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BRIEF COMMUNICATION

Callus production, somatic embryogenesis and plant regeneration of *Lycium barbarum* root explants

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Abstract

A new micropropagation system for *Lycium barbarum* (L.) was developed using root explants as starting material. Callus can be produced from root explants on Murashige and Skoog (MS) medium containing 0.2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid. After three subcultures on the same medium, callus was then transferred onto the MS medium supplemented with 500 mg dm^{-3} lactalbumin hydrolysate to induce somatic embryogenesis (SE). After 20 d, about 60 somatic embryos per 0.25 g(f.m.) of embryogenic callus were obtained but only about 10 % of embryos converted into plantlets. After acclimated in the greenhouse, all of the randomly selected plantlets had survived and were similar phenotypically to zygotic seedlings. In addition, the effects of irradiance, photoperiod, growth regulators, explant age and cold treatment on SE of root-derived callus were evaluated.

Additional key words: auxin, lactalbumin hydrolysate, medicinal plant, micropropagation.

Lycium barbarum (L.), a woody bush spread all over Eurasia and North Africa, is an ornamental and medicinal plant. *L. barbarum* polysaccharide has now been shown to prevent cancer and senescence (Yu *et al*. 2004, Zhang *et al.* 2005). Adventitious shoot production and plant regeneration in *L. barbarum* have been reported using diverse explant sources such as leaf, anther, hypocotyl, shoot top and ovary, either by organogenesis (Niu *et al.* 1983, Ratushnyak *et al*. 1989, 1990) or by somatic embryogenesis (SE) (Niu *et al.* 1985, Hu *et al.* 1998). The genetically stable transgenic plants have been regenerated from stem segments (Wang *et al*. 1993) and leaf explant (Hu *et al.* 2001, 2002) of *L. barbarum*. However, the use of root tissue as an explant for regeneration and transformation purposes has not been reported in *L. barbarum* so far.

Callus induction and plant regeneration *via* SE were also reported in culture of root segments of *Eragrostis tef* (Bekele *et al.* 1995), spinach (Komai 1996), *Lotus corniculatus* (Akashi *et al.* 1998), *Psoralea corylifolia* (Chand and Sahrawat 2002), *Chenopodium rubrum* (Milivojević *et al*. 2005) and *Areca catechu* (Wang *et al*. 2006). In this communication, we described a new, effective and reproducible micropropagation system for *L. barbarum* using root explants as starting material.

Seeds of *Lycium barbarum* (L.) cv. Ningji No. 1, were obtained from Ningxia Academia of Agricultural and Forest Science, P.R. China. Seeds were surface-sterilized in 70 % (v/v) ethanol (30 - 40 s) and 0.1 % (m/v) $HgCl_2$ (8 - 10 min), followed by 5 rinses with sterile distilled water and then germinated on hormone-free Murashige and Skoog (1962, MS) medium solidified with 0.7 % (m/v) agar at a temperature of 24 ± 1 °C, irradiance of 20 - 30 μmol m^{-2} s^{-1} and 16-h photoperiod.

About 3 weeks after seed germination, root segments (0.2 - 0.5 cm long) from aseptic seedlings of *L. barbarum* were placed on MS medium with 0.2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) (CI medium) at the same

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Abbreviations: BA - 6-benzyladenine; CI medium - the medium for callus induction; 2,4-D - 2,4-dichlorophenoxyacetic acid; EI medium - the medium for SE induction; GA_3 - gibberellic acid; MS - Murashige and Skoog's medium (1962); NAA - α-naphthalene-acetic acid; SE - somatic embryogenesis.
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temperature and photoperiod but at a higher irradiance of 300 μmol m^{-2} s^{-1} to induce callus formation.

To detect the effect of explant age on SE, calli were induced from root explants of 1, 2, 3, 4 or 5 weeks old *L. barbarum* seedlings. Also, to estimate the effect of 2,4-D concentration during callus induction and subculture on SE, root segments from 3-week-old seedlings were cultured onto MS medium contained 0.2, 0.5, 1, 2 or 4 mg dm^{-3} 2,4-D. After 20 d, calli induced by 0.2, and 0.5 mg dm^{-3} 2,4-D were then subcultured onto MS medium contained 0.2 mg dm^{-3} 2.4-D, and calli induced by 1, 2, 4 mg dm^{-3} 2,4-D were then subcultured onto MS medium contained 1 mg dm^{-3} 2.4-D.

After three subcultures, embryogenic calli were transferred onto the hormone-free MS basal medium supplemented with 500 mg dm^{-3} lactalbumin hydrolysate (EI medium) for SE induction. After 20 d, somatic embryos differentiated per callus were scored under a dissecting microscope and those calli with SEs were transferred onto the hormone-free MS basal medium for plantlet regeneration. All cultures were maintained at 24 ± 1 °C under a 16-h photoperiod with irradiance of 300 μmol m^{-2} s^{-1}.

