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Direct somatic embryogenesis from leaves, cotyledons and hypocotyls of *Hippophae rhamnoides*

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Abstract

Plant regeneration *via* direct somatic embryogenesis from cotyledons, hypocotyls and leaves in seabuckthorn (*Hippophae rhamnoides* L.) was achieved. The influences of basal media, carbon sources, plant growth regulators (PGRs) with different concentrations and combinations on embryogenesis capacity of explants were studied. The highest frequency of somatic embryos production and germination was obtained on Schenk and Hildebrandt medium (SH) supplemented with 1.0 mg dm^{-3} kinetin and 0.2, 0.5 mg dm^{-3} indole-3-acetic acid. Granulated sugar was the optimal carbon source. The embryo-derived plantlets with well-developed roots and shoots were transferred successfully to the greenhouse with a maximum survival rate of 55 %. Histological observation revealed that the somatic embryos were similar to those of zygotic embryos.

Additional key words: indole-3-butyric acid, *in vitro* morphogenesis, kinetin, plant regeneration, seabuckthorn.

Introduction

Seabuckthorn (*Hippophae rhamnoides* L.) is a shrub or small tree. It is being used as a source of medicine, wood, oil and fodder in Europe and China. Besides its economic value, due to its resistance to harsh conditions, the wide ecological adaptablity, the low cost and high benefits, it has firstly been selected among many kinds shrub tree species for soil and water conservation, wind-breaking and sand fixation in north China (Lu *et al.* 1997).

In vitro multiplication of seabuckthorn has been reported (Montpetit and Lalonde 1988, Mou 1995, Knyazev *et al.* 2003). However, some problems have been highlighted during clonal propagation, including low micropropagated ability, lower rooting rates and serious browning of explants (Yang *et al.* 2004). For these reasons, somatic embryogenesis is considered to be a commercial approach for large-scale micropropagation of seabuckthorn. Furthermore, knowledge on morphological changes of somatic embryogenesis is essential to understand organization of cell at different developmental stages. To our knowledge, there is no report on induction of somatic embryo from seabuckthorn.

The aims of present study were: *1*) to investigate the effects of the basal media and plant growth regulators on relative efficiency of embryogenic culture initiation from different explants, *2*) to assess the influence of different sugar types on somatic embryo formation, and *3*) to describe the histological analyses of somatic embryos at different stages from 3 different explants of seabuckthorn.

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Abbreviations: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; FAA - formalin acetic acid ethanol; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; KIN - kinetin; MS medium - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; NN69 medium - Nitsch and Nitsch medium; PGR - plant growth regulator; SH medium - Schenk and Hildebrandt medium; WPM medium - Lloyd and McCown medium.
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Materials and methods

Seeds of seabuckthorn were washed in water containing five drops of *Tween-20* with gauze to remove particles and oil membrane, and then surface sterilized by in 70 % ethanol (5 min). After washing in sterile distilled water, they were soaked for 1 h in 30 % sodium hypochlorite plus two drops of *Tween-20* per 100 cm^3, following by washing 5 times with sterile water. The sterilized seeds were inoculated into sterile glass jars (175 cm^3) containing 40 cm^3 Murashige and Skoog (1962; MS) medium with 3 % (m/v) granulated sugar and 0.65 % (m/v) agar (pH 5.8) and grown under a growth chamber under a 16-h photoperiod, a 55 µmol m^{-2} s^{-1} photosynthetic photon flux density provided by cool white fluorescent tubes and a 25 ± 2 °C temperature.

The shoot-tips of seabuckthorn were proliferated on Lloyd and McCown (1980, WPM) medium supplemented with 0.5 mg dm^{-3} 6-benzylaminopurine (BA) for 6 weeks and then transferred to fresh medium every 5 weeks.

The first fully expanded primary leaves from 30-d-old seedlings were cut aseptically into two segments (about 0.5 - 1.0 cm) consisting of the basal and apical parts of the leaves. Meanwhile, cotyledons (about 5 × 5 mm) and hypocotyls (about 2 ×3 cm) were excised from 20-d-old *in vitro* seedlings. Leaf segments were placed with the adaxial surfaced up, and cotyledons and hypocotyls horizontally in the media (a 175 cm^3 glass jar containing 40 cm^3 medium). The media for the experiments were full-strength MS, 1/2 MS (half-strength of MS major salts + full-strength of MS micronutrients), 1/4 MS (one-fourth strength of MS major salts + full-strength of MS micronutrients), Nitsch and Nitsch medium (1969, NN69) and Schenk and Hildebrandt medium (1972, SH). They were supplemented with BA, kinetin (KIN), 2,4-dichlorophenoxy-acetic acid (2,4-D), α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and filter-sterilized indole-3-acetic acid (IAA) in different concentrations and combinations. Additionally, the different explants were inoculated on the suitable media containing equimolar concentrations of granulated commercial sugar, sucrose, maltose, glucose, and combination of maltose or glucose. All the cultures were supplied with 0.65 % (m/v) agar and firstly maintained in darkness for 35 d and then in light in the growth chamber.

