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BRIEF COMMUNICATION

Effect of 6-benzyladenine and casein hydrolysate on micropropagation of *Amorpha fruticosa*

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Abstract

Using apical and axillary nodes as explants, a rapid and efficient method for propagation of *Amorpha fruticosa* L. has been developed. When grown on Murashige and Skoog (MS) medium supplemented with 8 mg dm^{-3} benzyladenine, 100 % explants responded with 4.94 shoots per explant after 6-weeks culture, and explants taken from the *in vitro* proliferated shoots subsequently produced multiple shoots when cultured on the same medium. The addition of casein hydrolysate (200 mg dm^{-3}) enhanced the number of shoots up to 8.77 per subculture, and coconut milk was found to promote the shoot elongation and make them grow more vigorously. 82.53 % excised shoots were rooted on half-strength MS medium containing 2.0 mg dm^{-3} indoleacetic acid after 3 weeks of incubation. After acclimatization, all of the rooted plantlets established in soil, exhibiting uniform morphological and growth characteristics.

Additional key words: acclimatization, false indigo, growth regulators, *in vitro* cultivation, plant regeneration.

Amorpha fruticosa L. (false indigo) is a widespread leguminous shrub native to the United States, now found in north Asia and in central and southeast Europe. Its seeds, roots, leaves, *etc.*, contain rotenone, a natural insecticide, which for a long time has been used to control seed beetles and as anti-microbial agents (Mitscher *et al.* 1981, Qu *et al.* 1998). Moreover, for its drought resistance and salt tolerance, it is most often used for reforestation in semiarid zones, such as northwest of China (Pei and Zhou 1993). In common with most temperate broadleaved trees, *A. fruticosa* has been planted traditionally as mixed seedling populations exhibiting a high degree of genetic variability, and enzyme electrophoresis analysis also showed its high genetic diversity both at the species and population level (Huh and Hong 1997). The conventional propagation by grafts and rooted cuttings is time consuming. Therefore, it is necessary to develop the essential micropropagation technology. As leguminous trees, which are relatively recalcitrant to regeneration *in vitro* (Datta and Datta 1985), there was only one report about the regeneration from callus of *A. fruitcosa*, and its frequency is very low (12.5 %) (An *et al.* 1992). To our best knowledge, no reports on micropropagation of *A. fruticosa* appeared until now. The present study describes a simple procedure for rapid multiple shoot formation at high frequency from apical and axillary nodes.

In the preliminary experiments, axillary node from adult trees, two-month-old seedlings and regenerated plantlets showed no significant difference of their regeneration potential. This seemed comparable with the results of Gagliardi *et al.* (2002), in their work no significant influence of the age of the donor plant was observed in wild *Arachis* species. So only those from seedlings and regenerated plantlets were used in our studies for decreasing explants contamination.

Dried mature seeds of *A. fruticosa* were collected from the campus of Lanzhou University, Gansu province, P.R. China. They were scarified, then surface-sterilized in 0.1 % $HgCl_2$ for 30 min, followed by four rinses with sterile distilled water and then germinated on half-strength MS medium (Murashige and Skoog 1962) devoid of growth regulators at temperature of 25 ± 1 °C under fluorescent tubes (irradiance of 30 μmol $m^{-2}s^{-1}$,

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Abbreviations: BA - 6-benzyladenine; CM - coconut milk; CH - casein hydrolysate; IAA - indoleacetic acid; IBA - indolebutyric acid MS medium - Murashige and Skoog's medium.

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16-h photoperiod). After two months, the apical and axillary buds with 1 - 2 mm of stem were excised from the whole plant, removing leaves, and used as explants.

The basal medium consisted of the mineral salts and organic nutrients of the MS medium, 3 % sucrose and 0.7 % agar. Depending on the experiment, the basal medium was supplemental with various combinations of 6-benzyladenine (BA), indoleacetic acid (IAA), indolebutyric acid (IBA) at different concentrations. In an attempt to enhance shoot proliferation, we added casein hydrolysate (CH) (50 - 500 mg dm^{-3}) and coconut milk (CM) (5 - 20 %, v/v) to the medium. All the supplements except IAA and IBA were added to the molten agar and adjusted to pH 5.8 prior to autoclaving at 121 °Ç, 104 kPa for 20 min; after autoclaving, the sterile IAA and IBA (0.1 - 2.0 mg dm^{-3}) were added into the media according to the design. All cultures were incubated at 25 ± 1 °C in a culture room under a 16-h photoperiod provided by cool-white fluorescent tubes, with irradiance of about 100 µmol $m^{-2}s^{-1}$. There were 10 - 15 explants per treatment and all experiments were repeated six times. One way *ANOVA* (*SPSS*) was used to compare the difference among treatments.

