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Micropropagation of Plantlets through Callus in Taranoki (*Aralia elata*)

タラノキ（*Aralia elata*）カルスからの幼植物体の大量増殖

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Abbreviations: BAP, 6-benzyl aminopurine; BT, broad-leaved tree medium (Chalupa 1984); 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kn, kinetin (6-furfurylamino purine); MS, Murashige and Skoog medium (1962); NAA, α-naphthaleneacetic acid; PGR, plant growth regulator.

Summary

In order to establish the micropropagation protocol of plantlets in Taranoki (*Aralia elata*), the present study investigated callus induction from petiole and leaf segments and subsequent plantlet regeneration. The highest frequency of callus induction (96.7%) was obtained in a BT medium supplemented with 5.0 μM 2,4-D using petiole explants. The highest regeneration rate of adventitious shoots, number of total shoots / culture, and average length of shoots / culture from petiole-derived calli were obtained in a PGR-free BT medium. The highest frequency (100%) of rooting was achieved when they were cultured in a BT medium containing 2.0 μM NAA. Rooted shoots were acclimatized successfully and established in the soil under greenhouse conditions with a final survival rate of 72%.

Keywords: *Aralia elata*, micropropagation, callus, adventitious shoot, acclimatization

要　旨

本研究では、タラノキ（*Aralia elata*）における幼植物体の大量増殖法の確立のために、葉柄及び葉からのカルス誘導及びカルスからの幼植物体の再生を検討した。最も高いカルス誘導率（96.7%）は、外植体に葉柄を用いて、5.0 μM 2,4-D を添加した BT 培地において得られた。葉柄由来カルスからの不定芽の再生率、シュート数及び平均シュート長は、植物成長調節物質を含まない BT 培地で培養した場合に最も高い値を示した。また、再生したシュートから根の誘導を行ったところ、2.0 μM NAA を添加した BT 培地で100％の発根率が得られた。発根した幼植物体は、温室下で順化を行ったところ、生存率は最終的に72％を示した。

キーワード：*Aralia elata*、大量増殖、カルス、不定芽、順化

Introduction

Taranoki (*Aralia elata*) is widely utilized in Japan. The fresh buds of *A. elata* are used as fresh edible vegetables due to their flavor and nutritional values [9]. The root bark of this plant has also been used for traditional folk medicine to cure cough, cancer, diabetes, gastric ulcer, hepatitis, and rheumatoid arthritis [13]. The extracts of this plant have been used for Korean traditional medicine to treat diabetes [4]. The young shoots and leaves are also used for the production of cosmetics.

Root cuttings have been used for the clonal propagation of *A. elata* as a conventional propagation method [9]. Although seeds can be used for multiplying this species, it is difficult for them to germinate, and they require a long time for stratification. The low viability and germination rate of seeds delay the rooting of seedlings. However, conventional propagation via seeds or stem and root cuttings still takes too much time. On the other hand, there are many reports on plant regeneration through tissue culture of *A. elata* [1,5,7,8,10,11,14]. However, when the breeding and micropropagation of plantlets were conducted in *A. elata*, more detailed regarding tissue culture would be needed.

In the present study, callus induction from petiole and leaf segments and plantlet regeneration from calli were investigated to establish the micropropagation protocol of *A. elata*.

Materials and Methods

Induction and culture of callus

Petioles and leaves of *A. elata* were collected from mature plants growing in Nikko Experimental Forest, Utsunomiya University, Japan. After washing with running tap water, they were cut into small pieces, 2 cm in length. They were surface-sterilized with 70% ethanol for 30 sec., further sterilized with a 3% sodium hypochlorite aqueous solution containing a few drops of Tween 80 for 10 min., and rinsed three times in sterilized distilled water. The explants were placed in an MS medium [12] and BT medium [3] solidified with 0.8% agar with the pH adjusted to 5.8. To examine the effects of PGRs on callus induction and shoot regeneration, different types of auxin (2,4-D, NAA, IBA, and IAA) and concentrations (1.0, 2.0, 5.0, and 10.0 μM) were used in this study. The data were collected as the percentage of contamination-free explants, callus induction, and death of explants after 8 weeks of culture. Cultures were maintained in an air-conditioned culture room at a 16-hr photoperiod with a light intensity of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 25 ± 1°C.

