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The Effect of Plant Growth Regulators and Sucrose on the Micropropagation and Microtuberization of *Dioscorea nipponica* Makino

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Abstract

The effects of sucrose, plant growth regulators, MS (Murashige and Skoog), and ½MS salt media formulations were investigated for the development of shoot cultures, microtuber induction, and plantlet regeneration in *Dioscorea nipponica*. The cytokinin N-benzyladenine (BA) in the range of 0.5–2.0 mg/l showed strong enhancing effects on microtuber induction only when used in conjunction with the auxin alpha-naphthalene acetic acid (NAA), with the effect that NAA increased from 0.5 to 2.0 mg/l. Murashige and Skoog salt media supplemented with sucrose at 3% (w/v) gave the highest frequencies of shoot induction (86%) when BA was present at 2.0 mg/l and NAA at 1.0 mg/l. Sucrose at 7% (w/v) was the single most significant medium constituent for microtuber growth. The heaviest microtubers were formed on media containing 1.0 mg/l BA and 2.0 mg/l (0.073 g), especially with 7% sucrose (3.46 g). With media containing ½MS, 2% sucrose, and 0.1% (w/v) activated charcoal, the percentage of rooting was maximal when supplemented with 1.0 mg/l BA and 0.5 mg/l NAA for the *in vitro* produced shoots (95%) and BA and NAA both at 0.5 mg/l for the microtubers (100%). When removed from culture flasks and transferred into sterilized soil in a greenhouse, most of the hardened plantlets survived (over 91% after 1 week), and they were suitable for field planting after 1 month.

Key words: *Dioscorea nipponica* Makino; Microtuber induction; Regeneration; Hormone; Sucrose

Introduction

Dioscorea nipponica Makino is a perennial herbaceous vine belonging to the family Dioscoreaceae. In China, *D. nipponica* is an important medicinal plant because its root stock is used as a Chinese crude

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drug. The essential component of *D. nipponica* is diosgenin, an important active substance used for the synthesis of steroid hormone medicine. Diosgenin can prolong clotting time, deplete thrombase reduction time, and treat measles (Gao and others 2003). It plays a role in protecting the liver (Nibbering and others 2001), inhibiting malignant cell multiplication (Moalic and others 2001), curing bone rarefaction (Higdon and others 2001), and improving diabetes (AI-Habori and others 2001). In recent years, as a result of industrial development in the synthesis of steroidal hormone medicine, the requirement for *D. nipponica* has increased in demand and the wild resources are rapidly being depleted. At present, *D. nipponica* is on the list as a secondary-level endangered plant species in China as its wild resource is facing extinction (Fu 1992).

Seed propagation and rhizome cutting are two routine propagation methods in *D. nipponica*, but the recovery rates and germination rates of natural seeds are poor. For seed propagation, the seedling stage takes about 25–28 days, and the rhizome is harvested 5 years after the initial growth. For vegetative propagation, a great deal of the rhizome is used, so the efficiency of vegetative propagation is low. In addition, in the process of storage and cultivation, the materials used for vegetative propagation are susceptible to diseases that cause tissue senescence and degeneration. Plant tissue culture techniques can be used to conserve germplasm resources and even to rapidly breed elite varieties (Han and others 2000). Some researchers have studied organ (Sedigeh and others 1998; Kohmura and others 1995; Martine and Mario 1991), callus (Yuan and others 2005; Chen and others 2003; Viana and Mantell 1989), and cell culture (Twyford and Mantell 1996; Osifo 1988) of other plants from the genus *Dioscorea*. Investigations on *D. nipponica*, however, have not yet been described. Plant tissue culture and cell culture have a number of advantages and have been called the third mode of production after wild growth and cultivation (Breat and Carlv 1985). In this article we report the results of a study on the optimization of cultural conditions for *in vitro* induction and production of shoots and microtubers in *D. nipponica*.