Within 7 - 10 d following inoculation, some buds differentiated from axillary nodes explants when grown on MS medium with or without BA, the addition of BA has significant effect on increasing the number of shoots. The largest number of shoots per explant (4.94) and the highest length (2.25 cm) was initiated on a medium containing 8.0 mg dm^{-3} BA (Table 1). The optimal concentration of BA for *A. fruticosa* was higher than that in some other trees such as linden (Sarvašová and Ďurkovič 2002). This may be due to phytohormone entatic state was difficult to be broken down for leguminous plant. At the time of subculturing, newly formed shoot nodes were separated and transplanted to fresh medium with the same composition and cultivated at the same condition with that of the initial. The shoots continued to proliferate through several subcultures, and this ability can be maintained in the next subcultures. Repeated subculturing of nodes and shoot tips helped to achieve continuous production of callus-free, healthy shoots at least through five subculture cycles. Similarly, a proliferation of *Spilanthes acmella* was achieved by repeatedly subculturing the nodal segments on shoot multiplication medium (Saritha *et al.* 2002).

Table 1. Effects of BA [mg dm^{-3}] on the number and length of shoots when the explants were cultured on MS medium. Means ± SE of 6 independent experiments, 10 - 13 explants were used for each experiment. Means in the column followed by different letters are different from each other (Least Significant Difference Test, $P \leq 0.05$).

BA	Number of explants	Cultures induced shoots [%]	Number of shoots [explant^{-1}]	Mean shoot length [cm]
0	67	37.14 ± 1.65a	1.24 ± 0.06a	1.11 ± 0.07a
1.0	72	97.33 ± 1.69b	2.86 ± 0.06b	1.22 ± 0.03ab
2.0	64	100.00 ± 0.00b	3.64 ± 0.09c	1.51 ± 0.09b
4.0	69	100.00 ± 0.00b	4.33 ± 0.09de	1.46 ± 0.06b
6.0	60	100.00 ± 0.00b	4.45 ± 0.14ef	1.81 ± 0.05bc
8.0	62	100.00 ± 0.00b	4.94 ± 0.42f	2.25 ± 0.09c
10.0	64	100.00 ± 0.00b	4.62 ± 0.13ef	1.64 ± 0.10bc

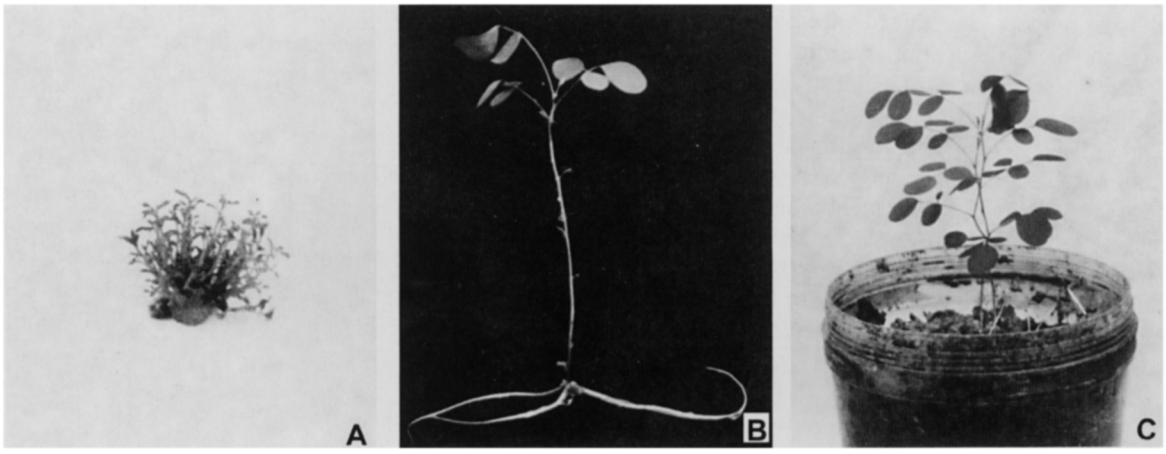

Fig. 1. The micropropagation of *A. fruticosa*: *A* - plantlets regeneration on MS medium with 8.0 mg dm^{-3} BA and 200 mg dm^{-3} CH after 6 weeks; *B* - root formation on MS medium with 2.0 mg dm^{-3} IAA after 3 weeks; *C* - establishment in soil of rooted plantlets .

The addition of 200 mg dm^{-3} of CH to the medium containing 8.0 mg dm^{-3} BA increased the number of new shoots per explant to 8.77 (Table 2, Fig. 1*A*); however, it inhibited shoot length to some extent. Although the addition of CM has no positive effect on increasing the shoots number per explant, it can improve shoots length and make them grow more vigorously. Roy *et al.* (1998) also reported that the addition of 100 mg dm^{-3} CH to the medium for the culture of shoot tips and nodal explants of *Elaeocarpus robustus* resulted in significant increase in growth.

