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Effects of Media Composition on Plant Regeneration and Callus Formation of *Abeliophyllum distichum* Nakai

Cheol Hee Lee, Yeon Hee Jin, Young Deug Chang and Ju Kwang Hwang*
Dept. of Horticultural Science, Chungbuk National University, Cheongju 360-763, Korea

Abstract - This experiments were carried out to find out the effects of different explant materials, kinds and concentration of plant growth regulators, and total nitrogen and sucrose contents on the *in vitro* regeneration of *Abeliophyllum distichum* Nakai. The effects of growth regulators on regeneration from 3 explant sources (leaf, internode and node) were more or less same. Leaf explants produced only callus with 2ip (Isopentenyladenine) and NAA (Naphthaleneacetic acid) treatment and other regulators had no effects. Test with internode explants yielded about same results but callus was obtained with 2,4-D (2,4-Dichlorophenoxyacetic acid). Node explants resulted in shoot regeneration by all regulator treatment except NAA and 2,4-D, but control also showed similar results. Callus formation from internode and node explants was vigorous by 2ip, zeatin, and 2,4-D treatments and high NAA concentration resulted in higher callus formation. In this experiment, various mixed treatment of growth regulators were also employed, using node as explant material. Shoot regeneration was obtained with BA (Benzyl adenine) + NAA treatments but the results were comparable with control. Generally shoot and root regeneration was poor with all combined treatment except 2ip + NAA and 2,4-D + NAA. However, callus was formed readily with all treatments. In this experiment, combined treatments of regulators were applied on the callus derived from singular regulator treatment. The results showed no shoot and root regeneration with any combination of 2,4-D, IAA (Indoleacetic acid) and NAA, but soft milky white callus was formed in all the treatments. No shoot and root regeneration was observed with any combination of 2iP, NAA and IAA, but somewhat hard, light green callus was formed in all the treatments. Callus formation decreased with high kinetin concentration in case of kinetin + NAA treatment. The experiments with total nitrogen content of media showed that low concentrations of 15 and 30mM were effective for the shoot and root regeneration. Sucrose experiment demonstrated shoot regeneration with 1~4% concentration, and root and callus formation with 2~4%. No root and callus formation was observed with 0 and 1% sucrose.

Key words - Oleaceae, Masspropagation, Tissue culture, Auxin, Cytokinin, Callus

Introduction

"Misun tree" whose scientific name is *Abeliophyllum distichum* Nakai belongs to the family Oleaceae and grows at Jincheon and Goesan country of Chungbuk province. It is a shrub, about 1.5m tall, and is the only species in the genus *Abeliophyllum*. It is classified as one of a rare species because of its very limited habitat, and, therefore, is protected as Natural Monument no. 147, 220, and 221 (Lee, 1976, 1990). However, natural habitat of Jincheon have been so much destroyed that it is not on the Monument list anymore (Kim *et al.*, 2002).

In Korea, Central Council of the Preservation of Nature was formed on 1979 for the nationwide movement on the conservation of nature, but legal and technical measures for the enforcement of the conservation have not been followed afterwards (Woo, 1990; Lee, 1990). Plant tissue culture technologies have been used effectively for the preservation and propagation of rare and endangered plant and tree species (Miller, 1993; Youn *et al.*, 1992; Moon *et al.*, 1997; Withers, 1991), but reports on *A. distichum* Nakai is very scanty (Moon *et al.*, 1999). Present studies were carried out to establish methods for the micropropagation of *A. distichum* Nakai, one of endangered tree species in Korea.

Materials and Methods

Axillary buds of *A. distichum* Nakai grown at Cheongju were collected, cut into about 2cm pieces and sterilized according to Moon *et al.* methods (1997) during April. Basal medium was MS (Murashige and Skoog, 1962) containing 3% sucrose and 0.8% agar, and its pH was adjusted to 5.8. Three replicates were employed in

*Corresponding author. E-mail : jkhwang@chungbuk.ac.kr

each experiment under the culture conditions of 25±1℃ and 16 hour day-length of 40μmol · m^{-2} · s^{-1} intensity. Derived plantlets were subcultured on MS media once every 4 weeks for mass propagation.

