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Formation of Adventitious Shoots and Plant Regeneration by Culture of Cotyledon Segment in *Astragalus sinicus* (Chinese Milk Vetch)

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Cultivation and subsequent plowing of leguminous plants increases soil fertility. Chinese milk vetch (*Astragalus sinicus*), known as renge-sou (*A. sinicus* cv. Japan) in Japanese, is one of the most popular legumes used as a green manure crop in China, Korea and Japan; in China it is cultivated over an area of nearly 5 million ha. The plant has a symbiotic relationship with soil bacteria, namely *Rhizobium huakuii*[1] or *R. huakuii* bv. renge[2], which results in the formation of nodules on its roots and fixation of atmospheric nitrogen in the nodules. Besides being used as a green manure, renge-sou can also be used as fodder for animals and a source of honey for bees[3].

Regeneration of plants from cell and tissue cultures has been reported in many species including legumes, for example *Vicia*[4], *Cajanus*[5], *Coronilla*[6], *Arachis*, *Glycine*, *Melilotus*, *Trifolium*, *Phaseolus*, *Stylosanthes*, *Lotus* and *Medicago*[7]. However, to our knowledge, no reports of morphogenic responses to plant growth regulators or of shoot regeneration in *A. sinicus in vitro* has been published.

This report describes the effects of growth regulators on the regeneration of adventitious shoots in cotyledon explants of *A. sinicus*.

Seeds of *A. sinicus* cv. Japan (Yutouobansei Renge; Takayama Seed Co., Ltd., Kyoto, Japan) were surface-sterilized by immersion in concentrated sulfuric acid for 5 min. in a Erlenmeyer flask and washed 5 times with sterilized distilled water. The sterilized seeds were allowed to germinate on 1% agar, 0.5% sucrose plates for one week at 25°C in the light (*ca.* 1000 lx).

Cotyledons, hypocotyls and roots were excised from one-week-old seedling. Cotyledons were routinely divided into four to six segments, and hypocotyls and roots were cut into 3- to 5-mm-long segments. The nutrient medium consisted of MS (Murashige and Skoog) salts[8] with vitamins and 3% sucrose, and the pH was adjusted to 5.5. The medium was solidified with 3 g/*l* Gellan Gum. The growth regulators used in the experiments were auxins, namely, naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D), and cytokinins, namely, benzyladenine (BA) and kinetin (Kin). Segments were cultured on MS medium that contained various combinations and concentrations of 2, 4-D, NAA, Kin and BA that ranged from 0 to 10 mg/*l*. The plates were sealed with Parafilm TM and grown at 25°±2°C under fluorescent light (*ca.* 1000 lx) with a 16-h photoperiod. The explants that produced apical shoots within one week in culture were discarded to avoid contamination of apical meristem parts in the explants.

Table 1. Morphogenic responses of cotyledon explants of *A. sinicus* to different combinations of auxins and cytokinins.

Cytokinin (mg/*l*)		Auxin (mg/*l*)										
		NAA						2, 4-D				
BA	Kin	0	0.1	0.5	1	5	10	0.1	0.5	1	5	10
0	0	—	CR	CR	CR	CR	CR	CR	CR	C	C	C
0.1	0	—	CR	CR	CR	CR	CR	CR	C	C	C	C
0.5	0	—	C	CR	CR	CR	CR	C	C	C	C	C
1.0	0	—	SC	C	SC	SCR	SCR	C	C	C	C	C
5.0	0	—	C	SC	C	C	SC	SC	C	C	C	C
10.0	0	—	SC	C	C	SC	—	—	—	—	—	—
0	0.1	—	CR	CR	CR	CR	CR	CR	C	C	C	C
0	0.5	—	CR	CR	CR	CR	CR	CR	C	C	C	C
0	1.0	—	C	C	CR	CR	CR	CR	C	C	—	—
0	5.0	—	—	C	—	—	—	SCR	C	C	—	—
0	10.0	—	—	—	—	—	—	—	—	—	—	—

S, Initiation of shoots; C, initiation of callus; R, initiation of roots; —, poor response. At least 3 independent experiments with 25 to 30 explants each were analyzed after five weeks of culture.

Table 2. Influence of concentration of NAA and BA on shoot regeneration from cotyledon explants cultured *in vitro*.

	Average frequency of shoot regeneration (%)					
	NAA (mg/*l*)					
BA (mg/*l*)	0	0.1	0.5	1.0	5.0	10
0	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.5	0	0	0	0	0	0
1.0	0	22	0	22	15	50
5.0	0	0	21	0	0	9
10	0	7	0	0	20	0

Each value is the mean from at least 3 independent experiments with 25 to 30 explants each, as determined after five weeks of culture.

Morphogenic responses of five-week-old cultures of cotyledon, hypocotyl and root explants to varying combinations of NAA, 2, 4-D, Kin and BA are shown in **Table 1**. Direct regeneration of shoots from cotyledon explants was obtained reproducibly in media that contained mainly BA (1.0 mg/*l*) and NAA (0.1 to 10 mg/*l*). Although the frequency of shoot regeneration and the number of shoots produced per cotyledon explant varied, the best results in terms of the frequency of shoot regeneration were obtained with medium supplemented with 10 mg/*l* NAA and 1 mg/*l* BA, on which about 50% of cotyledon explants developed multiple shoots after five weeks (**Table 2**). The cut surfaces of cotyledon swelled gradually within a week, green shoot primordia and limited callus formation appeared on the swollen parts after two to three weeks. Many adventitious shoots were formed directly from green primordial parts after culture of more than two weeks (**Fig. 1-A**). No adventitious shoots formed when treated with cytokinin alone in the MS basal medium or in the absence of growth regulators. These results indicate that both auxin and cytokinin are essential for induction of shoots in *A. sinicus*. In our results, the combination of NAA and BA was better than that of other phytohormones for formation of shoots on cotyledon explants.