Table 2. Effects of coconut milk, CM [%] and casein hydrolysate, CH [mg dm^{-3}], on the number and length of shoots when shoots were cultured on MS medium with 8 mg dm^{-3} BA. Means ± SE of 6 independent experiments, 10 - 13 explants were used for each experiment. Means in the column followed by different letters are different from each other (Least Significant Difference Test, $P \leq 0.05$).

CH	CM	Number of explants	Number of shoots [explant^{-1}]	Mean shoot length [cm]
0	0	62	4.94 ± 0.42a	2.25 ± 0.09bc
0	5	62	3.08 ± 0.08ab	2.02 ± 0.14bcd
0	10	62	4.17 ± 0.33a	2.35 ± 0.11b
0	20	61	2.70 ± 0.37b	3.57 ± 0.20a
100	0	63	5.65 ± 0.29ac	1.97 ± 0.30cd
100	5	61	7.15 ± 0.73d	1.44 ± 0.04fg
100	10	60	5.30 ± 0.36a	1.34 ± 0.12fg
100	20	61	6.38 ± 0.27cd	2.34 ± 0.20bc
200	0	60	8.77 ± 0.66e	1.96 ± 0.15cde
200	5	61	6.66 ± 0.29cd	1.65 ± 0.11def
200	10	61	4.79 ± 0.24a	1.20 ± 0.11g
200	20	61	4.35 ± 0.06a	1.73 ± 0.07def
500	0	61	6.77 ± 0.29d	1.51 ± 0.05f
500	5	61	6.41 ± 0.29cd	1.80 ± 0.03def
500	10	60	5.68 ± 0.45ac	1.60 ± 0.09ef
500	20	62	4.81 ± 0.07a	1.73 ± 0.02def

For root induction, well-developed shoots (> 1.5 cm) were excised from the culture tube and implanted individually on root induction medium containing half-strength MS basal medium with different concentrations of IAA and IBA (0 - 2.0 mg dm^{-3}) (Table 3). The first root began to emerge after 9 d. After 3 weeks culture, 2.0 mg dm^{-3} IAA was found to be the best choice of auxins, providing 82.53 % rooting in 3 weeks, with 2 - 3 roots 1 - 2 cm long in each plantlet (Fig. 1*B*). This seemed not as efficient as that of *Spilanthes acmella* (Saritha *et al.* 2002). The differential morphogenetic potential of *in vitro* rooting may be a consequence of different growth regulators endogenous levels or receptor affinity in different species. However, both IAA and IBA concentration have not significant effect on the shoot length and root number per explant.

Three to four-week-old plantlets with well-developed roots were taken into a culture room for hardening, and maintained at 15 ± 1 °C under 16-photoperiod with an irradiance of 30 μmol $m^{-2}s^{-1}$. After 7 - 10 d, they were removed from the culture tubes, washed free of agar, and subsequently transplanted into a pot containing alluvial soil and river sand (1:1). Potted plantlets were covered with polyethylene foils to ensure high humidity, and misted everyday in this room for another week, then they were brought to the culture room with the same irradiance and temperature 25 ± 1 °C. After the week, they were transferred to mild soil and further cultivated in greenhouse. The shoots elongated and grow vigorously (Fig. 1*C*). The technique described here provides a promising method for propagation on a commercial scale as well as for the conservation and selection of superior genetic strains or transgenic plants.

Table 3. Root induction in shoots of *A .fruticosa* on 1/2 MS medium with various auxins after 3 weeks culture. Means ± SE of 6 independent experiments, 10 - 13 explants were used for each experiment. Means in the column followed by different letters are different from each other (Least Significant Difference Test, $P \leq 0.05$).

Auxin	[mg dm^{-3}]	Number of explants	Shoots rooted [%]	Number of roots [explant^{-1}]	Length of roots [cm explant^{-1}]
Control	0	82	62.82 ± 5.62ab	2.36 ± 0.20ab	1.70 ± 0.22a
IAA	0.1	62	75.24 ± 3.94cd	2.46 ± 0.25a	1.69 ± 0.06a
IAA	0.5	64	63.39 ± 2.17ae	2.73 ± 0.33a	1.55 ± 0.31a
IAA	1.0	61	61.02 ± 4.67abf	2.09 ± 0.20ab	0.96 ± 0.08b
IAA	2.0	60	82.53 ± 4.04c	2.47 ± 0.27a	1.34 ± 0.06bc
IBA	0.1	65	79.85 ± 2.14cd	2.36 ± 0.45a	1.61 ± 0.09a
IBA	0.5	62	70.37 ± 6.76de	2.59 ± 0.13a	2.28 ± 0.19c
IBA	1.0	63	49.86 ± 4.27fg	1.67 ± 0.11b	1.77 ± 0.20a
IBA	2.0	62	40.41 ± 1.20g	2.11 ± 0.36ab	1.56 ± 0.18a

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