To evaluate the effects of plant growth regulators on plant regeneration, 7 kinds of regulators with concentration of 0～20 mg · L^{-1} were added to the media. Tissues of leaves, internodes, and nodes were used as explant materials. The size of explants was 0.5 ×0.5cm for leaf tissues, and 1cm for internodes and nodes including axillary buds. After selecting the best explant source, and the best concentration and kind of regulator by singular application experiments, mixed treatments of regulators were conducted. When callus were used as an explant material, 100mg tissue was employed. Explants were placed on 30ml media in 100ml culture vessels with 3 replicates of 4 samples / replication. After 8 weeks of culture, fresh weight, shoot numbers and length, root numbers and length, and callus weight were measured.

To asses the effects of total nitrogen contents on the plant regeneration, 4 levels of nitrogen concentration ranging from 1/4X, 1/2X, 1X, to 2X of basic MS medium were employed in this experiment. Sucrose concentrations used were 0, 1, 2, 3, and 4%.

All experiments were laid on a Completely Randomized Design (CRD). Treatment means were compared using Duncan's Multiple Range Test (DMRT) at 5% level using Statistical Analysis System (SAS) Program.

Results and Discussion

For mass propagation of *A. distichum* Nakai, the rare tree species growing only in Korea, various plant materials such as leaves, internodes and nodes were employed to select the best explant sources firstly. The regeneration of shoots, and roots was not observed when leaf materials were used as plants, except in case of 5 mg · L^{-1} NAA (Naphthaleneacetic acid) treatment which produced 0.3 roots per explant (Data not shown). Treatment of 2iP (Isopentenyladenine) and zeatin produced no shoot and root formation, but active callus formation was observed. The highest yield of green callus was obtained with 10mg · L^{-1} 2iP treatment. Treatment of 2,4-D (2,4-Dichloro phenoxyacetic acid) produced light white callus. It is generally known that high concentration of NAA and kinetin induced callus from leaf tissues (Seong *et al,* 1993; Lee *et al,* 2002), but in this experiment callus was not induced by NAA and low rate of root formation was obtained by 5mg NAA (Data not shown). Experiments with cytokinins showed different results - kinetin did not induce callus formation while 2iP and zeatin did.

When internode explants were used, growth regulators produced no improvement in shoot regeneration over control (Data not shown). Root regeneration was not obtained in all treatment except in NAA and IAA (Indoleacetic acid). Maximum root formation (1 root / explant) was found in 20mg · L^{-1} IAA. Shoot regeneration (1.7 shoots / explant) was observed on the medium containing 20mg · L^{-1} 2iP only. Callus formation decreased as the concentration of 2,4-D, zeatin and 2iP increased (Data not shown). Like the case of leaf explants, 2,4-D produced soft light white callus, and 2iP somewhat light green callus which were also reported in *Lycium chinense* Mill. by treatment of NAA and BA (Benzyl adenine) (Kim *et al.,* 2001). In *Actinidia arguta* Planch., combined treatments of zeatin, NAA and BAP on internode culture produced callus with roots, but zeatin alone yielded only shoots, suggesting the influence of NAA and BA on callus induction (Kim *et al.,* 1997). In our experiments internode culture on NAA containing media yielded small few roots and callus, but internode died out on BA. Vigorous callus and shoots were induced by 2iP treatment.

The use of nodes as explants resulted in increased fresh weight in 2iP, zeatin, NAA, 2,4-D, compared to control (Fig. 1, Table 1). However BA, kinetin and IAA treatment had no effects on fresh weight and high concentration rather inhibited growth. At low concentration of BA, shoot regeneration was better than other treatments, optimum concentration being 1mg · L^{-1} (3.0 shoots / explant). The number of roots regenerated was 1.9～2.0 per explant in 1～5mg · L^{-1} NAA, but it was no better than control. Treatment of BA and 2,4-D produced no roots, and addition of regulators tended to inhibit root formation. No callus was formed with kinetin and IAA treatments, but 1mg · L^{-1} 2,4-D yielded 2.1g of callus. It was reported that treatment of zeatin or kinetin on the node culture of *A. disticum* Nakai resulted in shoot proliferation with poor callus formation (Moon *et al.,* 1999). Consequently they let shoots grow and used shoot segments as explant for mass propagation. They also mentioned on the further research on shoot growth because shoot growth was influenced by explant location and timing of explant extraction. In *Hovenia dulcis* Thunb. high shoot regeneration was obtained by node culture on MS and WPM media containing 0.1 mg · L^{-1} of BA and NAA (Eom *et al.,* 2002), but in our experiments high shoot formation was obtained with 1mg · L^{-1} BA, and high callus formation with 2,4-D. Treatment of BA 1mg · g^{-1} with 0.5 mg · g^{-1} TDZ resulted in best shoot formation in shoot formation in