Induction of adventitious shoots from callus

After 8 weeks of culture, calli that appeared regenerable and were yellow greenish in color were used for the induction of adventitious shoots. Calli were cultured in a BT medium with different concentrations, types, and combinations of PGR: 0.5 or 1.0μM cytokinin (BAP or Kn) alone; combinations of a cytokinin (BAP or Kn) and an auxin (IBA or NAA) at 1.0 + 0.5 or 1.0 + 1.0μM; or without PGRs. The culture conditions were the same as those for callus culture. After 8 weeks of culture in the media, the percentage of calli that formed shoots, number of total shoots per culture, and average length of shoots were recorded.

Rooting from shoots

After 10 - 12 weeks of culture initiation, the usable shoots were excised from proliferated multiple shoots. Then, individual shoots were placed into rooting media supplemented with different concentrations (0.5, 1.0, 2.0, and 5.0 μM) of auxin (NAA, IBA, and IAA) or without auxin. After 7 weeks of culture, the root formation rate, number of total roots, and average length of roots were recorded.

Acclimatization

Prior to transplantation, individual regenerated plantlets were removed from the culture medium, and roots were washed by continuous flashing with running tap water to remove the remaining gel. Plantlets were then transferred to small plastic pots (6 × 9 cm) containing vermiculite. For the first 7 - 15 days of transfer, potted plants were kept covered with transparent plastic pots to maintain high humidity. This practice facilated gradual acclimatization of transplants in an *ex vitro* environment. The transplants were maintained under culture room condition. The potted plants were watered regularly. After one month of transfer, plantlets were transferred to larger pots (18 × 24 cm) with Kanuma soil (Kanuma Bonsai Potting Medium Co., Japan) and maintained under greenhouse conditions. In this way, the potted plantlets were successfully acclimatized to natural conditions through a gradual increase in their exposure time to sunlight.

Results

Callus induction from petiole and leaf explants

Table 1 shows the callus induction rate recorded after 8 weeks of culture. Of the two different media, the BT medium was better for callus induction than the MS medium. The frequencies of the highest callus initiation in the BT medium supplemented with 5.0 μM 2,4-D were 96.7% and 83.3% for petiole and leaf explants, respectively. These calli were yellow-greenish in color and appeared regenerable. Among different types of auxin, 2,4-D showed the best performance for callus induction from both explants (Fig. 1 A, B). On the other hand, in the MS medium supplemented with 5.0 μM 2,4-D, the maximum frequencies of callus induction were 81.7% and 68.3% for petiole and leaf explants, respectively. These calli were hard, compact, and deep green in color; they were not able to regenerate any shoots when cultured.

Table 1 Callus induction from petiole and leaf explants after 8 weeks of culture.

PGR (μM)		MS		BT	
		Callus formation from petiole (%)	Callus formation from leaf (%)	Callus formation from petiole (%)	Callus formation from leaf (%)
Free		0.0	0.0	0.0	0.0
2,4-D	1.0	50.0	36.7	58.3	48.3
	2.0	65.0	53.3	76.7	65.0
	5.0	81.7	68.3	96.7	83.3
	10.0	38.3	31.7	48.3	40.0
NAA	1.0	36.7	31.7	45.0	38.3
	2.0	46.7	38.3	55.0	48.3
	5.0	71.7	58.3	81.7	71.7
	10.0	43.3	35.0	50.0	40.0
IBA	1.0	33.3	23.3	38.3	31.7
	2.0	25.0	16.7	35.0	28.3
	5.0	46.7	33.3	58.3	48.7
	10.0	25.0	21.7	35.0	30.0
IAA	1.0	18.3	11.7	26.7	18.3
	2.0	26.7	21.7	33.3	26.7
	5.0	33.3	20.0	40.0	28.3
	10.0	16.7	11.7	25.0	16.7