Materials and Methods

Tender vines about 30–40 days old were used to obtain the original explants and were cut into stem pieces with a single axillary bud. The tender vines were grown from wild-grown *D. nipponica* tubers collected from their natural habitat, the Changbai Mountain in Northeast China. The identity of the tubers was confirmed by the expert in the School of Life Sciences, Northeast Normal University. After the tender stem cuttings were defoliated and thoroughly washed with tap water for 30 min, their surfaces were sterilized with 75% alcohol for 30–60s and 0.1% mercuric chloride solution (approximate 1% active chlorine) for 3–5 min and then rinsed 5–6 times with sterile distilled deionized water. The shoot segments were then cut into single node explants about 4–7 mm long. They were inoculated vertically in a flask (100 ml) with 30 ml of initiation medium containing MS (Murashige and Skoog 1962) salts supplemented with the macroelements, microelements, and vitamins at full concentration, 3% (w/v) sucrose, and 0.7% (w/v) agar according to a standard yam nodal segment culture protocol (Mantell and others 1978; Mantell and Hugo 1989). There were 10–16 nodal explants per flask.

In experiments to test the effect of various concentrations of different plant growth hormones, the initiation medium mentioned above was supplemented with BA at various concentrations (0, 0.2, 0.5, 1.0, 2.0, or 4.0 mg/l), alone or in combination with NAA (0, 0.2, 0.5, 1.0, or 2.0 mg/l). For the microtuber induction medium, the MS medium was supplemented with 0.1% (w/v) activated charcoal and 5%, 7%, and 8% sucrose, plus the various hormone combinations. For rooting, the medium contained ½MS medium with 2% (w/v) sucrose, 0.1% (w/v) activated charcoal, and the various hormone combinations. In all cases, the pH of the medium was adjusted with 1 mol/l NaOH to 5.8 before being autoclaved at 121°C for 15 min.

One hundred and fifty ml flasks were used to culture the nodal explants, each containing 30 ml of the respective initiation medium and covered with a cotton plug and kraft paper (thicker than normal paper with increased gas permeability). Two hundred and fifty ml flasks were used for root induction and plantlet regeneration, each containing 50 ml of media. These were also covered with a cotton plug and kraft paper. All cultures were incubated under 60 μmol m^{-2} s^{-1} light provided by a white, cool fluorescent tube at a photoperiod of 16 h at 18°–26°C.

When the explants developed from the nodal explants on initiation media to 30–40-mm-long shoots, they were removed, and the bases with microtubers attached were transferred onto microtuber induction media. Whole plantlets developed on this medium after 28 days, with and without microtubers, were transferred intact onto rooting medium for further development.

The regenerated plantlets were acclimated in the flasks in the culture room for 2 days by removing

