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ARTICLES

PLANT REGENERATION FROM CALLUS CULTURES DERIVED FROM MATURE ZYGOTIC EMBRYOS OF *SOPHORA ALOPECUROIDES* LINN. IN MONGOLIA

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Abstract: *The aim of this study is to determine the effect of hormones and selection of the most effective medium using callus cultures derived from mature zygotic embryos of Sophora alopecuroides Linn. for plant regeneration. After 8 weeks of culture, the highest callus induction medium (93.3%) was obtained on MS medium supplemented with 0.2 mglL Zeatin and 2.0 mg/L α-naphthaleneacetic acid (NAA). The best callus proliferation was observed on the same medium.*

Shoots regenerated at the highest frequency of 50.0% with 5.8 shoots when calli were cultured on MS medium with 2.0 mg/L BA. Therefore, this protocol provides a basis for future studies on genetic improvement and could be applied to large-scale multiplication systems for commercial nurseries of S.alopecuroides L.

Keywords: *Sophora alopecuroides Linn.; Zygotic embryo; Plant growth hormones; Callus; Shoot regeneration;*

INTRODUCTION

Sophora alopecuroides Linn., a perennial shrub of *Fabaceae* family has been used in traditional Mongolian medicine to treat fever, bacterial infection, heart disease, and rheumatism [6]. *Sophora* is a genus encompassing about 45 species, and two species of this genus have been recorded in Mongolia. The population of this genus is declining [8] owing to overharvesting of the roots of this herb of rare plant used for medicinal purposes,. This species has been assessed as endangered due to poor regeneration in the nature and very limited geographic range in Mongolia [5].

Direct shoot propagation using stem disc has been implemented successfully in this plant [1, 7, 15, 17]. However, development of shoot regeneration from callus is still required. Production of regenerated plant through indirect organogenesis is one possible way to contribute to *S.alopecuroides* L. genetic improvement since there are some advantages of shoot regeneration from callus over direct shoot regeneration. A callus phase is commonly included in tissue culture protocols with the objectives of generating variability to introduce new desirable traits, such as

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abiotic stress tolerance in *Sophora* crops and generating transgenic plants [16]. Moreover, callus production is also a necessary step for obtaining protoplasts used in protoplast fusion, a useful tool in genetic improvement of vegetatively propagated *Sophora*'s species for introducing useful genes or producing new crops [4].

To our knowledge, although there are some previous reports on callus induction and shoot regeneration in some species of other Leguminosae family plants, to date no successful method has been reported for shoot regeneration from callus of *S.alopecuroides* L, [2, 3, 9-11, 13]. In these species, callus differentiation and shoot regeneration were determined by growth regulators [2-3, 9-10].

In addition, other studies on Astragalus species also described various calli types that were formed during callus induction, associated with shoot regeneration. Those studies were useful in selecting suitable calli for further subculture [14]. Thus we aimed to study the effect of culture media and select the most effective medium from callus cultures derived from mature zygotic embryos of Sophora alopecuroides Linn. for plant regeneration.

MATERIALS AND METHODS

Plant material: The mature seeds of *S.alopecuroides* L. were collected in August 2011 from Ekhiin Gol, in Bayankhongor aimag. They were identified by taxonomist Mönkh-Erdene T. and voucher samples are kept at our institute.

The seeds were injured mechanically and washed in 70% ethanol for 3 minutes, followed by surface sterilization in 1% NaOCl with 2-3 drops Tween-20 for 10 minutes and then rinsed five times with sterilized distilled water.

Effect of culture medium on callus induction, proliferation and shoot regeneration: The mature zygotic embryos from sterilized seeds were isolated in Laminar flow and cultured in 100-ml glass jars. All medium contained 3% sucrose, they were solidified with 0.2% gellan gum, 250 mg/L casein hydrolizate, L-proline and were adjusted to pH 5.8 with 1N KOH or 1N HCl prior to autoclaving at 121^0C for 20 minutes. All cultures were maintained at 25 ± 2^0C under light in a 16h photoperiod.

For callus induction, isolated mature zygotic embryos were cultured in 100-ml glass jars containing MS medium supplemented with various concentrations and combinations of cytokinins such as BA (Benzyladenin), TDZ (Tidiazuron), Zeatin, Kin (kinetin) (0.2, 2.0 mg/L) and an auxin, NAA (Naphtaleneacetic acid) (0.2, 2.0 mg/L) (Table 1). Calli derived from zygotic embryos with good growth were excised, divided into small pieces at 0.5 cm and were subcultured in the same medium 4 times every 3 weeks for further proliferation. Callus proliferation was evaluated in total 4 passages.

