In vitro shoot proliferation of *Alnus japonica* (Thunberg) Steudel

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ABSTRACT

***In vitro* proliferation system was achieved by using nodal segment excised from greenhouse grown juvenile stock plants of *Alnus japonica*. Stem explants were cultured on MS medium supplemented with different plant growth regulators of cytokinin and/or their combinations. The most effective cytokinin source was the combination of zeatin 2.0 mg/L and TDZ 0.05 mg/L producing the average number of shoots (16.8 ± 3.6). In addition, healthy roots were formed after small clumps of shoots were transferred to half strength of MS medium containing IBA 0.02 mg/L with optimal rooting capacity. Soil acclimatization was successfully conducted in cell tray containing artificially mixed soil with 92% survival rate.**

Key words **:** Acclimation, *Alnus japonica*, plant growth regulators, shoot proliferation

INTRODUCTION

In vitro proliferation is an alternative method for cloning and for gene manipulation. There is a unique method for shoot proliferation through organogenesis of axillary bud or adventitious bud formation. For inducing axillary buds, shoots can be produced from either shoot tips or nodes as the explant materials. Adventitious shoots can be formed from leaf or root segment. Among explant types, nodal culture as the simplest method can induce axillary bud for shoot proliferation.

Shoot proliferation derives from the three different original cell types; the apical meristem of the axillary bud in node cultures, the vascular cambium around the several ends of the node, and the bark cambium or phellogen in the vicinity of lenticels which are structurally differentiated portions of the periderm that serve a respiratory function (Kang and Hall, 1996). Most reports of plant regeneration are based on apical meristem or axillary bud proliferation.

Alder species have received high attention for soil amelioration and biomass production because of its nitrogen fixing symbiotic character with the actinomycete *Frankia* (Dawson, 1983). The species have been identified as a woody crop for plantations on marginal land. Vegetative propagation of alder is possible to use softwood internodal cuttings (Hall and Maynard, 1979). Clonal propagation from mature trees has been difficult, time consuming, and of limited success.

In vitro micropropagation has been reported for

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seedlings and juvenile trees of *A. glutinosa, A. crispa,* and *A. rubra* (Garton *et al.*, 1981; Read *et al.*, 1982; Louis and Read, 1983; Perinet and Lalonde 1983; Tremblay *et al.*, 1984; Barghchi, 1988). However, *Alnus japonica* is hard to propagate in *in vitro* system. A few research has been focused on *in vitro* regeneration of *A. japonica* among Alder family (Tremblay and Lalonde 1984).

The objectives of the present studies were to enhance the efficiency of micropropagation with suitable medium containing plant growth regulators and to develop a technique to maximize shoot formation from suitable explant.

MATERIALS AND METHODS

Plant materials

Nodal segments (3-4cm) containing axillary buds were collected from 2 month-old-stock plants of *Alnus japonica*, which were maintained under greenhouse conditions. The explants were obtained from the top branches of stock plants with succulent tissues. Stock plants were grown at 25℃ under natural day light supplemented to 16h photoperiod by cool white fluorescent lamps. The stock plants were watered once a day and fertilization was performed on a monthly basis with a 20N : 10P : 20K water soluble product. The collected succulent nodal segments were immersed in distilled water overnight at room temperature, dipped in 70% ethanol for 30 seconds, disinfected with a solution of 2% sodium hypochlorite for 40 minutes, and then rinsed 4 times with sterilized deionized water.

Shoot induction

In vitro cultures were conducted for shoot proliferation on MS medium (Murashige and Skoog, 1962) supplemented with different types and various concentrations of plant growth regulators. For shoot induction, three major cytokinins, BA, zeatin, and TDZ were applied for shooting in *in vitro* condition. BA was added directly into the medium before autoclaving, whereas zeatin and TDZ was added through membrane filters (0.2 ㎛ pore size) after the medium was sterilized. The medium was adjusted to pH 5.8 with 0.1N NaOH or HCl before the addition of 0.7% Sigma agar and autoclaved at 1.05 kg cm^{-2} and 121℃ for 20 minutes. The nodal cultures were maintained at 25 ± 2 ℃ with 16h photoperiod and a photosynthetically active photon flux rate of cool white fluorescent tubes. The total number of shoots and their length were observed after four weeks of initial culture. The collected data were analyzed for the average number of shoots with standard errors. Each treatment was composed of five replications with nodal explant.

Rooting test

Shoot proliferated from the explant were tested for rooting ability on the half strength of MS medium supplemented with IBA, IAA, NAA with concentration of 0.02 mg/L and kinetin and 2,4-D with concentration of 0.2 mg/L.

Hardening to soil

Rooted plantlets were transferred to cell tray after agar was removed with tap water. The plantlets were acclimated under shade, intermittent mist for two weeks, transferred to a regular greenhouse bench under shade, and grown for one month. Survival data were collected after one month period in the greenhouse.