Table 1. Effect of growth regulators on shoot regeneration from nodes of *Abeliophyllum distichum* Nakai after 8 weeks in culture

Growth regulators		Fresh wt.	Shoot		Root		Callus wt.
(mg · L^{-1})		(g)	No.	Length (cm)	No.	Length (cm)	(g)
Control		0.78fgh[z]	1.3cdefg	0.9fgh	0.8b	1.7a	0
BA	1	0.19fgh	3.0a	4.6cd	0	-	0.01h
	2	0.11fgh	2.3b	2.3e	0	-	0.01h
	5	0.08gh	2.0bc	1.5efgh	0	-	0.01h
	10	0.04gh	1.3defg	0.4gh	0	-	0
	20	0.01h	0	-	0	-	0
Kinetin	1	0.14fgh	1.8bcde	0.5fgh	0.2c	0.1c	0
	2	0.13fgh	2.0bc	0.6fgh	0.1c	0.3c	0
	5	0.12fgh	1.8bcde	0.7fgh	0	-	0
	10	0.01h	0.8h	0.1h	0	-	0
	20	0.01h	0	-	0	-	0
2iP	1	0.75d	1.7bcdef	7.7a	0.1c	0.1c	0.56de
	2	0.72d	1.5cdefg	7.2a	0	-	0.53ef
	5	0.73d	1.9bcd	5.7bc	0	-	0.55de
	10	0.61de	1.0fg	1.1efgh	0	-	0.52ef
	20	0.04gh	0	-	0	-	0.02h
Zeatin	1	1.07c	1.5cdefg	5.9b	0.1c	0.1c	0.60de
	2	0.63de	1.6bcdefg	5.9b	0.1c	0.1c	0.50ef
	5	0.75d	1.7bcdef	6.5ab	0	-	0.58de
	10	0.63de	1.3cdefg	4.2d	0	-	0.52ef
	20	0.58de	1.1efg	1.7efg	0	-	0.46ef
NAA	1	0.35efg	0.1h	0.1h	2.0a	1.9a	0.08gh
	2	0.40ef	0	-	1.9a	1.1b	0.29fg
	5	1.09c	0	-	2.0a	1.5ab	0.91c
	10	1.08c	0	-	0.3bc	0.4c	0.93c
	20	0.57de	0	-	0	-	0.54e
IAA	1	0.07gh	1.4cdefg	1.5efg	0.1c	0.1c	0
	2	0.08gh	2.0bc	1.9ef	0.2c	0.1c	0
	5	0.07gh	1.7bcdef	2.4e	0.3bc	0.5c	0
	10	0.04gh	1.3cdefg	0.6fgh	0	-	0
	20	0.04gh	0.9g	0.8fgh	0	-	0
2,4-D	1	2.10a	0	-	0	-	2.10a
	2	1.24bc	0	-	0	-	1.24b
	5	1.41b	0	-	0	-	1.41b
	10	0.78d	0	-	0	-	0.78cd
	20	0.24fgh	0	-	0	-	0.23gh

[z]Mean separation within columns by Duncan's multiple range test, 5% level.