Morphologically well-developed plantlets 2.5 cm high were removed from culture jars, washed gently under running water and planted into a white plastic pots containing a sterile mixture of sand : *Perlite* : soil (1:1:1, v/v). Plantlets were covered with grass beakers for 1 week before growing in greenhouse (temperature of 30 ± 2 °C and relative humidity 85 %. Percentage of survival was recorded two months after transfer.

The number of explants for each treatment varied from 80 to 100, and three replicates were conducted. Frequency of somatic embryo production on different explants (45 d after inoculation) and somatic embryo germination (60 d after inoculation) were recorded. The data were analyzed using analysis of variance (*ANOVA*) on the statistical package of *SPSS* (version *10*, *Software Release 10.01*). Means were compared using Duncan's Multiple Range Test (DMRT) at the 5 % probability level and the standard errors of means were calculated.

Embryogenic tissues for histological studies were taken from the 20 to 60-d-old cultured hypocotyls at a 5-d interval. The tissues were vacuum-infiltrated for 30 min, fixed with FAA (formalin : acetic acid : absolute ethanol : distilled water (5:5:45:45; v/v) for 4 h at room temperature, and then washed with running water for 10 min. After dehydrating in graded ethanol and infiltrating in xylene, they were embedded in paraffin wax at 60 °C and cut into 5 µm thick sections by using a *LKB 1508* (Zhejiang, China) rotary microtome. The sections were transferred and affixed to glass slides with Meyer albumen. After staining with safranin and fast green, mounting in neutral balsam and covering with a cover glass, they were observed and photographed under an *Olympus RX 51* (Tokyo, Japan) photomicroscope.

Results

Morphological and histological observations of somatic embryogenesis: On the WPM medium supplemented with 0.5 mg dm^{-3} BA in absence of IBA, the number and the length of shoots per explant were reached approximate 5.72 and 6.48 cm after 40 d in culrure, respectively. Explants of *H. rhamnoides* L. were expanded and curved after 10 d culture on the seven initial media. Early globular somatic embryos formed at the edges of cotyledon and leaf sections (Fig. 1*A*,*B*) and over the surface of hypocotyls (Fig. 1*C*) without callus formation after 20 d in culture. Somatic embryos proliferated and produced secondary embryos or embryo masses when transferred onto fresh media (Fig. 1*D*) and cotyledonary embryos formed after about 45 d (Fig. 2*A*). Some somatic embryos continued to proliferate and others germinated during 2 months sub-culturing (Fig. 2*B-D*).

At the beginning, globular embryos had a dense vascular cylinder form in the center after 20 d culturing (Fig. 3*A*), and then not only enlarged but also covered with some stellate hairs from their surfaces (Fig. 3*B*). Secondary globular embryos formed on the surfaces of the later globular embryos after 30-d culturing (Fig. 3*C*,*D*). Then somatic embryos pass through typical heart-shaped (Fig. 3*E*,*F*), torpedo-shaped (Fig. 3*G*), embryos with two or three cotyledons (Fig. 3*H*,*I*) and embryos exhibiting radicle development and cotyledon swelling (Fig. 3*J*). Histological analysis revealed that they had bipolar structures and distinct physiological isolations with the mother tissues at their developmental stages.

Fig. 1. Macroscopic observation of direct globular embryos production from specific region of explants cultivated on SH medium supplemented with 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA in seabuckthorn: *A* - globular embryos erupting through cotyledon epidermis in cotyledon section for 20 d; *B* - early globular embryogenesis at cut edge of the leaf for 20 d; *C* - globular embryogenesis from the surface of hypocotyls for 25 d; *D* - multiplication of globular embryo from the section of cotyledon for 30 d.