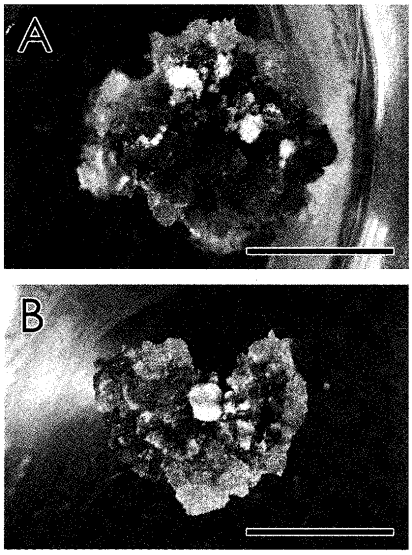

Fig. 1 Callus induction from petiole (A) and leaf (B) explants.
Note: Callus induction after 4 weeks of culture on BT media containing 5.0 μM 2,4-D; scale bar = 1 cm.

Therefore, petiole- or leaf-derived calli induced in the BT medium supplemented with 5.0 μM 2,4-D were used for the subsequent plant regeneration process.

Adventitious shoot regeneration from petiole-derived callus

Table 2 shows the effects of combinations and concentrations of PGR on adventitious shoot regeneration from petiole-derived callus. It was observed that adventitious shoot buds were formed on the calli within 2 weeks of culture, and they elongated accompanied with leaf development within 4 weeks of culture. The calli cultured in the BT medium without any PGRs produced the highest percentage of shoots, 96.7%, with a total number of 39.1 ± 1.2 shoots and 6.1 ± 0.3 cm average length of shoots per culture (Fig. 2 A, B). Adventitious root formation was also observed during the initiation of shoots in a PGR-free BT medium. Thin and long roots were induced from the base of shoot clusters. In the presence of different concentrations of BAP in a BT medium, 0.5 μM BAP showed better results for shoot regeneration from petiole-derived calli. In this medium, the shoot formation frequency was 86.7 %, and the total number and average length of shoots per culture were 30.2 ± 1.7 and 5.1 ± 0.3 cm, respectively.

Of the different concentrations and combinations of a cytokinin and an auxin, the BAP-NAA formulation showed better performance than the other combinations (Table 2). The combination of 1.0 μM BAP + 0.5 μM NAA showed the highest frequency of shoot regeneration (80%), the greatest number (28.6 ± 1.5), and the greatest length (6.0 ± 0.5 cm) of shoots per callus (Fig. 2 C, D).

Table 2 Adventitious shoot regeneration from petiole-derived callus after 8 weeks of culture.

PGR (μM)		Shoot formation (%)	Number of total shoots/culture	Average length of shoots/culture (cm)
Free		96.7	39.1 ± 1.2 a	6.1 ± 0.3 a
BAP	0.5	86.7	30.2 ± 1.7 b	5.1 ± 0.3 b
	1.0	73.3	24.2 ± 1.3 c	4.2 ± 0.4 b
Kn	0.5	46.7	12.1 ± 1.1 f	4.2 ± 0.2 b
	1.0	70.0	19.0 ± 1.7 d	5.0 ± 0.3 b
BAP + IBA	1.0 + 0.5	66.7	16.2 ± 1.2 e	5.9 ± 0.5 a
	1.0 + 1.0	60.0	12.1 ± 1.0 d	4.6 ± 0.3 b
BAP + NAA	1.0 + 0.5	80.0	28.6 ± 1.5 b	6.0 ± 0.5 a
	1.0 + 1.0	56.7	23.4 ± 1.0 c	4.9 ± 0.4 b
Kn + IBA	1.0 + 0.5	63.7	11.9 ± 1.0 f	4.9 ± 0.3 b
	1.0 + 1.0	50.0	9.8 ± 1.0 f	4.4 ± 0.4 b
Kn + NAA	1.0 + 0.5	70.0	24.0 ± 1.2 e	5.8 ± 0.3 a
	1.0 + 1.0	53.3	18.2 ± 1.8 d	4.6 ± 0.2 b

Values represent means ± standard errors of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple range test at 0.05 probability level.