The next experiment was carried out to evaluate the effects of culture medium on the callus potential for shoot regeneration. After 4 subculture passages, callus with best growth were transferred onto MS medium with various concentrations of TDZ (0.5, 1.0, 2.0 mg/L), BA (0.5, 1.0, 2.0 mg/L) (Table 2). Each treatment contained 20 explants with three replicates. Shoot regeneration was estimated by percentage of callus forming shoots and number of shoots formed by callus after 4 weeks of culture.

After removing agar and washing in tap water, healthy plants with a well-developed root system were transferred to pots with soil containing organic materials and acclimatized under greenhouse condition.

Statistical analysis: Results were analyzed statistically using the Statistical Analysis System program (SAS, 2008). Mean values were calculated and were compared by Student's tests ($P < 0.05$).

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RESULTS AND DISCUSSION

Effect of culture medium on callus induction from mature zygotic embryos

Callus formation started to initiate callus from mature zygotic embryos after 2 weeks of culture. These calli were compact, globular and yellowish. It was calculated that callus induction occurred after 8 weeks. However, lower callus induction (3.3-13.3%) was achieved when MS medium supplemented NAA with various concentrations and combinations of BA and TDZ, whereas callus induction (30-93.3%) was relatively higher when MS medium supplemented NAA with various concentrations and combinations of Zeatin and Kinetin (Table 1).

These derived calli were subculture in the same medium 4 times, with an interval of 3 weeks. After 4 subculture passages, various concentrations and combinations of BA and TDZ with NAA induced callus was light green, compact but the various concentrations and combinations of Kinetin with NAA induced was whitish, mucilaginous, smooth and sloppy.

Most of the calli induced in the MS medium with various concentrations and combinations of Zeatin with NAA, were yellowish and globular. The obtained results are in agreement with other reports that for some plant of Leguminosae family such as *Astragalus Chrysochlorus* [13], *Astragalus Maximus Wild*, the most effective medium to induce organogenic callus was Zeatin [14].

We observed that mucilaginous surface appeared to negatively affect callus growth and subsequent shoot regeneration produced within the callus in *S.alopecuroides*.

Table 1. Effect of different growth hormone combinations on callus induction from mature zygotic embryos of S. alopecuroides L.

Growth hormone (mg/L)		Callus induction(%)	Growth hormone (mg/L)		Callus induction(%)
TDZ 0.2	NAA 0.2	$10\pm3.2^{g,h}$	Zeatin 0.2	NAA 0.2	$50\pm5.7^{c,d}$
TDZ 2.0	NAA 0.2	$3.3\pm0.0^{g,h}$	Zeatin 2.0	NAA 0.2	$33.3\pm3.3^{e,f}$
TDZ 0.2	NAA 2.0	0.0 ± 0.0^{h}	Zeatin 0.2	NAA 2.0	93.3 ± 3.3^{a}
TDZ 2.0	NAA 2.0	6.6 ± 1.3^{h}	Zeatin 2.0	NAA 2.0	76.7 ± 3.3^{b}
BA 0.2	NAA 0.2	10 ± 0.0^{h}	Kin 0.2	NAA 0.2	$63.3\pm8.8^{b,c}$
BA 2.0	NAA 0.2	$3.3\pm0.0^{g,h}$	Kin 2.0	NAA 0.2	$46.7\pm8.8^{d,e}$
BA 0.2	NAA 2.0	0.0 ± 0.0^{h}	Kin 0.2	NAA 2.0	$30\pm5.7^{f,g}$
BA 2.0	NAA 2.0	13.3 ± 0.0^{h}	Kin 2.0	NAA 2.0	$60\pm5.7^{c,d}$

Data collected after 8 weeks of culture. Explants were cultured in MS medium. Values represent the mean_S.E. Means following the same letter within columns are not significantly different, according to Student's test ($P<0.05$).

Callus proliferation and shoot regeneration

There are no reports for shoot regeneration from callus of *S.alopecuroides.* Therefore, we carried out the experiment according to some researcher's reports on shoot regenerated from callus of some plants of Leguminosae family [2, 3, 9-11, 13].