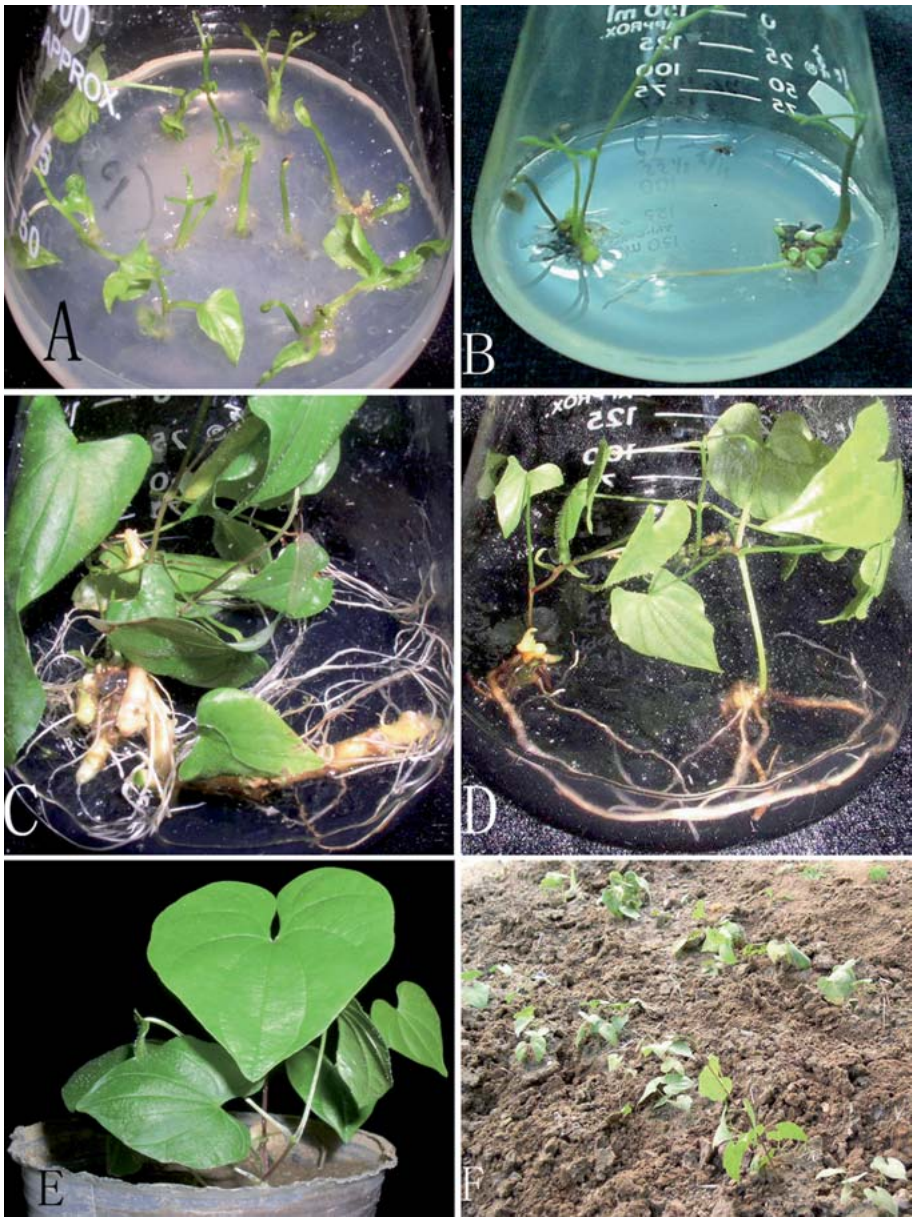

Figure 1. Plant regeneration of *D. nipponica* via segment cutting with a node. **A.** Shoot formed from a nodal explant cultured on MS medium supplemented with 3% (w/v) sucrose, NAA 0.5 mg/l and BA 2.0 mg/l for 28 days. **B.** Multiple buds and shoots developed gradually after nodal explants were cultured on MS medium supplemented with 3% (w/v) sucrose, NAA 1.0 mg/l and BA 2.0 mg/l after 42 days. **C.** Microtubers growing on MS medium supplemented with 7% (w/v) sucrose, 0.1% (w/v) activated charcoal, NAA 2.0 mg/l and BA 1.0 mg/l after 60 days. **D.** Rooting in ½MS media supplemented with 2% (w/v) sucrose, 0.1% (w/v) activated charcoal, BA 2.0 mg/l, and NAA 0.5 mg/l after 28 days. **E.** Regenerated plantlets transplanted into sterilized soil in a greenhouse after 30 days. **F.** Regenerated plantlets transplanted into the field after 28 days.

the covers. The plantlets were then transplanted into sterilized soil in the greenhouse. The plantlets were sprayed with water 2–3 times each day for the first 2 weeks; then they were transplanted to the field. The number of explants contaminated at each stage throughout these experiments was included in the tabulated data.

Results

Tissue Culture of Nodal Explants from Tender Stem Cuttings

Nodal explants of tender stem cuttings of *D. nipponica* Makino were planted on basal media supplemented with hormone combinations at different concentrations. After 4–7 days in culture, the lateral buds displayed visible growth, and most of them grew into 35–50-mm-long shoots within 4 weeks (Figure 1A). Buds inducted by the different treatments were single or multiple (Figure 1B), and microtubers were observed within 4 weeks after initial cultivation. Multiple buds growing on a suitable initiation medium developed into plantlets, with part of the plantlet producing microtubers.