Fig. 2. Development and rooting of somatic embryogenesis on SH medium including 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA in seabuckthorn: *A* - cotyledonary embryogenesis of the cotyledon for about 45 d; *B* - direct somatic embryo rooting at the edge of cotyledon for 2 months; *C* - direct somatic embryo rooting in the middle region of the hypocotyls for 2 months; *D* - individual plantlets with complete cotyledon, plumular axis and radicle; *E* - plantlets grew vigorously after transplanting into a soil-less substrate on the shaded greenhouse for 4 months.

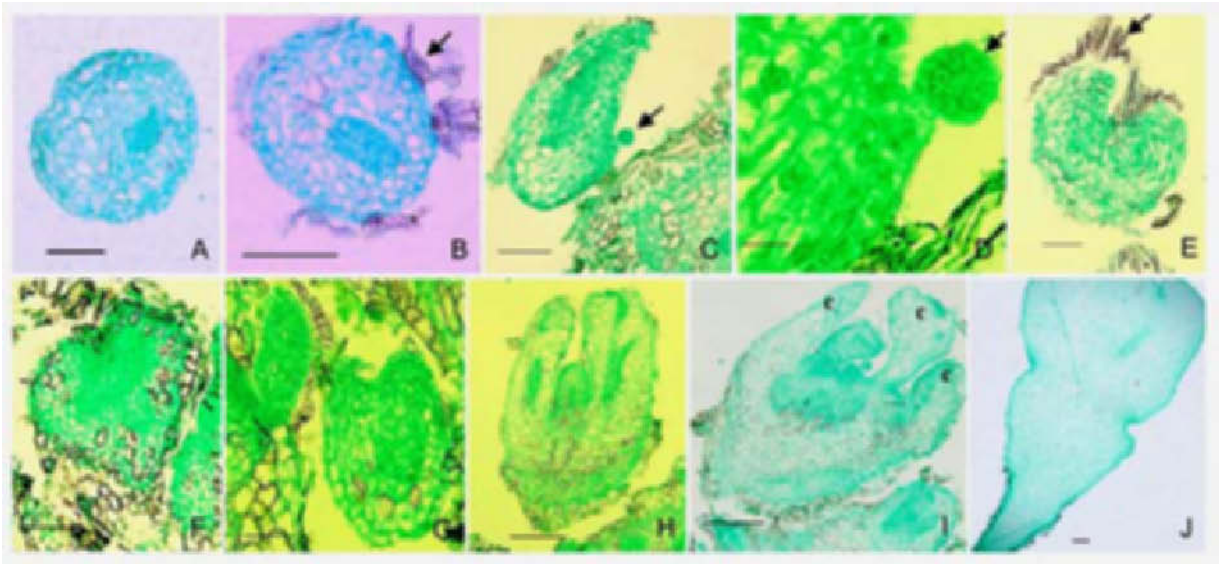

Fig. 3. Histological observation of somatic embryos development on SH medium supplemented with 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA in seabuckthorn (fixation: FAA, staining: safranin, fast green filter): *A* - early globular embryo for 20 d (*bar* = 100 μm); *B* - middle globular embryo covered with stellate hairs (arrow) from its surface for 25 d (*bar* = 100 μm); *C* - later globular embryo with secondary embryoids (arrow) developed from its surface for 30 d (*bar* = 100 μm); *D* - magnified view of a part of Fig. 3*C* (*bar* = 20 μm); *E* - heart-shaped embryo formed covered with epidermic hairs (arrow) from its surface for about 35 d (*bar* = 50 μm); *F* - heart-shaped embryo formed covered with epidermic hairs (arrow) from its surface for about 35 d (*bar* = 100 μm); *G.* - torpedo-shaped embryo covered densely with hairs on its surface for about 40 d (*bar* = 50 μm); *H* - two cotyledon embryos for about 45 d (*bar* = 200 μm); *I* - cotyledon embryo with three cotyledons (c) for about 45 d (*bar* = 200 μm); *J* - germination of somatic embryo with complete cotyledon, plumular axis and radicle for 60 d (*bar* = 200 μm).

Table 1. Frequency of different explants of seabuckthorn with somatic embryos produced (45 d of culture) and germinated (60 d of culture) on different media. Means ± SE of three replicates with 80 - 100 explants per treatment. Mean with different letters were significantly different according to Duncan's Multiple Range Test (DMRT) at $P = 0.05$. [a] - Including buds, shoots and small plantlets developed from somatic embryos.

