 15 months, 10% of regenerated plantlets could flower in vitro (Fig. 1f). The developing inflorescences appeared in the shoots, and the in vitro florets were morphologically comparable to the ex vitro florets, although smaller in size (Fig. 1g). The average flower lasted 5–7 days and only a few flowers could produce mature seeds, whereas most of them senesced after 6–8 days. The most

remarkable characteristic observed in our cultures was that some vitrified embryos and embryos without root formation could also produce plantlets with flowers and fruit in vitro (Fig. 1h).

The ability to flower could not be regained over the next 3 months under the same conditions. Further studies showed that a low concentration of PP333 (range = 0.34–1.36 μM) or ETH (range = 1–4 μM) could promote in vitro flowering of up to 26 or 21%, respectively. However, high concentrations of these compounds prohibited the development of the plantlet, even causing death (Table 3).

The transition to flowering in vitro is regulated by an array of both external and internal factors (Bernier and others 1993; Kinet 1993). Especially important factors are carbohydrates, growth regulators, light, and pH of the culture medium. Many studies have shown the effects of the sucrose concentration on in vitro flower induction (Carson and Leung 1994). Cultures of *S. divaricata* were kept on media containing 4-5% sucrose which may have facilitated flower induction. However, the role of sucrose was not studied in any detail. Similarly, cytokinin, alone or in combination with auxin, may promote in vitro flowering (Scorza 1982; Peeters and others 1991; Kostenyuk and others 1999; Chang and Chang 2003; Naor and others 2004), but their role in control of in vitro flowering in *S. divaricata* remains to be shown.

Endogenous ethylene levels have been correlated with flowering (Abeles and others 1992; Buer and others 2003). The low permeability of the cap on the culture jars may have resulted in accumulation of ethylene gas in the jars. The results with an open system suggest a role for ethylene in induction of flowering in *S. divaricata*. Growth retardants such as paclobutrazol (PP333), which prevent gibberellin biosynthesis, inhibit elongation growth and may promote flowering (for example, Zheng and Pang 2006). PP333 was also shown to promote in vitro flowering in *Dendrobium* (Wang and others 2006). However, the effects of these two PGRs were strictly dose-dependent in our experiment; high concentrations inhibited flower formation, and even caused the death of the plantlets.

Normal and abnormal somatic embryos usually appear simultaneously in tissue culture. Some studies (Zhang and others 1996; Liu and others 1999; Junaid and others 2007) indicated that abnormal somatic embryos could be transformed into normal seedlings by regulating the kinds and concentrations of PGRs used. In particular, the present results showed, perhaps for the first time, that abnormal somatic embryos, most of them rootless, could have the same reproduction ability as the normal embryos. In bamboo, flowers could be induced only in the absence of roots when the shoots were cultured on medium containing BA (Joshi and Nadgauda 1997). Root pruning has also been shown to promote flowering in well-known woody species (Meilan 1997) and in *Cymbidium niveo-marginatum* (Kostenyuk and others 1999). This effect possibly could be attributed to the presence in the roots of substances that inhibit flowering, as McDaniel (1996) reported a specific signal in tobacco roots that prevented flowering. Thus, lack of roots in abnormal somatic embryos could have enhanced flowering. Development of such abnormal embryos should be studied more closely.

The available data on in vitro flowering in *S. divaricata* is still insufficient and we do not know what the dominate controlling factors of in vitro flowering and fruiting in *S. divaricata* could be. Our results suggest a significant effect of ethylene and paclobutrazol, but the effects of other treatments (sucrose, cytokinins, auxins) cannot be excluded because the retarding PGRs were supplied only in the last phase in tissue culture. Hence, more data, including physiologic and molecular evidence, should be collected in further studies on in vitro flowering and fruiting in *S. divaricata*.

Table 3 Effects of PP333 and ETH at different concentrations on flowering in vitro, death percentage, plant height after 4 weeks, and time to flowering

PGRs treatments (μM)	Flowering (%)	Death percentage (%)	Plant height after 4 weeks (cm)	Time to flowering (days)
0 (control)	2 f	0 a	6.3 a	51 d
0.34 PP333	12 d	0 a	3.8 b	41 bc
0.68 PP333	17 c	0 a	2.9 c	40 b
1.02 PP333	26 a	7 b	2.8 c	40 b
1.36 PP333	21 b	16 d	2.2 d	39 b
1.70 PP333	8 e	67 e	1.4 e	43 c
1 ETH	11 d	0 a	3.8 b	36 a
2 ETH	12 d	0 a	3.7 b	38 ab
3 ETH	15 c	0 a	2.3 d	37 a
4 ETH	21 b	11 c	2.2 d	39 b
5 ETH	12 d	17 d	1.5 e	37 a

Basal medium is MS medium. Each value represents the mean of 50–60 replications. Different letters within columns indicate significant differences ($P < 0.05$, LSD)

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