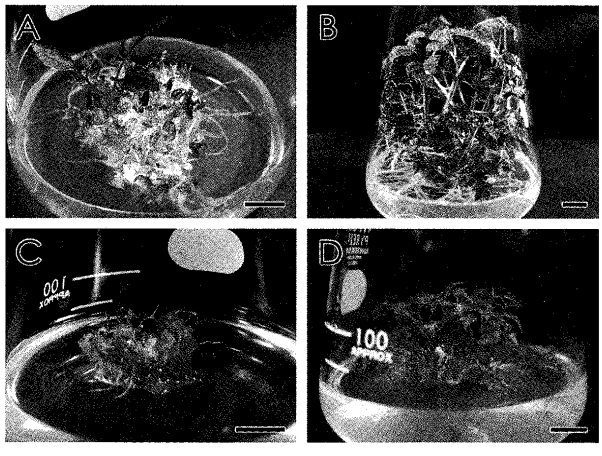

Fig. 2 Adventitious shoots developed from petiole-derived callus on BT media.
Note: A&B, after 4 weeks (A) and 10 weeks (B) of culture without any PGRs; C&D, after 5 weeks (C) and 9 weeks (D) of culture with 1.0 μM BAP + 0.5 μM NAA; scale bar = 1 cm.

Adventitious shoot regeneration from leaf-derived callus

Table 3 shows the effects of the combinations and concentrations of PGR on adventitious shoot regeneration from leaf-derived calli. In this study, it was observed that adventitious shoot buds were formed from calli within 3 weeks of culture initiation and elongated accompanied with leaf development within 5 weeks of culture. The percentage of shoot formation, number of total shoots per culture, and average shoot length were recorded after 8 weeks of culture. The calli produced the highest percentage of shoots per culture, 86.7%, with a total 27.6 ± 1.8 shoots per culture and 5.5 ± 0.4 cm average length of shoots, in a PGR-free BT medium (Fig. 3 A, B). Adventitious root formation was also observed during the initiation of shoots in the same BT medium; however, the induced roots were very thin.

Of the different combinations of PGR, Kn + NAA showed better results for shoot regeneration than other combinations, such as BAP + NAA, BAP + IBA, and Kn + IBA. The highest percentage of shoot regeneration in this combination was 76.7%, and the highest number of shoots per culture and average length of shoots per culture were 21.2 ± 1.4 and 5.6 ± 0.5 cm, respectively, which were recorded in the BT medium with 1.0 μM Kn + 0.5 μM NAA (Fig. 3 C, D).

Table 3 Adventitious shoot regeneration from leaf-derived callus after 8 weeks of culture.

PGR (μM)		Shoot formation (%)	Number of total shoots/culture	Average length of shoots/culture (cm)
Free		86.7	27.6 ± 1.8 a	5.5 ± 0.4 a
BAP	0.5	70.0	12.5 ± 1.1 d	4.2 ± 0.3 b
	1.0	66.7	10.0 ± 0.8 d	3.3 ± 0.3 c
Kn	0.5	60.0	17.7 ± 1.3 c	4.2 ± 0.4 b
	1.0	80.0	23.1 ± 1.5 b	5.1 ± 0.4 a
BAP + IBA	1.0 + 0.5	60.0	12.1 ± 1.2 d	4.0 ± 0.4 b
	1.0 + 1.0	46.7	8.2 ± 0.9 e	3.2 ± 0.3 c
BAP + NAA	1.0 + 0.5	68.0	17.0 ± 1.2 c	4.3 ± 0.4 b
	1.0 + 1.0	53.3	13.1 ± 1.0 d	4.1 ± 0.4 b
Kn + IBA	1.0 + 0.5	66.7	16.6 ± 1.3 c	5.6 ± 0.5 a
	1.0 + 1.0	50.0	12.4 ± 1.0 d	4.3 ± 0.4 b
Kn + NAA	1.0 + 0.5	76.7	21.2 ± 1.4 b	5.6 ± 0.5 a
	1.0 + 1.0	60.0	17.2 ± 1.2 c	4.5 ± 0.5 b

Values represent means ± standard errors of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple range test at 0.05 probability level.