Table 1 shows that, on media containing only BA hormonal supplements, the rate of shoot induction and shoot height increased with increasing hormone concentration up to 2.0 mg/l and then dropped at 4.0 mg/l as the BA became superoptimal. At

Table 1. Effect of N-benzyladenine (BA) on Shoot Formation and Microtuber Induction of *D. nipponica* Makino Nodal Explants Cultured *in vitro* for 28 Days

Concentration of BA in media (mg/l)	Number of explants cultured	Number of explants contaminated	Percentage of nodes forming shoots (%)	Average shoot height (mm)	Number of microtubers
0	66	7	21.21	26[d]	0
0.2	73	4	26.03	31[c]	0
0.5	78	0	35.90	38[b]	0
1.0	65	4	47.69	41 [a]	0
2.0	89	8	52.81	42[a]	0
4.0	86	5	18.60	29[c]	0

Means were compared by using the least significant difference (LSD) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$. For this experiment, the MS medium was supplemented with 3% sucrose; the nodal explants came from wild-grown D. nipponica tubers. After culture in vitro after 28 days, height per shoot uncontaminated was measured shoot by shoot.
Percentage of nodes forming shoots (%) = number of explants forming shoots/number of explants initiated × 100%. If more than one shoot was generated, it was calculated as one.

Table 2. Effect of Alpha-Naphthalene Acetic Acid (NAA) on Shoot Formation and Microtuber Induction of *D. nipponica* Makino Nodal Explants Cultured *in vitro* for 28 Days

Concentration of NAA in media (mg/l)	Number of explants cultured	Number of explants contaminated	Percentage of nodes forming shoots (%)	Average shoot height (mm)	Percentage of shoots forming microtubers (%)	Average fresh weight of microtubers (g)
0	66	7	21.21	26[d]	0	0
0.2	37	0	45.95	29[c]	0	0
0.5	38	4	55.26	34[b]	6.90	0.022
1.0	32	3	62.50	41[a]	12.50	0.028
2.0	34	0	55.89	39[a]	20.59	0.039

Means were compared by using the least significant difference (LSD) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$. For this experiment, the MS medium was supplemented with 3% sucrose; the nodal explants came from wild-grown D. nipponica tubers. After culture in vitro after 28 days, per shoot uncontaminated was washed to remove adherent medium and water was swabbed before the microtubers of each shoot were weighed.
Percentage of shoots forming microtubers (%) = number of explants forming microtubers/number of explants initiated × 100%. If more than one microtuber was generated, it was calculated as one.

4.0 mg/l BA, rosette buds alone were produced within 4weeks. Explants did not form microtubers in media with only BA and without NAA. N-benzyladenine of excessively high or low concentrations could result in formation of fewer shoots and shorter shoots, or no shoot at all.

When the media were supplemented with only NAA, shoot induction was similar to media supplemented with BA alone, with the rate of shoot induction and shoot height increasing with NAA to 1.0 mg/l and then decreasing at 2.0 mg/l as NAA became superoptimal (Table 2). When the concentration of NAA was at 0.5–2.0 mg/l, microtubers were also produced. The percentage of microtubers produced and the average fresh weight of these microtubers increased with NAA from 0.5 to 2.0 mg/l. When NAA was at excessively high or low concentrations, formation of fewer shoots and shorter shoots could result, but NAA of high concentrations promoted the formation of more and heavier microtubers.

On the basis of single hormone experiments, the combinations of BA (0.5–2.0 mg/l) and NAA (0.2∼2.0 mg/l) supplements were tested. The data presented in Table 3 indicate that the highest shoot induction frequency was obtained on media with 2.0 mg/l BA and 1.0 mg/l NAA. Microtuber production increased with increasing NAA concentration. The heaviest microtubers were obtained on media with hormonal supplements of 1.0 mg/l BA and 2.0 mg/l NAA. Overall, the percentage of explants forming microtubers was lower than that obtained with NAA alone (Table 2), but their weight was greater on media with both BA and NAA.

At any BA concentration, increasing the NAA concentration tended to favor shoot induction, shoot elongation height, and microtuber production (Table 3). According to the data presented in the

Table 3. Effect of BA and NAA Synergism on Shoot Formation and Microtuber Induction of *D. nipponica* Makino Nodal Explants Cultured *in vitro* for 28 Days