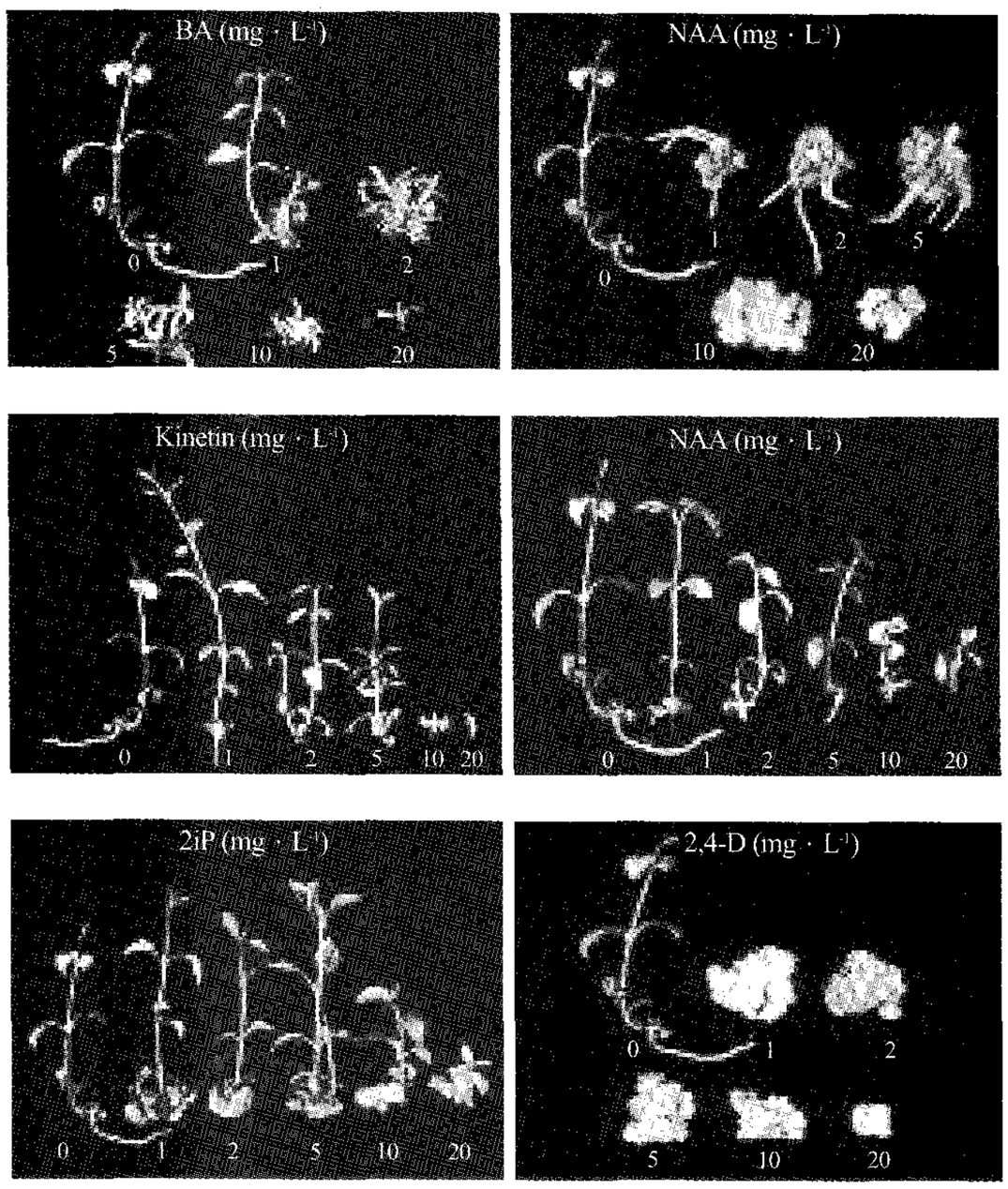

Fig. 1. Cultural response from nodes explant of *Abeliophyllum distichum* Nakai on MS medium containing different concentration of growth regulators for 8 weeks.

Withania sonifera (L.) Dunal. (Lee *et al.,* 2005). Therefore, addition of BA to node culture media is critical to shoot regeneration in many tree species, and the effects would be enhanced by combined treatment with other auxin and cytokinin regulators.

Shoot formation of *A. distichum* Nakai was possible only with node as explants. Therefore, mixed treatments of regulators to enhance the shoot regeneration were carried out with node as explants. Table 2 shows that fresh weight increased with 5 and 10 mg · L^{-1} BA mixed with various NAA concentrations. However, number of shoot formation was lower than singular treatment of growth regulators. Root regeneration was not observed at higher BA concentration. Trend of callus formation was similar to fresh weight in 2 and 5mg · L^{-1} BA concentration, and high concentration of NAA reduced callus formation (Fig. 2). The importance of IBA on rooting of *A. distichum* Nakai was reported by other workers (Moon *et al.,* 1999), but in our experiments it was not so. The callus induction is known to be greatly influenced by many factors such as plant species, plant parts, and kinds and concentration of plant growth regulators added to culture media (Rai and Chandra, 1988), and even with same plant callus type is different due to explant materials and concentration of regulators (Tao and Sugiura, 1992). So it is very important to find out adequate plant materials and optimum concentration and kind of growth regulators for mass propagation of rare plant species.