RESULTS AND DISCUSSION

Shoot proliferation

The explant was tested for shoot proliferation with various concentrations of the cytokinins. Shoot formation was highly affected by the types and concentrations of PGRs. After 2 weeks of initial culture, the nodal segments on proliferation medium

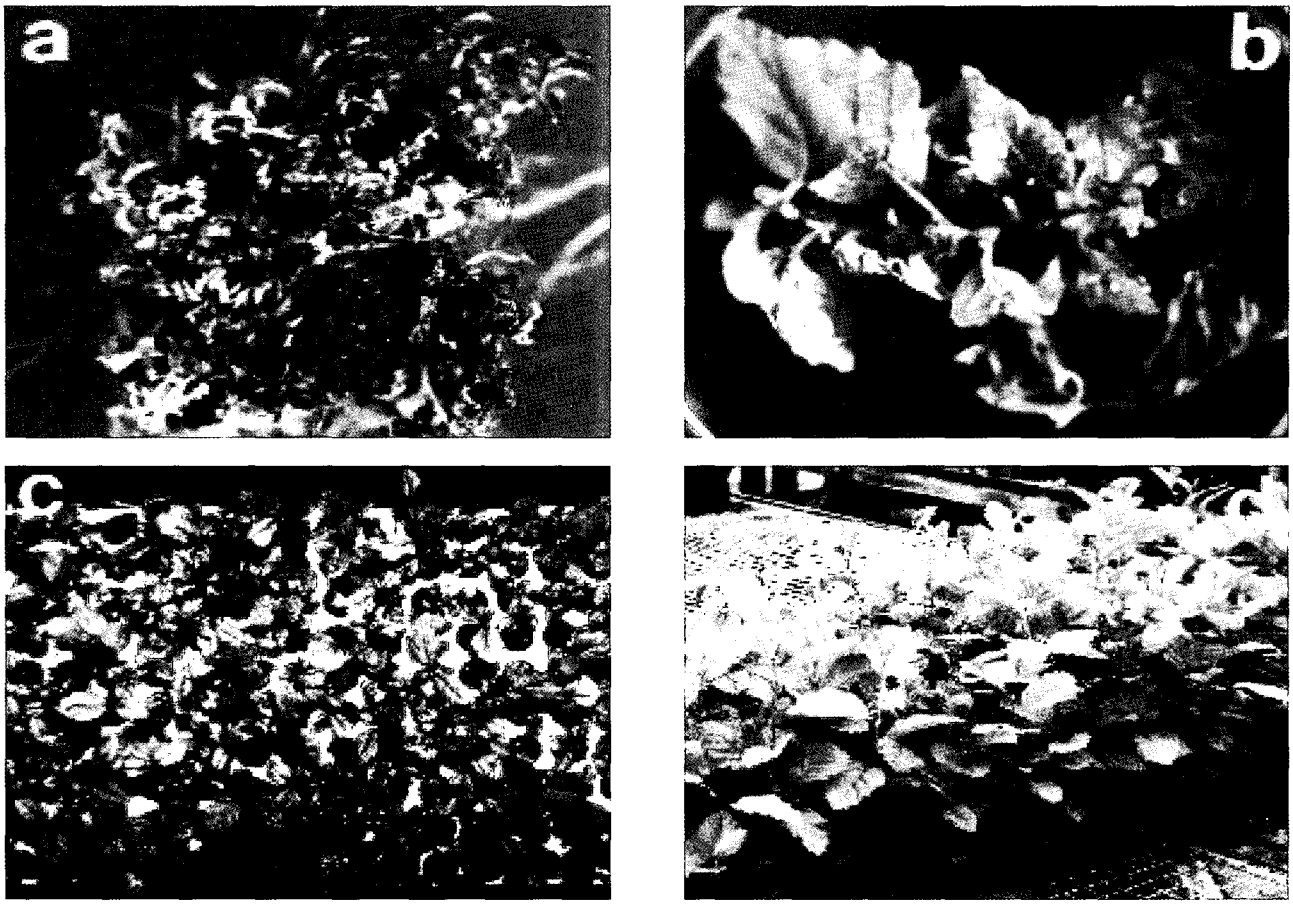

Fig. 1. Plantlet regeneration from nodal explant of *Alnus japonica* (Thunberg) Steudel. (a) Plant regeneration from nodal culture under *in vitro* condition with zeatin 2.0 + TDZ 0.05 mg/L, (b) shoot growth in the half strength of elongation medium containing IBA 0.02 mg/L, (c) hardening in spongy mini-tray under intermittent mist, (d) hardening to soil mix in regular bay under greenhouse condition.

supplemented with cytokinins produced multiple shoots more than two shoots per each explant. Most shoots were formed from axillary buds of nodal segment. The highest number of shoots (16.8 $\pm$ 3.6) was obtained from the culture of nodal explant supplemented with the proliferation medium containing zeatin 0.2 mg/L and TDZ 0.05 mg/L (Fig. 1 and Table 1).