Concentration of hormone in media (mg/l)		Number of explants cultured	Number of explants contaminated	Percentage of nodes forming shoots (%)	Average shoot height (mm)	Percentage of shoots forming microtubers (%)	Average fresh weight of microtubers (g)
BA	NAA						
0.5	0.2	25	0	28.00	32^{g}	0	
0.5	0.5	32	2	37.50	35^{f}	0	
0.5	1.0	38	5	50.00	39^{e}	2.63	0.043
0.5	2.0	41	4	34.15	43^{d}	4.88	0.051
1.0	02	39	0	48.72	33^{fg}	0	
1.0	05	37	2	56.76	45^{cd}	2.70	0.058
1.0	1.0	36	0	63.89	46^{bc}	5.56	0.062
1.0	2.0	35	4	68.57	49^{b}	5.71	0.073
2.0	0.2	39	3	71.79	48^{bc}	0	
2.0	0.5	31	0	77.42	52^{a}	3.23	0.053
2.0	1.0	28	1	85.71	54^{a}	3.57	0.066
2.0	2.0	34	0	73.53	55^{a}	5.88	0.061

Means were compared by using the regression sum of squares (SSR) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$. For this experiment, the MS medium was supplemented with 3% sucrose; the nodal explants came from wild-grown D. nipponica tubers.

Table 4. Effect of BA, NAA, and Sucrose on Microtuber Fresh Weight of *D nipponica* Makino Nodal Explant Base Subcultured *in vitro* for 60 Days The nodal explant base used in this experiment had buds and microtubers.

Combination of hormone	Average fresh weight of microtubers (g)		
Concentration of BA and NAA (mg/l)	Sucrose (w/v) 5%	Sucrose (w/v) 7%	Sucrose (w/v) 8%
BA0.5 + NAA0.5	(***26–3***) 1.562^{h}	(***25–0***) 1.998^{i}	(***28–3***) 1.778^{i}
BA0.5 + NAA1.0	(***28–2***) 1.692^{f}	(***27–2***) 2.135^{f}	(***27–0***) 1.862^{g}
BA0.5 + NAA2.0	(***26–0***) 1.811^{c}	(***28–3***) 2.487^{c}	(***24–1***) 2.018^{e}
BA1.0 + NAA0.5	(***23–3***) 1.689^{f}	(***25–1***) 2.015^{h}	(***27–2***) 2.156^{c}
BA1.0 + NAA1.0	(***25–6***) 1.736^{d}	(***27–0***) 2.630^{b}	(***28–2***) 2.363^{b}
BA1.0 + NAA2.0	(***24–2***) 1.885^{a}	(***26–0***) 3.456^{a}	(***26–6***) 2.482^{a}
BA2.0 + NAA0.5	(***26–4***) 1.628^{g}	(***23–1***) 2.109^{g}	(***24–0***) 1.857^{h}
BA2.0 + NAA1.0	(***28–5***) 1.702^{e}	(***26–1***) 2.157^{e}	(***23–0***) 1.973^{f}
BA2.0 + NAA2.0	(***24–1***) 1.829^{b}	(***24–4***) 2.196^{d}	(***26–2***) 2.116^{d}

Means were compared by using the regression sum of square (SSR) test ($p \leq 0.05$). Data within each column followed by dissimilar letters differ significantly at $p \leq 0.05$. In this table, the bold, italic digits within parentheses represent, respectively, the number of microtubers planted and the number of microtubers contaminated.
A part of the shoots with microtubers forming in Tables 2 and 3 was used as the starting material for this experiment. The sizes of these microtubers were almost the same. These whole shoots were randomly planted into the MS media supplemented with 0.1% (w/v) activated charcoal.

tables, the optimal hormonal combination for shoot induction (2.0 mg/l BA and 1.0 mg/l NAA) was different from that for microtuber production (1.0 mg/l BA and 2.0 mg/l NAA).

Microtuber growth

Whole plantlets with microtubers formed on initiation media were planted onto the microtuber growing medium. After 20 days, heavier and more microtubers formed gradually. Thin light-brown or yellowish-white strips appeared on the surface of the microtubers. There were a lot of buds that would potentially develop into plantlets. The inner part of the microtuber was white (Figure 1C). The data presented in Table 4 indicate the effect of MS media supplemented with hormone and sucrose at different concentrations and 0.1% (w/v) activated charcoal on microtuber growth after the shoots were planted for 60 days. The results indicated that, for a given concentration of hormonal combination, the average fresh weights of microtubers growing on 7%

(w/v) sucrose were heavier than those growing on the other two concentrations, except for the hormonal combination of BA 1.0 + NAA 0.5. When the MS media were simultaneously supplemented with BA 1.0 mg/l, NAA 2.0 mg/l, 7% (w/v) sucrose and 0.1% (w/v) activated charcoal, microtuber growth was better than in any of the other treatments; the average fresh weight reached 3.46 g (Table 4).