Mixed treatment of 5mg · L^{-1} kinetin + 1mg · L^{-1} NAA resulted in highest fresh weight increase (0.58g), and in general 2～5mg · L^{-1} kinetin produced high fresh weight. Higher shoot regeneration

Table 2. Effect of BA and NAA concentration on plant regeneration from *Abeliophyllum distichum* Nakai nodes cultured on MS medium for 8 weeks

Growth regulators (mg · L^{-1})		Fresh wt.	Shoot		Root		Callus wt.
BA	NAA	(g)	No.	Length (cm)	No.	Length (cm)	(g)
2	0.5	0.54a[z]	0.9ab	0.6a	0.1ab	0.1b	0.46a
	1.0	0.21bc	0.1c	0.1a	0	-	0.17bc
	2.0	0.57a	0.3bc	0.2a	0.7a	0.7a	0.47a
5	0.5	0.55a	1.4a	0.7a	0	-	0.38ab
	1.0	0.53a	0.4bc	0.4a	0.4ab	0.2b	0.41ab
	2.0	0.56a	0.3bc	0.4a	0.2ab	0.1b	0.52a
10	0.5	0.57a	0.8abc	0.5a	0	-	0.52a
	1.0	0.49a	0.7bc	0.4a	0	-	0.42ab
	2.0	0.46ab	0.8abc	0.5a	0	-	0.33abc
20	0.5	0.41ab	0.8abc	0.5a	0	-	0.35abc
	1.0	0.36abc	0.4bc	0.3a	0	-	0.29abc
	2.0	0.14c	0.4bc	0.2a	0	-	0.12c

[z]Mean separation within columns by Duncan's multiple range test, 5% level.

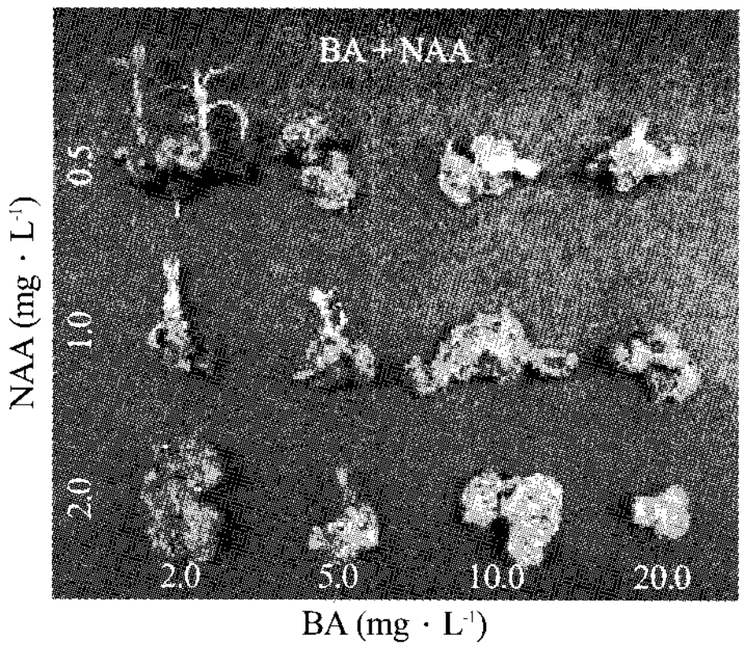

Fig. 2. Cultural response of *Abeliophyllum distichum* Nakai nodes cultured on MS media supplemented with BA and NAA for 8 weeks.

was obtained with 2~10mg · L^{-1} kinetin + 0.5mg · L^{-1} NAA but still lower than singular treatment. Relatively high root regeneration was observed with 2mg · L^{-1} kinetin + 0.5mg · L^{-1} NAA, but high kinetin concentration produced no root regeneration, regardless of NAA concentration levels. Best callus formation was 0.53g by 2mg · L^{-1} kinetin + 1mg · L^{-1} NAA treatment (Table 3). Combined treatment of 2ip and NAA generally resulted in fresh weight and callus increase, but shoot and root regeneration was poor (Table 4). Shoot regeneration was lower than other combined treatments. Root formation was also poor with high 2iP concentration.