Shoot formation on hypocotyl segments was observed on both of the cut edges and surface of the

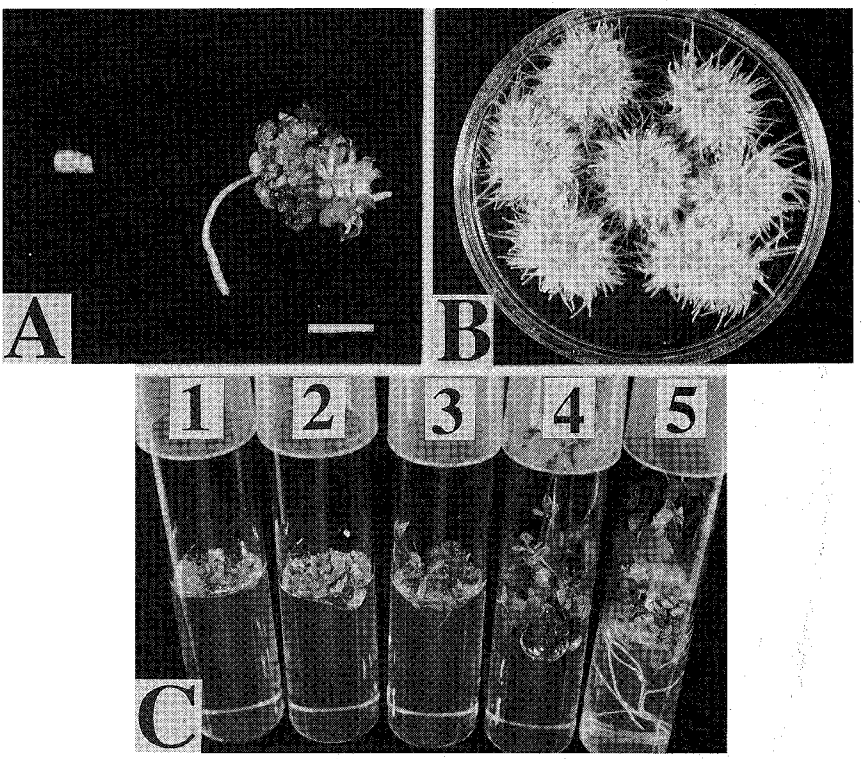

Fig. 1 (A) A cotyledon explant on hormore-free medium (left) and differentiation of adventitious shoots on medium supplemented with 0.1 mg/*l* NAA and 1 mg/*l* BA (right), after five weeks of culture in each case. Bar=5 mm. (B) Formation of callus and adventitious roots from cotyledon explants on medium supplemented with 5 mg/*l* NAA, after five weeks of culture. (C) Formation (tube 1, after three weeks of culture) and elongation (tube 2, after four weeks of culture; 3, after five weeks of culture; 4, after six weeks of culture) of shoots from a cotyledon explant on medium supplemented with 0.1 mg/*l* NAA, 1 mg/*l* BA and a shoot developing normal roots (tube 5, after eight weeks of culture) on hormone-free medium.

explants, and its frequency was much lower than that on cotyledon explants. During our experiments, we never observed any direct regeneration of shoots from root segments. The best results for shoot regeneration were obtained with the cotyledon explants (data not shown). Thus, distinct differences in morphogenic response to phytohormones *in vitro* were observed between explants of different tissues, even within a single species.

The production of adventitious roots by cultures was noted on several occasions (**Table 1**). Adventitious roots were easily obtained (at a frequency above 90%) from cultured cotyledon, hypocotyl and root explants on MS medium supplemented only with NAA. The optimal concentration of NAA for root formation was 5 mg/*l* (**Fig. 1-B**).

The adventitious shoots excised from explants were transferred for root formation to MS medium without growth regulators. Some shoots grew well (shoot elongation), formed adventitious roots at their basal parts, and subsequently developed into entire plants (**Fig. 1-C**). About 40% of adventitious shoots had rooted on the medium within two to three weeks of inoculation. About eight weeks were required to obtain plantlets from initial cultures of cotyledon explants; five weeks for generation of adventitious shoots and three weeks for generation of roots on hormone-free medium.

In this report, we have described a protocol for direct regeneration and hormonal control of

morphogenesis that gave reproducible results with different explants. Since plants regenerated from callus cultures often showed genetic variability[9,10], direct shoot regeneration without an intermediate callus phase in cultures of explants can be an advantage as it avoids culture-induced genetic variability. Since formation of shoots on the dissected cotyledons was closely associated with the cut or injured tissue, infection of *A. sinicus* by *Agrobacterium in vitro*, with subsequent transformation of whole shoots, may be possible.

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《和文要約》

レンゲソウ(*Astragalus sinicus*)の子葉切片における不定芽の形成と植物体の再生

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マメ科の冬季増殖植物であるレンゲソウの子葉，胚軸，根の切片を用いて，不定芽形成の最適条件と種々の植物ホルモン処理に対する形態応答について検討を行った．不定芽の形成は子葉切片より良好な結果が得られ，NAA 10 mg/l+BA 1 mg/l を含む培地で約 50% の効率が認められ，さらにその約 40% より根が形成され幼植物が得られた．今回初めて，レンゲソウの組織断片よりの再生条件を確立した．