Rooting and Growth of Plantlets after Transplanting

When vigorous plantlets via subculture grew to 40∼60 mm long (included 3–4 unfolded leaves), some of the plantlets showed fibrous roots. All of the vigorous plantlets were transferred into a ½MS medium containing 2% (w/v) sucrose and 0.1% (w/v) activated charcoal. After about 7–10 days, some plantlets (without fibrous roots) produced short roots at their base, whereas some plantlets (with fibrous roots) produced longer roots when all of the vigorous whole plantlets were cultured on the same ½MS medium. After being cultured for 4 weeks, these plantlets (with or without fibrous roots) produced roots of various sizes. The plantlets regenerated via subculture had developed a root system and their branches and leaves were flourishing (Figure 1D). Some plantlets with microtubers also produced many fibrous roots when they were cultured on rooting medium. The effect of hormones at different concentrations on the rooting of plantlets with and without a microtuber is presented in Table 5. Compared with the plantlet without a microtuber, the plantlets with microtubers produced roots more easily with the highest rate being 100% (with 0.5 mg/l BA and NAA supplements). Maximum rooting of the plantlets without microtubers (94.67%) was on a medium with 1.0 mg/l BA and 0.5 mg/l NAA. Compared with the control, low concentrations of BA could increase the rooting frequency in regenerated plantlets without microtubers, but 2.0 mg/l BA was optimal only for plantlets without microtubers, and thus decreased the overall rooting frequency.

The acclimated plantlets with 5–8 roots showed obvious growth 1 week after being removed from culture flasks and transferred to sterilized soil in the greenhouse (Figure 1E). One month after transferring, the survival rate of the plantlets was greater than 91%, and the young plants grew vigorously in the greenhouse. Each surviving plant increased in height and number of leaves and could be transplanted into the field after 4 weeks (Figure 1F).

Discussion

The Effect of Plant Growth Regulators on Shoot Induction and Tuberization of D. Nipponica

The auxin NAA regulates not only vegetative growth but also organ growth, whereas the cytokinin BA facilitates cell division and sprouting (Pan 2001). In the present study, BA and NAA at different concentrations were tested for their ability to effect both shoot growth and the frequency of microtuber induction in *D. nipponica* and to optimize the medium composition for plantlet regeneration. On MS media containing 3% (w/v) sucrose and BA without NAA, no microtubers were induced, whereas they were induced on the 0.5 mg/l to 2.0 mg/l NAA treatments (Tables 1 and 2). The result indicated that the growth regulators BA and NAA could ensure *in vitro* regeneration of *D. nipponica*, with the efficiency depending on their concentrations. It also indicated that the synergism of BA and NAA in their proper concentration is extremely favorable for the tissue culture of *D. nipponica*. The absence of both hormones or only a single phytohormone is not suitable for *in vitro* regeneration of *D. nipponica*. The results reported in this article support the findings that the interaction of auxin and cytokinin is necessary for plant *in vitro* organogenesis, and cytokinin of high concentrations and auxin of low concentrations were prerequisites for differentiation of adventitious buds (Skoog and Miller 1957). Our experimental results were in concordance with this, such that BA (2.0 mg/l) and NAA (0.5–1.0 mg/l) jointly effected the formation of multiple shoots from the *D. nipponica* explants. In contrast, in the current investigation on microtuber induction in *D. nipponica* culture, the enhancing effect of NAA on inducing microtubers was obvious, and this effect increased gradually with increasing NAA concentration, both alone (Table 2) and with BA, except when NAA and BA were both supplied at 2.0 mg/l or above. Our investigation indicated that the absence or excessively high or low concentrations of hormones were not at optimum for *D. nipponica* to form shoots and produce microtubers. Suitable combinations of hormones at different concentrations are very important for *D. nipponica* culture. As with tissue culture of other plants, the case mentioned above relates to physiology, multiformity of plant acceptors, and metabolism and synthesis of endogenous hormones (Song 1985; Ni and Deng 1992).