Based on the results of singular and mixed treatments of

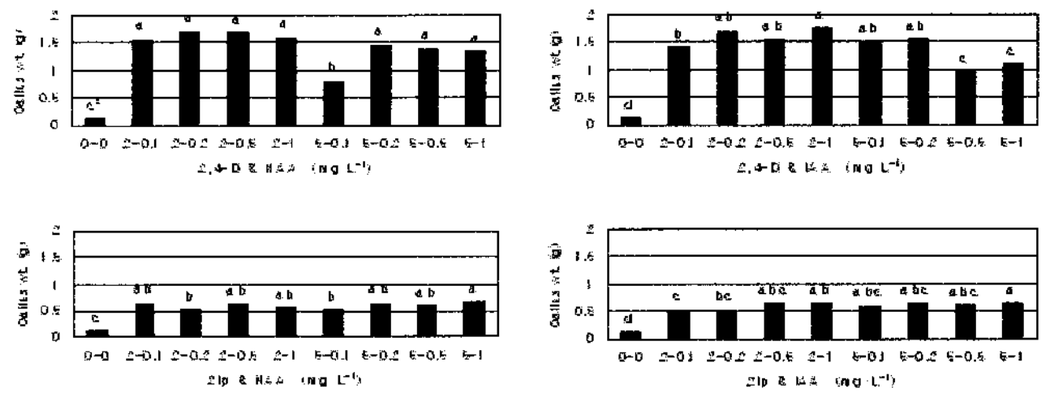

Fig. 3. Cultural response of *Abeliophyllum distichum* Nakai callus cultured on MS media supplemented with 2,4-D, 2ip and NAA, IAA for 8 weeks.
[z]Mean separation within treatments by Duncan's multiple range test, 5% level.

regulators, various combined regulator treatments were carried out to induce good callus formation and shoot regeneration. In this experiments, callus derived from 5mg · L^{-1} 2ip + 1mg · L^{-1} NAA treatment and from 2mg · L^{-1} 2,4-D were used and results are shown in Fig. 3. Callus from 2mg · L^{-1} 2,4-D treatment showed much higher proliferation rate than the control. The highest fresh weight and callus formation was 1.76g obtained by 2mg · L^{-1} 2,4-D + 1mg · L^{-1} IAA treatment. However, no shoot or root regeneration was observed. The callus formation was favorable with 2iP + NAA treatment. In treatment of 2iP + IAA, callus formation was promoted with the increase of IAA concentration. The high callus formation was 0.66g obtained by 5mg · L^{-1} 2iP + 1mg · L^{-1} NAA. As above mentioned case, no shoot or root regeneration was

Table 3. Effect of kinetin and NAA concentration on plant regeneration from *Abeliophyllum distichum* Nakai nodes cultured on MS medium for 8 weeks

Growth regulators (mg · L^{-1})		Fresh wt.	Shoot		Root		Callus wt.
Kinetin	NAA	(g)	No.	Length (cm)	No.	Length (cm)	(g)
2	0.5	0.50abcd[z]	0.6a	1.6a	1.7a	0.6a	0.39abc
	1.0	0.57ab	0.5a	0.4bc	0.9ab	0.7a	0.53a
	2.0	0.54abc	0.3a	0.2c	0.5b	0.1a	0.46a
5	0.5	0.51abcd	0.7a	0.7abc	0.6b	0.5a	0.40abc
	1.0	0.58a	0.4a	1.4ab	0.3b	0.4a	0.50a
	2.0	0.36bcd	0.4a	0.2c	0.3b	0.1a	0.22cd
10	0.5	0.29de	0.8a	1.1abc	0.3b	0.6a	0.20cde
	1.0	0.37abcd	0.1a	0.1c	0	-	0.25cd
	2.0	0.34cd	0.3a	0.2c	0.1b	0.1a	0.29bc
20	0.5	0.04f	0.3a	0.1c	0	-	0.02e
	1.0	0.04f	0.1a	0.1c	0	-	0.02e
	2.0	0.12ef	0.3a	0.1c	0	-	0.08de

[z]Mean separation within columns by Duncan's multiple range test, 5% level.