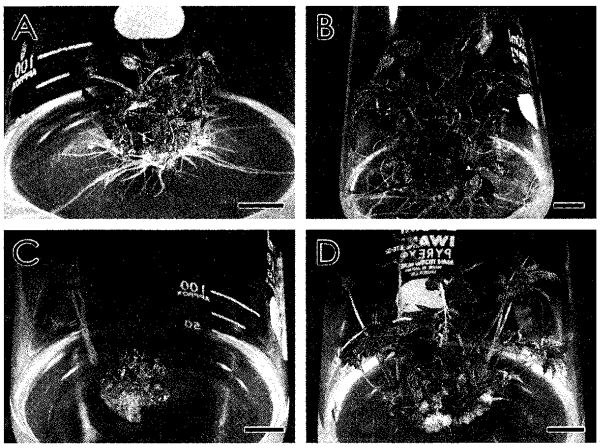

Fig. 3 Adventitious shoots developed from leaf-derived callus on BT media.

Note: A&B, after 5 weeks (A) and 9 weeks (B) of culture without any PGRs; C&D, after 4 weeks (C) and 9 weeks (D) of culture with 1.0 μM Kn + 0.5 μM NAA; scale bar = 1 cm.

Rooting of in vitro-regenerated shoots

The percentage of root formation, number of roots per microshoot, and average length of roots were recorded after 7 weeks of culture, and the results are presented in Table 4.

Of the different types of auxin in a BT medium, the NAA-containing media showed better results for root induction. The highest frequency (100%) of root formation with a total number of roots of 12.4 ± 0.3 and average length 6.2 ± 0.3 cm was obtained in the BT medium containing 2.0 μM NAA (Fig. 4). On the other hand, the MS medium showed lower performance for the induction of roots than the BT medium (Table 4). In addition, in the MS medium, roots were mulformed, and callus formation was observed at the cut base of microshoot (Fig. 4). The maximum frequencies of root induction were 86.7, 73.3, and 60.0% for NAA, IBA, and IAA, respectively, in MS media containing 2.0 μM of each auxin. No root formation was observed in the auxin-free medium.

Table 4 Root formation from *in vitro* grown microshoots after 7 weeks of culture.

Basal media	PGR (μM)		Root formation (%)	Number of total roots/shoot	Average length of roots/shoot (cm)
MS	Free		0.0	-	-
	NAA	0.5	80.0	4.4 ± 0.7 d	3.4 ± 0.1 d
		1.0	76.7	5.4 ± 0.7 d	4.8 ± 0.2 c
		2.0	86.7	8.3 ± 0.6 b	5.6 ± 0.2 b
		5.0	70.0	2.1 ± 0.3 e	3.3 ± 0.2 d
	IBA	0.5	63.3	3.1 ± 0.2 e	2.3 ± 0.3 e
		1.0	66.7	3.7 ± 0.9 e	2.6 ± 0.2 e
		2.0	73.3	4.7 ± 0.3 d	4.8 ± 0.2 c
		5.0	56.7	2.0 ± 0.3 e	2.3 ± 0.3 e
	IAA	0.5	46.7	2.1 ± 0.9 e	2.2 ± 0.3 e
		1.0	50.0	2.7 ± 0.7 e	3.1 ± 0.3 d
		2.0	60.0	3.8 ± 0.3 e	4.1 ± 0.3 c
		5.0	30.0	1.6 ± 0.6 f	1.9 ± 0.2 e
BT	Free		66.7	6.6 ± 0.5 c	5.4 ± 0.3 b
	NAA	0.5	93.3	7.4 ± 0.6 b	5.3 ± 0.2 b
		1.0	96.7	7.9 ± 0.4 b	5.1 ± 0.3 b
		2.0	100.0	12.4 ± 0.3 a	6.2 ± 0.3 a
		5.0	93.3	6.3 ± 0.8 c	4.1 ± 0.3 c
	IBA	0.5	86.7	4.3 ± 0.9 d	3.3 ± 0.2 d
		1.0	90.0	5.1 ± 1.0 d	4.2 ± 0.3 c
		2.0	96.7	8.1 ± 0.5 b	5.1 ± 0.3 b
		5.0	80.0	3.9 ± 0.5 d	3.3 ± 0.3 d
	IAA	0.5	60.0	2.2 ± 0.9 e	1.9 ± 0.4 e
		1.0	70.0	3.5 ± 0.7 d	2.1 ± 0.2 e
		2.0	83.3	5.2 ± 0.3 d	4.0 ± 0.3 c
		5.0	50.0	2.5 ± 0.3 e	2.0 ± 0.2 e