Table 5. Effect of BA and NAA Synergism on Root Formation of *D nipponica* Makino Plantlets With or without Microtubers Cultured *in vitro* for 60 Days

	Plantlets without microtubers			Plantlets with microtubers		
Hormone concentration (mg/l)	Number	C_1	Percentage rooting (%)	Number	C_2	Percentage rooting (%)
No growth regulators	27	4	29.63	24	0	41.67
NAA0.2	78	9	44.87	34	2	76.47
NAA0.5	56	5	58.93	31	5	87.10
NAA1.0	73	4	52.05	41	3	82.92
BA0.5 + NAA0.2	68	4	48.53	38	4	89.47
BA0.5 + NAA0.5	66	2	60.61	36	3	100.0
BA0.5 + NAA1.0	71	6	54.93	40	6	92.50
BA1.0 + NAA0.2	59	5	64.4	33	9	78.79
BA1.0 + NAA0.5	75	8	94.67	36	7	83.33
BA1.0 + NAA1.0	76	9	67.11	35	5	85.71
BA2.0 + NAA0.2	54	6	53.70	43	8	62.79
BA2.0 + NAA0.5	63	9	55.56	39	6	69.23
BA2.0 + NAA1.0	47	5	48.94	37	0	70.27

In this Table, $C_1$and C_2 represent the number of explants contaminated after planting.
For this experiment, ''Plantlets without microtubers'' came from a part of the shoots without microtubers forming in Tables 2 and 3. ''Plantlets with microtubers'' came from a part of the shoots with microtubers forming in Tables 2 and 3. These shoots (with or without) were subcultured onto the same MS medium containing 3% sucrose, 2.0 mg/l BA, and 2.0 mg /l NAA, because the highest average shoot height and the highest frequency of microtuber induction had been obtained (Table 3).
When these whole shoots via subculture (with or without fibrous roots) grew to 40–60 mm long (including 3–4 leaves unfolded), they were transferred onto ½MS medium supplemented with 0.1% (w/v) activated charcoal and 2% (w/v) sucrose.

The Effect of Sucrose at Different Concentration on Tuber Growth of *D. Nipponica*

In a similar manner to the phytohormones, sucrose has been shown to play an important role on *in vitro* microtuber formation of *D. nipponica* (Table 4). As a carbon and energy source, sucrose provides a carbon frame and energy for shoot induction and microtuberization in *D. nipponica*. Meanwhile, as an osmotic pressure agent, sucrose could maintain a stable osmotic pressure environment for culture (Kuhlmann and Foroughi-Wehr 1989; Finnie and others 1989) and support vigorous growth of *in vitro* tissue culture of most plants (Lu 1981), so it was widely used as a standard carbon source for plant tissue culture. Furthermore, at different growth stages of *in vitro* culture of *D. nipponica*, sucrose at different concentrations (as carbon sources) and different osmotic pressure environments was needed. In this article, starting with microtubers, high microtuber induction levels were observed when 7% (w/v) sucrose was used for culture of *D. nipponica*. The present investigation indicated that at a high concentration of sucrose the production of heavier microtubers of *D. nipponica* culture *in vitro* was promoted (Table 4). In reference to the *in vitro* shoot induction and microtuber formation of several *Dioscorea* spp, some authors have pointed out the need to increase sucrose and decrease the photoperiod time for microtuber production of yam, but these authors started their regeneration protocol with nodal pieces. It was reported that, for example, when 8% or 10% sucrose was supplied with 2.5 mM kinetin, the percentage of *D. rotundata* microtuberization was only 37.5% and 12.5% (Ng 1988), respectively, whereas sucrose at 8% (w/v) was the single most significant medium constituent for microtuber induction of the steroid yam *D. composita* (Sedigeh and others 1998).

D. nipponica is a medicinal plant, but traditional breeding for rearing heavy-producing and high-grade *D. nipponica* tubers takes considerable time. A combination of tissue culture and the genetic engineering technique is likely an available process for the improvement and selection of *D. nipponica*. The key is to establish a plant regeneration system to suit genetic transformation. The present study established a regeneration system for *D. nipponica* by inducing shoots and microtubers *in vitro*. This is also the foundation for immediate propagation of elite high-steroid-yielding varieties and for the conservation of *D. nipponica*.

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