Medium	PGRs	Conc. [mg dm^{-3}]	Somatic embryos produced [%] leaves	cotyledons	hypocotyls	Somatic embryos germinated [%][a] leaves	cotyledons	hypocotyls
MS	BA+KIN+IAA	0.5+0.5+1.0	5.3 ± 1.9f	3.4 ± 0.2e	0 e	2.4 ± 0.5f	2.0 ± 0.9e	0 g
1/2MS	BA+KIN+IAA	0.1+0.3+2.0	15.3 ± 2.7e	15.3 ± 2.7d	20.5 ± 7.3d	7.5 ± 2.3e	8.2 ± 1.2d	12.3 ± 2.9f
1/4MS	BA+KIN	1.0+0.1	21.0 ± 6.0d	16.4 ± 2.5d	24.6 ± 5.0d	16.5 ± 3.3d	28.5 ± 3.8c	27.1 ± 3.2e
NN69	BA+IAA	0.3+0.2	44.2 ± 12.0b	50.9 ± 3.4b	39.8 ± 1.3c	52.7 ± 3.5b	43.2 ± 2.5b	56.1 ± 5.5c
SH	KIN+IAA	1.0+0.5	76.9 ± 4.2a	86.3 ± 2.5a	74.3 ± 3.7a	79.1 ± 3.2a	88.3 ± 4.2a	92.6 ± 3.7a
SH	BA+KIN	0.5+0.3	3.6 ± 1.6f	3.3 ± 1.0e	27.3 ± 6.7d	8.3 ± 1.9e	9.1 ± 3.1d	31.3 ± 3.4d
SH	BA+KIN+IBA	1.0+0.5+0.2	29.1 ± 5.5c	37.7 ± 4.5c	47.1 ± 9.7b	32.4 ± 4.0c	41.0 ± 5.5b	68.9 ± 8.5b

Table 2. Effects of sugars on somatic embryogenesis (45 d of culture) and germination of embryos (60 d of culture) from different explants of seabuckthorn grown on SH medium supplemented with 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA. Means ± SE; experiments were repeated three times, and each replicate consisted of 80 - 100 explants; means followed by the same letter were not significantly different at $P = 0.05$. [a] - Including buds, shoots, and small plantlets developed from somatic embryos.

Sugars	Somatic embryos produced [%] leaves	cotyledons	hypocotyls	Somatic embryos germinated [%][a] leaves	cotyledons	hypocotyls
Granulated sugar	76.0 ± 3.4a	87.4 ± 2.5a	75.2 ± 3.7a	81.4 ± 7.8a	90.3 ± 4.6a	90.1 ± 4.6a
Sucrose	52.8 ± 4.9b	57.3 ± 4.0b	45.6 ± 14.4 b	53.8 ± 5.3b	67.7 ± 4.6b	51.7 ± 6.5b
Maltose	24.3 ± 5.3c	50.6 ± 7.7b	18.7 ± 5.8c	34.5 ± 5.3c	40.8 ± 4.7c	23.5 ± 4.3c
Glucose	21.4 ± 2.7c	18.9 ± 4.2c	10.6 ± 3.0c	11.2 ± 1.8d	10.3 ± 2.7e	3.2 ± 0.7d
Maltose + glucose	21.6 ± 3.7c	27.1 ± 4.3c	13.2 ± 3.5c	17.4 ± 3.8d	24.8 ± 4.6d	10.5 ± 2.7d

Effects of basal media and PGRs on somatic embryo induction and germination: Different combinations of basal media and plant growth regulators resulted in different responses of different explants. From the 25 different combinations of basal media and plant growth regulators used, only 7 treatments induced somatic embryogenesis and embryos germination (Table 1). The highest frequency of somatic embryogenesis (76.9, 86.3 and 74.3 %) and germination (79.1, 88.3 and 92.6 %) from leaves, cotyledons and hypocotyls, respectively, was obtained on SH medium containing 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA (Table 1). This medium was used in further studies. The frequency of somatic embryo produced and germinated on the other media ranged from 0 to 50.9 % and 0 to 68.9 %, respectively. On full strength MS medium many explants turned brown (data not shown) within 3 weeks and led to lowest average frequency of somatic embryogenesis and germination. NN69 basal medium, 1/4 MS and 1/2 MS were better, but the lowest extent of browning of explants and highest frequency of somatic embryos produced and geminated was observed on SH medium. PGRs are indispensable for the induction of somatic embryogenesis. The results revealed that KIN was more effective than BA at same concentration (1.0 mg dm^{-3}) in induction of somatic embryos. Among the tested auxins, the highest frequency of somatic embryo was obtained on medium containing 0.2 or 0.5 mg dm^{-3} IAA.