Table 4. Effect of 2iP and NAA concentration on plant regeneration from *Abeliophyllum distichum* Nakai nodes cultured on MS medium for 8 weeks

Growth regulators (mg · L^{-1})		Fresh wt.	Shoot		Root		Callus wt.
2iP	NAA	(g)	No.	Length (cm)	No.	Length (cm)	(g)
2	0.5	0.96ab[z]	0.7a	0.8a	0.2a	0.1a	0.91ab
	1.0	0.84ab	0.3ab	0.3abc	0.3a	0.1a	0.83ab
	2.0	0.64b	0.3ab	0.2bc	0.1a	0.1a	0.63ab
5	0.5	1.05a	0.4ab	0.7ab	0.1a	0.1a	0.93a
	1.0	0.78ab	0	-	0	-	0.77ab
	2.0	0.89ab	0.1b	0.1c	0	-	0.88ab
10	0.5	0.81ab	0.3ab	0.1c	0	-	0.80ab
	1.0	0.77ab	0.2ab	0.3abc	0	-	0.77ab
	2.0	0.72ab	0.3ab	0.2bc	0	-	0.72ab
20	0.5	0.60b	0.1b	0.1c	0	-	0.56ab
	1.0	0.87ab	0.3ab	0.2bc	0	-	0.85ab
	2.0	0.61b	0	-	0	-	0.51b

[z]Mean separation within columns by Duncan's multiple range test, 5% level.