In single treatment of cytokinins, more shoots were produced in the medium containing TDZ 0.1 mg/L. The combination of either BA and TDZ or zeatin and TDZ responded well for shoot formation with greenish small plantlets (Fig. 1-a). However, Small shoot clumps like callus were formed in higher concentrations of BA 5.0 mg/L, zeatin 5.0 mg/L, TDZ 0.5 mg/L shown yellowish or reddish shoots.

Similar studies have been reported with *A. glutinosa* and *A. crispa* by Tremblay and Lalonde (1984). They suggested that multiple shoots were produced from axillary buds on full strength MS medium supplemented with BA 0.5 mg/L. They recommended that half strength MS salts supported growth of cultures for the first 3 weeks, but after that time, cultures showed numerous deficiency symptoms. Another paper mentioned that subsequent transfers of *A. japonica* on full strength MS medium brought a higher frequency of shoot malformations and basal necrosis, together with a

Table 1. Effect of plant growth regulators on shoot proliferation from nodal segment of *Alnus japonica* after 1 month of *in vitro* culture.

Treatment(mg/L)	% of shoot development	In vitro shoot formation		
		No. of shoot	Length (cm)	Type
Control	80	1.0 ± 0.7	6.3 ± 0.7	Dark green
BA 0.2	100	4.8 ± 1.1	3.1 ± 0.2	Green
0.5	100	2.4 ± 0.5	1.8 ± 0.3	Green
2.0	100	4.0 ± 0.7	1.3 ± 0.2	Light green
5.0	40	0.4 ± 0.2	0.9 ± 0.3	Reddish
Zea 1.0	80	0.8 ± 0.4	3.6 ± 0.4	Green
2.0	100	3.8 ± 0.8	1.7 ± 0.2	Light green
5.0	80	5.6 ± 0.9	0.9 ± 0.3	Yellowish
TDZ 0.05	100	5.4 ± 0.9	0.7 ± 0.2	Green
0.1	100	9.8 ± 2.2	0.8 ± 0.3	Green
0.2	100	6.6 ± 0.9	0.9 ± 0.3	Light green
0.5	80	2.0 ± 0.7	0.7 ± 0.2	Yellowish
BA 0.2 + TDZ 0.05	100	10.6 ± 3.8	1.0 ± 0.4	Green
BA 0.5 + TDZ 0.1	100	7.8 ± 2.2	1.1 ± 0.4	Green
BA 1.0 + TDZ 0.2	80	3.6 ± 0.9	0.5 ± 0.2	Green
BA 2.0 + TDZ 0.5	80	0.8 ± 0.4	Tiny shoots	Yellowish
Zea 2.0 + TDZ 0.05	100	16.8 ± 3.6	0.9 ± 0.3	Green
Zea 2.0 + TDZ 0.1	100	11.6 ± 2.7	1.2 ± 0.4	Green
Zea 2.0 + TDZ 0.2	80	4.8 ± 0.8	Tiny shoots	Yellowish
Zea 2.0 + TDZ 0.5	67	1.2 ± 0.4	Tiny shoots	Reddish

decrease of shoot production. They concluded that modification of the nitrogen amount was necessary for *in vitro* tissue culture of alder species (Perinet and Tremblay, 1987). The presence of auxin in combination with cytokinin in shoot inducing medium was reported to be deleterious as callusing on shoots in *Alnus species*.

In present study, we enhanced shooting capacity on full strength MS medium supplemented with the combination of zeatin and TDZ or BA and TDZ, respectively. The highest number of shoots was shown in full strength of MS medium supplemented with the combination of zeatin 2.0 mg/L and TDZ 0.05 mg/L. It means that higher concentration of zeatin and lower concentration of TDZ worked well for shoot proliferation in *in vitro* tissue culture of *Alnus japonica*. To avoid shoot malformation, subsequent cultures were conducted on half strength of MS medium containing low concentrations of auxin.

In *Alnus japonica*, TDZ of cytokinins was highly affected for shooting in *in vitro* condition. In aspect of shoot length, a few shoots formed in shoot proliferation medium, showed longer multiple shoots in *in vitro* condition. Even though shoot growth promoted in control medium without any PGR sources, the best proliferation for shoot growth were full strength MS medium with zeatin 1.0 mg/L showing mean number of

3.6 ± 0.4 shoots (Table 1).