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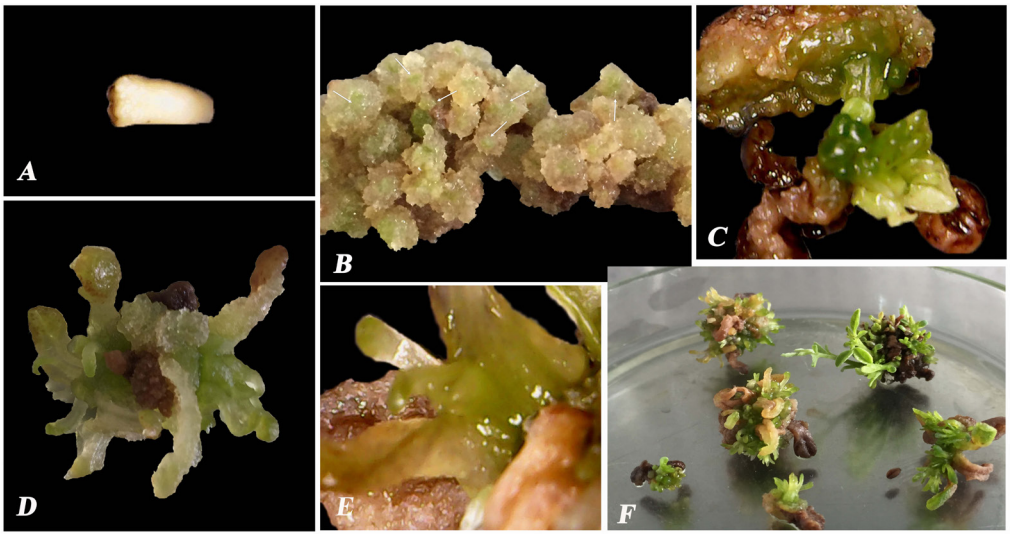

Figure. 1. Plant regeneration from callus derived from mature zygotic embryos of S.alopecuroides L. (A) Isolated zygotic embryo from seed. (B) After an 8 week-culture, induced meristem with green spot after it was transferred to MS medium with 0.5 mg/L TDZ.(C, D and E) Shoot induced in MS medium with 2.0 mg/L BA after 3 weeks of culture.(F) Shoot formed

The highest callus growth rate (93.3±3.3) was obtained when MS medium was supplemented with 0.2 mg/L Zeatin and 2.0 mg/L NAA (Table 1).

Furthermore, calli were proliferated to subculture in the same medium. Proliferated globular, yellowish calli were transferred to MS medium supplemented with various concentrations of BA and TDZ to investigate their potential for shoot regeneration (Table 2). When these calli were transferred to MS medium supplemented with 0.5 mg/L of TDZ, each calli appeared to induce meristem with green spot (Fig. 1B). However, shoot regeneration rate from calli with green spot (18.3±1.66) was very low when MS medium was supplemented with 0.5 mg/L of TDZ.

Table 2. Effect of BA and TDZ on shoot regeneration from callus of S.alopecuroides L

Growth hormone (mg/L)		Percentage of explants forming shoot (%)	No. of shoots/explant
TDZ	0.1	$6.60\pm1.66^{c,d}$	$1.66\pm0.33^{c,d}$
	0.5	18.3 ± 1.66^{b}	$2.00\pm0.26^{c,d}$
	1.0	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
	2.0	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
BA	0.5	$11.6\pm1.66^{b,c}$	$3.00\pm0.30^{b,c}$
	1.0	15.0 ± 2.88^{b}	3.44 ± 0.29^{b}
	2.0	50.0 ± 5.77^{a}	5.86 ± 0.23^{a}
	3.0	16.6 ± 3.33^{b}	$2.10\pm0.23^{c,d}$

Data collected after 3 weeks of culture. Explants were cultured in MS medium. Values represent the mean_S.E. Means following the same letter within columns are not significantly different, according to Student's test ($P<0.05$).

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In this callus we observed that with the addition of BA alone, there was an increase in shoot regeneration. Particularly, the highest shoot regeneration rate (50.0±5.77) was achieved when each callus was cultured with 2.0 mg/L of BA that produced 5.86±0.23.

After 2 weeks of culture, most of the calli with light green color, green spot, and globular structure gradually turned to dark green during culture. After 3 weeks of culture, numerous shoots were derived from calli that were formed (Fig. 1 C, D, E). BA plays a key role in shoot regeneration *in vitro* [1, 12, 15, 17].

When BA concentrations at 3 mg/L was added to MS medium, abnormal shoot increased and shoot regeneration rate (16.6±3.33%), 2.10±0.23 shoots per callus decreased. These results agree with the reports that some plant of Leguminosae family such as *A. adsurgens* Pall, *A. melilotoides* Pall, *A. aquilonius* Barneby, *A. amblytropis* Barneby, *A. cariensis* Boiss have higher rate of shoot generation from callus [2-3] with a broader application of BA.

CONCLUSION

This regeneration protocol will be useful not only for further research, such as Agrobacterium mediated genetic transformation or protoplast fusion studies, but will also have benefits for commercial nurseries that would use virus-free plants and agricultural practices to reduce pesticide use and increase yield production.

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