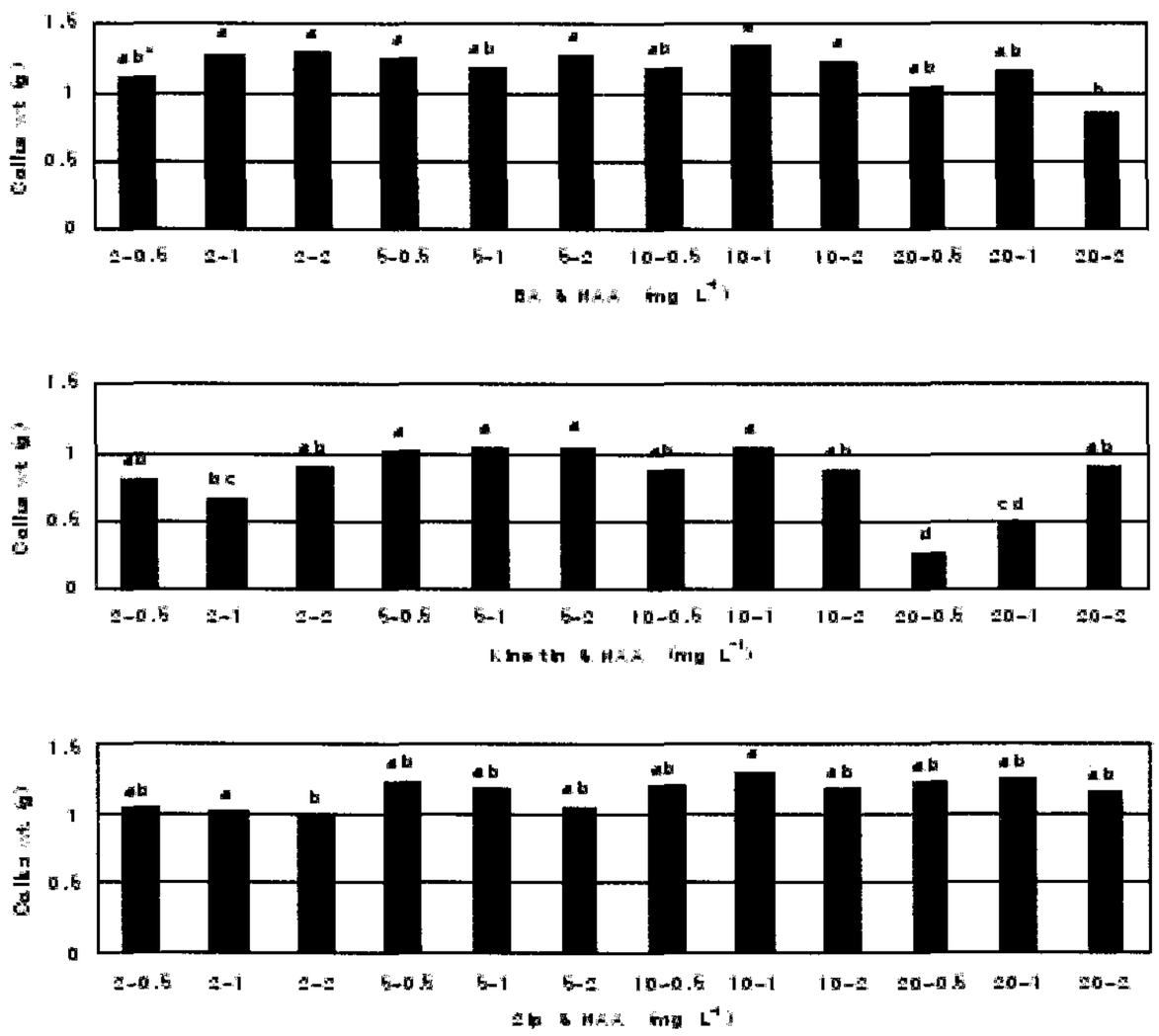

Fig. 4. Cultural response of *Abeliophyllum distichum* Nakai callus cultured on MS media supplemented with BA, kinetin, 2ip and NAA for 8 weeks.

[z]Mean separation within treatments by Duncan's multiple range test, 5% level.

observed.

To evaluate the effect of cytokinin on callus formation, BA, kinetin and 2iP were combined with NAA, and result were shown in Fig. 4. Callus formation was favorable by BA + NAA treatments, while treatment of kinetin + NAA produced lower callus formation. Treatment of 2iP + NAA resulted in similar callus formation, comparable to BA + IAA treatment. Best callus proliferation was 1.33g obtained by 10mg · L^{-1} BA + 1mg · L^{-1} NAA.

Table 5 show the results of experiment that evaluated the effect of total nitrogen concentration on plant regulation. Node tissue was

Table 5. Effect of total nitrogen concentration on plant regeneration from *Abeliophyllum distichum* Nakai nodes cultured on MS medium[z] for 8 weeks

Total nitrogen concentration	Fresh wt. (g)	Shoot		Root		Callus wt. (g)
		No.	Length (cm)	No.	Length (cm)	
× 1/4 of MS	0.47a[y]	1.8b	5.8a	2.2a	3.6a	0.19a
× 1/2 of MS	0.67a	2.8a	6.9a	1.6ab	2.8a	0.26a
× 1 of MS	0.58a	1.8b	4.9a	0.9bc	3.4a	0.31a
× 2 of MS	0.47a	0.9c	0.8b	0.1c	0.1a	0.30a

[z]MS basal media contained BA 5mg · L^{-1} and NAA 0.5mg · L^{-1}.
[y]Mean separation within columns by Duncan's multiple range test, 5% level.

Table 6. Effect of sucrose concentration on plant regeneration from *Abeliophyllum distichum* Nakai nodes cultured on MS medium[z] for 8 weeks

Sucrose (%)	Fresh wt. (g)	Shoot		Root		Callus wt. (g)
		No.	Length (cm)	No.	Length (cm)	
0	0.01d[y]	0c	-	0	-	0c
1	0.31c	0.1b	0.1b	0	-	0c
2	0.46bc	1.6a	4.5a	0.7b	3.6ab	0.22bc
3	0.58c	1.8a	4.9a	0.9ab	3.4ab	0.31b
4	0.83a	1.7a	4.8a	1.3a	3.8a	0.57a

[z]MS basal media contained BA 5mg · L^{-1} and NAA 0.5mg · L^{-1}.
[y]Mean separation within columns by Duncan's multiple range test, 5% level.

used as explant sources. Best shoot formation was obtained by 30 mM nitrogen (2.8 shoots / explant) and 120mM resulted in lowest shoot and root regeneration. Root regeneration decreased as the nitrogen content increased, but callus formation increased with higher nitrogen contents.

In the process of plant tissue culture, sucrose is added to the media as a carbon source. Here concentrations of 0~4% were used and the results were shown on Table 6. Sucrose of 3% produced best shoot formation (1.8 shoots / explant), but results were about same except in 0 and 1%. Generally root and shoot formation increased with higher sucrose content.

Acknowledgements

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