Shoot elongation

Shoots proliferated from nodal segments were excised and transferred to Magenta GA-7 boxes (7.6 × 7.6 × 10.2 cm) containing half strength MS medium supplemented with IBA, IAA, NAA, kinetin, and 2,4-D. The optimal concentrations was 0.02 mg/L for IBA, IAA, and NAA and kinetin and 2,4-D was 0.2 mg/L for shoot elongation. After 2 months of subculture, the plantlets fully elongated in *in vitro* condition (Fig. 1-b). The best concentration of plant growth regulator was 0.02 mg/L of IBA. When higher concentrations of PGRs were tested, the induced roots showed malformations such as thicker diameter and stunted length.

Similar research has been conducted with *A. cordata* for shoot elongation (Tremblay and Lalonde, 1984; Barghchi, 1988) They suggested that the treatment of IBA promoted rooting for shoot elongation. In case of *A. crispa*, full strength MS medium completely inhibited rooting compared with half strength. They mentioned that the addition of IBA in the rooting medium was essential to root microshoots of *A. crispa*, *A. glutinosa*, *A. incana*, and *A. japonica*.

Hardening to soil

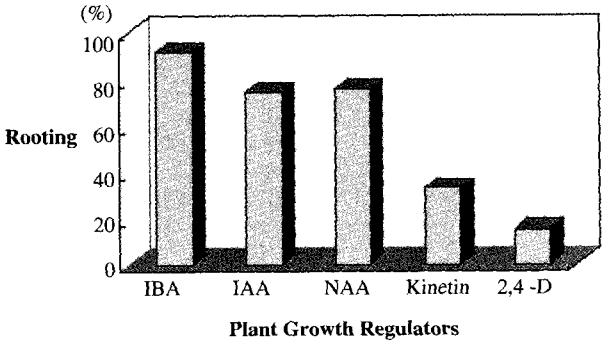

Fig. 2. Shoot elongation from proliferated shoots on half strength of MS medium in *Alnus japonica* (Thunberg) Steudel. The concentration of IBA, IAA and NAA was 0.02 mg/L and kinetin and 2,4-D were 0.2 mg/L.

In vitro proliferated shoots started rooting in mini-cell tray. After removing the agar, the expanded plantlets were transferred to mini-cell trays containing spongy soil in a shaded mist bench under greenhouse conditions (Fig. 1-c). After 2 weeks, these elongated plantlets were moved to regular cell tray in greenhouse (Fig. 1-d). The survival rate was 92% in *in vivo* condition of greenhouse.

In conclusion, the system for shoot proliferation was developed and the regeneration frequency was substantially increased by the application of zeatin and TDZ. The protocol described could prove to be useful for shoot proliferation of alder species. In addition, it could be applied for gene expression in further studies.

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LITERATURE CITED

Barghchi, M. 1988. Micropropagation of *Alnus cordata* (Loisel) Loisel. Plant Cell, Tiss. Org. Cult. 15: 233-244.

Dawson, J. O. 1983. Dinitrogen fixation in forest ecosystems. Can. J. Microbiol. 29: 979-992.

Garton S., M. A. Hosier, P. E. Read, R. S. Farnham. 1981. *In vitro* propagation of *Alnus glutinosa* Gaertn. Hort Sci. 16: 758-759.

Hall, R. B. and C. A. Maynard. 1979. Considerations in the genetic improvement of alder. In: Symbiotic Nitrogen Fixation in the Management of temperate forests. Eds. J. C. Gordon, C. T. Wheeler and D. A. Perry. Oregon State University. Corvallis, OR. pp 322-344.

Kang, H. and R. B. Hall. 1996. Shoot proliferation from *in vitro* Nodal cultures of cottonwood hybrid

(*Populus deltoides X P. nigra*). Kor. J. Plant Tiss. Cult. 23: 39-44.

Louis, K. and P. E. Read. 1983. The effect of 2ip, BA and GA on *in vitro* recultured *Alnus*. (Abstract). Hort Sci. 18: 617.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

Perinet P. and M. Lalonde. 1983. *In vitro* propagation and nodulation of the actinorhizal host plant *Alnus glutinosa* Gaertn. Plant Sci. Lett. 29: 9-17.

Perinet P. and F. M. Tremblay. 1987. Commercial micropropagation of five *Alnus* species.

Read P. E., S. Garton, K. Louis, E. S. Zimmerman. 1982. *In vitro* propagation of species for bioenergy plantations. In: Akio Fujiwara (ed) Proc. 5th Intern Congress Plant Tissue and Cell Culture, pp 757-758.

Tremblay F. M. and M. Lalonde. 1984. Requirements for *in vitro* propagation of seven nitrogen-fixing *Alnus* species. Plant Cell Tiss. Org. Cult. 3: 189-199.

Tremblay F. M., X. Nesme, M. Lalonde. 1984. Selection and micropropagation of nodulating and non-nodulating clones of *Alnus crispa* Pursh. Plant and Soil 78: 171-180.

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