Effect of carbon source on somatic embryo induction and germination: To optimize the somatic embryogenesis of seabuckthorn, different kinds of sugars were added to SH basal medium containing 1.0 mg dm^{-3} KIN and 0.5 mg dm^{-3} IAA (Table 2). The results showed that maltose and glucose alone or in combination were far less efficient in induction and germination of somatic embryos than sucrose at the same concentration. Granulated sugar was the best one for somatic embryogenesis of seabuckthorn, resulting in the highest frequencies of somatic embryos induction (76.0, 87.4 and 75.2 %) and germination (81.4, 90.3 and 90.1 %) from leaves, cotyledons and hypocotyls, respectively (Table 2). In general, the frequency of somatic embryogenesis and germination in cotyledon explants was higher than in leaf or hypocotyl explants on the same medium.

Shoot elongation and plant growth: Most of somatic embryos induced from leaves, cotyledons and hypocotyls were generally able to germinate and develop into complete plantlets on the initial culture media. The survival rates of the normal plantlets ranged from 30 to

55 % after growing in the greenhouse for 2 months (Fig. 2*E*). However, several abnormal embryos including jointed embryos, abnormal bud embryos and hyperhydric embryos were also observed in the process of somatic embryogenesis. From these only about 5 % could be recovered into normal plantlets when they were transferred to the greenhouse.

Discussion

Effects of basal medium: Composition and strength of basal media strongly affected somatic embryogenesis. Vooková and Kormuťák (2001) reported that there was no significant differences between the MS and SH medium on germination of *Abies numidica* somatic embryos. In this study, however, the results were not in accordance with their report. SH basal medium was proven to be the best among the five kinds of basal media tested. The different responds of somatic embryogenesis to basal medium may be due to the use of different cultivars and cultural conditions.

Effects of sugar: Somatic embryogenesis was decreased when granulated sugar was replaced by sucrose, maltose, glucose, and maltose + glucose. Since the ability of a somatic cell to undergo embryogenesis relied on reprogramming to embryogenic cells (Merkie *et al.* 1995), such reprogramming could involve the activation of genes for specific carbon source metabolism. The influence of carbon source on somatic embryogenesis has been reported for other plant species (Strickland *et al.* 1987, Parrot and Bailey 1993, Daigny *et al.* 1996, Abdoulaye 2000). The effectiveness of a carbon source in plant tissue culture depended on the plant species, the genotype, and the explants used. Abdoulaye (2000) reported that glucose, sucrose and fructose resulted in higher somatic embryogenesis than maltose and sorbitol in cacao, while maltose was more efficient than sucrose, fructose, glucose in apple and cassava somatic embryogenesis (Daigny *et al.* 1996, Li *et al.* 1999). However, maltose was unsuitable for cucumber somatic embryogenesis (Ladyman and Girard 1992).

Effects of PGRs: Most experiments demonstrated that 2,4-D play a crucial role in the induction and maintenance of the embryogenic cultures in most fruit and ornamental tree species (Vieitez *et al.* 1992). In this study, however, 2,4-D or NAA + IBA (in ratio 1:1) could not induce somatic embryos in seabuckthorn. Similarly, the somatic embryos of *Zizyphus mauritiana* were not induced but recovery of plantlets was very rare on 2,4-D medium (Raj Bhansali 1989). The combination between 2,4-D and other phytohormones significantly reduced the number of somatic embryo of guava (Akhtar *et al.* 2000) and pre-mature cucumber fruits (Hassanein 2003). In our experiments, the best combination was KIN + IAA for somatic embryogenesis of seabuckthorn which was quite similar to what was reported in *Citrus aurantifolia* and *Carica papaya* (Litz and Gray 1992).

The histology: The developments of somatic embryo were similar to that of zygotic embryos of monocotyledonous plants (Yang *et al.* 2000, Palacios *et al.* 2003, Sun *et al.* 2003). However, abnormal somatic embryos with one or multiple cotyledons reduced the percentage of plants recovered. Similar observations were made in dicotyledonous plants such as cotton (Ganesan and Jayabalan 2004), *Aralia cordata*, soybean, peanut, melon, and carrot (Soh 1996). Therefore, further research should focus on improving the survival rate of the plantlets in the greenhouse.

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