In order to evaluate the effect of additional growth regulators on SE, calli derived from root explants of 3-week-old seedlings were cultured on EI medium or on EI medium supplemented with 0.25 - 4.0 mg dm^{-3} 6-benzyladenine (BA), α-naphthalene-acetic acid (NAA) or giberellic acid (GA_3). In addition, for investigating the effect of cold pretreatment on SE, calli that had subcultured at 24 °C for 7 d were then transferred and subcultured at low temperature (4 °C) in the dark. After 1, 2, 3, 4 or 5 weeks of cold pretreatment, calli were transferred directly to 24 ± 1 °C to induce SE.

When the plantlets that were produced from the germinating embryos were approximately 4 cm long, they were randomly selected and washed with water, then transferred to the greenhouse in plastic bags filled with sand. A plastic film was kept over the plantlets for 15 d to facilitate a gradual adaptation to greenhouse conditions, and then the air humidity was gradually decreased.

Experiments were set up as completely random designs with three replicates (Petri dishes) per treatment and each treatment consisting of about 10 calli. Each experiment was repeated at least twice.

In our preliminary experiments, root explants from 3-week-old seedlings of *L. barbarum* did not grow and form callus onto hormone-free MS medium. When cultured on the MS medium with indole-acetic acid (IAA), indole-butyric acid (IBA) or NAA root segments could elongate and branch. The addition of 1 mg dm^{-3} IBA into the MS medium was the most suitable for long-term *in vitro* culture of *L. barbarum* root (Hu *et al*. 2006). When cultured onto the MS medium with higher concentration of auxins, root segments produced much more calli which had low ability of SE. In this present paper, the MS medium containing 0.2 mg dm^{-3} 2,4-D was chosen as the best for callus induction and subculture from *L. barbarum* root explant.

When cultured onto CI medium, callus formed at the cut of the root explant after 10 d of culture. By 3 weeks of culture, callus had grown to 4 - 6 mm in diameter. Then friable, nodular and yellowish callus (embryogenic callus) was selected and subcultured onto the same medium every 20 d and used for study of SE and plantlet regeneration of *L. barbarum*.

After being cultured onto EI medium for a week, embryogenic calli began to be differentiated. Under a dissecting microscope, globular embryos could be observed from these differentiating calli. With continued culture onto EI medium in light, their further development through later stages of somatic embryo (heart stage, torpedo stage and cotyledon embryo) into plantlets could be realized. After 20 d of culture onto EI medium, about 60 somatic embryos per 0.25 g(f.m.) of embryogenic callus were obtained. In *L. barbarum,* somatic embryo formed and developed asynchronously.

Calli originated from 3- or 4-week-old roots were more responsive than that from 1- or 5-week-old roots. Following transfer onto EI medium, calli that derived from root explants of 3-week-old seedlings had the highest frequency of SE. Irrespective of light conditions, somatic embryos could be induced from root-derived calli, while light irradiation was indispensable to both future development and conversion of somatic embryo into plantlet.

Fig. 1. Effect of age of root explant on number of somatic embryos.

Table 1. Effect of continuous light or continuous darkness for 20 d on somatic embryogenesis of root-derived callus and plant regeneration. Means ± SD, $n = 3$.

SE induction	Regeneration	Number of somatic embryos [callus^{-1}]	Number of regenerated plants [callus^{-1}]
light	light	63.2 ± 3.8	5.0
	dark	41.3 ± 2.9	0
dark	light	54.0 ± 4.8	3.2
	dark	46.3 ± 4.9	0

When cultured onto MS medium contained 0.2 to 4 mg dm^{-3} 2,4-D, all of root segments from three-week-old seedlings appear to be callusing. After three or fifteen subcultures onto MS medium contained 0.2 or 1 mg dm^{-3} 2.4-D, these calli were then transferred onto EI medium

and had different abilities for somatic embryo formation. Calli that formed at lower concentrations of 2,4-D (0.2, 0.5 mg dm^{-3}) and had sucultured 15 times at 0.2 mg dm^{-3} 2,4-D had only a lower frequency of SE compared to three subcultures. But with increasing the number of subcultures and prolonging of culture time, calli that formed at higher concentrations of 2,4-D (1 - 4 mg dm^{-3}) and then subcultured at 1 mg dm^{-3} 2,4-D still kept their high abilities of SE. In our experiment, calli that derived from *L. barbarum* root explants at lower concentrations of 2,4-D and subcultured three times at 0.2 mg dm^{-3} 2,4-D, had the highest frequency of somatic embryogenesis (reached about 60 embryos per callus) (Table 2). Similar results were reported from SE of other species (Carman 1990, Jayasankar *et al.* 1999). However, during the long-term subculture at high concentrations of 2,4-D, the aberrance of callus chromosome has occurred easily and consequently the embryogenic capability of callus decreased or was lost entirely (Sharma and Kumar 1994).