Values represent means ± standard errors of 10 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different by Tukey's multiple range test at 0.05 probability level.

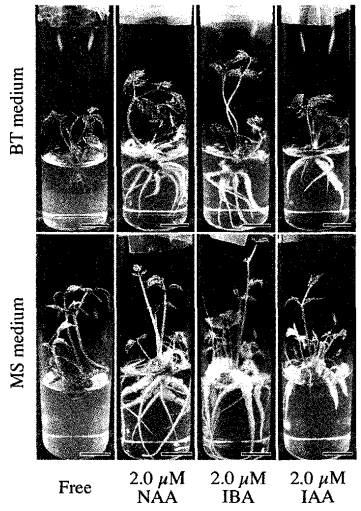

Fig. 4 Rooting of *in vitro* grown microshoots on MS and BT media after 8 weeks of culture.
Note: scale bar = 1 cm.

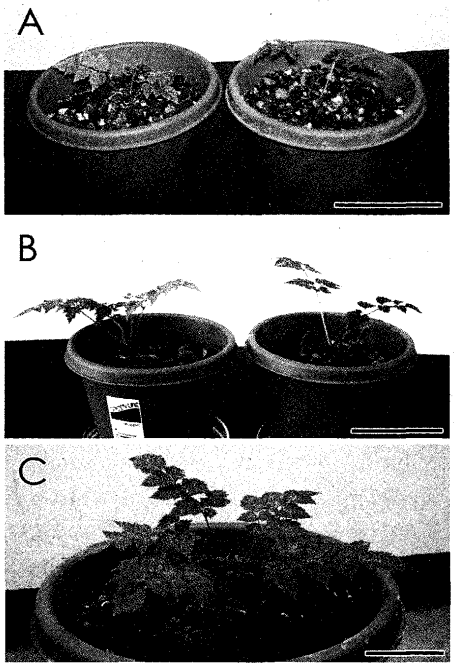

Fig. 5 *Ex vitro* establishment of in *vitro* regenerated plantlets.
Note: Plantlets growing on vermiculite under culture room conditions after 2 weeks (A) and 5 weeks (B) of transfer, and plantlets grown under green house conditions on soil after 8 weeks (C) of transfer; scale bar = 5 cm.

Acclimatization and establishment of in vitro-regenerated plantlets in the soil

The 40 *in vitro*-regenerated plantlets were transferred to small pots containing vermiculite. Plantlets were initially established in small pots (Fig. 5 A, B) for handling during transplantation. Using this treatment, 90% of plantlets survived and were able to overcome the surrounding environmental conditions. It was noticed that the prevailing atmospheric conditions of the transplanting season largely influenced the initial survival of potted plantlets. After one month, when the initially established plantlets were transferred to larger pots containing soil (Fig. 5 C) and placed in the greenhouse, the final survival rate was 72% for rooted shoots.