Table 2. Effect of 2,4-D concentration during root-derived callus induction and during further subcultures on number of somatic embryos. Means ± SD, $n = 3$.

2.4-D [mg dm^{-3}]		Number of somatic embryos [$callus^{-1}$]	
induction	subculture	3 subcultures	15 subcultures
0.2	0.2	58.0 ± 4.8	17.7 ± 0.8
0.5	0.2	61.7 ± 7.6	19.7 ± 1.3
1.0	1.0	47.0 ± 3.6	41.0 ± 4.3
2.0	1.0	42.3 ± 4.4	43.3 ± 3.9
4.0	1.0	39.7 ± 2.3	42.3 ± 5.0

In *L. barbarum*, SE of callus was inhibited by the addition of BA or NAA. The inhibitory effect of NAA on SE was observed only at higher concentrations (2 - 4 mg dm^{-3}), while BA at all concentrations tested significantly inhibited the somatic embryo formation. After 3 weeks of culture onto the EI medium supplemented with 4 mg dm^{-3} BA, the abilities of callus SE were completely lost. Addition of exogenous GA_3, like NAA, only at higher levels resulted in a decrease in somatic embryo formation from callus induced on a 2,4-D-supplemented medium, while it was useful for somatic embryo maturation. After 20 d of culture onto EI medium containing 4 mg dm^{-3} GA_3, root-derived callus produced 65 % of total embryos and 300 % of torpedo-stage somatic embryos compared to the control medium without GA_3 (Table 3). These results confirmed that GA_3 plays a significant stimulatory effect on improvement of embryo conversion into plants.

Cold treatment was used as an environmental stimulus to promote SE in different species (Bergmann *et al.* 1996, Hess and Carman 1998). In cell suspension cultures of *Astragalus adsurgens*, cold-treatment combined with a proper Ca^{2+} level in the medium enhanced SE (Luo *et al.* 2003). In this paper, SE of *L. barbarum* the capacity of

Table 3. Effects of growth regulators BA, NAA and GA_3 at different concentrations [mg dm^{-3}] on total number of somatic embryos [$callus^{-1}$]. Means ± SD, $n = 4$. Number of somatic embryos on hormone free medium was 70.5 ± 16.2.

	0.25	0.5	1.0	2.0	4.0
BA	16.7± 4.5	13.7± 2.5	6.3± 1.5	5.7±2.1	1.0± 1.0
NAA	58.7±12.3	58.3± 8.3	49.3± 2.3	26.7±5.1	28.0±10.8
GA_3	71.8±13.5	66.8±11.6	57.5±14.7	52.5±6.8	46.0± 6.2

calli to produce somatic embryos was influenced considerably by the duration of cold pretreatment. Calli pretreated for one- and three-weeks displayed only a slight increase in the amount of somatic embryos formed, but cold pretreatment for two weeks significantly enhanced SE in comparison with the control. The maximum capacity for somatic embryo formation was obtained from calli pretreated for 2 weeks, in which embryogenic capacity was 260 % higher than that without cold pretreatment. The longer cold pretreatment (≥ 4 weeks) resulted in the inhibition of SE. Compared to those calli without cold pretreatment, the higher amount of endogenous polyamines was found in cold pretreated calli (data not shown). This result implied that the enhanced embryogenic response of calli to the cold pretreatment was associated with accumulation of endogenous polyamines.

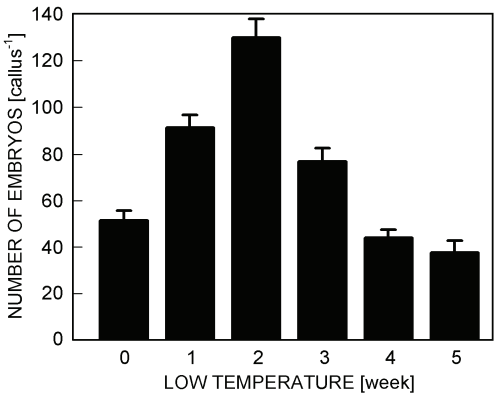

Fig. 2. Effect of low temperature (4 °C) treatment on SE.

In *L. barbarum*, somatic embryos germinated and formed plantlets (Fig. 1*E*) on MS medium free of growth regulators. Usually, about 5 plantlets per callus could be regenerated, and the conversion frequency of somatic embryos into plantlets was lower at approximately 10 %.

When plantlets with well-developed roots reached about 4 cm high, 20 plantlets (with 8 - 10 leaves) were randomly selected, washed very well and transferred then to the greenhouse in plastic bags filled with sand. After one month of the acclimation in the greenhouse, all of the plantlets survived and grew normally without showing any morphological variation.

The paper here presented a new, effective and

reproducible micropropagation system for *L. barbarum*. Plantlets could be obtained through SE using root as explants. And some factors that affect SE from root-derived calli were evaluated. Further investigations will be needed to modify this protocol for mass propagation and gene transformation of this species.

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