Discussion

Induction and growth of callus

Chalupa [3] reported that, as nutrient media, BT and WP (a woody plant medium) were found to be the best for the growth and development of shoots for most explants of *Quercus* robur. As shown in Table 1, the BT medium was better for callus induction than the MS medium. Yoshizawa *et al.*[14] reported that, when petiole explants were cultured in a BT medium with 1.0 mg/l (5.37 μM) NAA or 1.0 mg/l (4.52 μM) 2,4-D, calli were effectively induced. In a BT medium, nitrogenous compounds (NH_4NO_3 and KNO_3) exist in a ten-fold lesser amount than in an MS medium, although the BT medium contains $(NH_4)_2SO_4$ and $Ca(NO_3)_2 \cdot 4H_2O$ as additional nitrogenous elements [3, 12]. Such differences in the amounts of nitrogenous compounds between MS and BT media may have resulted in the induction and growth of calli in *A. elata.*

In general, it is well known that phenoxy auxins (e.g. 2,4-D) are strong promoters of callus induction and growth [2]. In callus induction from various types of explants in *A. elata*, 2,4-D, either alone or in combination with other PGRs, was an effective promoter of callus induction and growth [1,5,7,11,14]. For example, Moon and Youn [13] reported that all concentrations (0.9, 2.3, and 4.5 μM) of 2,4-D tested induced calli successfully, whereas the growth of calli was retarded as the levels of the 2,4-D concentration increased. In the present study, of the different types of auxin, 2,4-D showed the best performance for callus induction from petiole explants.

Adventitious shoot formation from petiole- and leaf-derived calli

It is well known that adventitious shoot formation requires treatment with auxin and cytokinin [2]. However, auxin should be used sparingly because excessive auxin application favors callus growth but frequently causes shoot abnormalities [2]. On the other hand, in the present study, regarding adventitious shoot regeneration from petiole- and leaf-derived calli, the PGR-free BT medium showed the best result for shoot induction (Tables 2, 3). In paticular, a 100% shoot formation rate was observed in petiole-derived callus cultured in a PGR-free BT medium (Table 2). Amemiya *et al.* [1] succeeded in plant regeneration through petiole-derived calli cultured in MS media supplemented with 2,4-D and BAP. Yoshizawa *et al.* [14] reported that both the adventitious roots and the buds regenerated within 10 days of culture when the calli were induced in a BT medium

containing 0.1 mg/l (4.52 μM) 2,4-D and then subcultured in a PGR-free medium. In addition, Furuya and Hosoki [5] also reported that somatic embryos and adventitious shoots were induced in a PGR-free MS medium from root-derived calli cultured in MS media containing 2,4-D. In *A. elata*, therefore, when calli were induced in a medium containing 2,4-D, adventitious shoots could be easily formed from callus cultured in media containing any kinds of PGR.

Rooting of adventitious shoots and acclimatization of in vitro-grown plantlets

There are many physical and chemical factors that favor rooting *in vitro* [2]. The auxins IBA and NAA are often more effective for rooting than IAA [6]. In the present study, among the different concentrations and combinations of PGR, the BT medium containing NAA showed the best results for root formation, number of total roots / culture, and average length of roots / culture (Table 4).

Micropropagated plants are generally susceptible to transplantation shock [2]. Therefore, plants should be properly acclimatized when they are transferred from an *in vitro* environment to soil [2]. In *A. elata, Jhang et al.* [7] reported callus induction and plant regeneration from leaf tissue cultures. However, they achieved only 20% plantlet regeneration from calli with hormone-free media. In the present study, when the initially established plantlets were transferred to larger pots containing soil (Fig. 5 C) and placed in a greenhouse, after one month, the final survival rate was 72% for rooted shoots. It can be said that the survival rate of plantlets during acclimatization was relatively high compared to the results obtained by Jhang *et al.* [7]. However, further research is needed to increase the survival rate of *in vitro*-rooted plantlets during acclimatization.

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