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STUDIES ON BOTANICAL CHARACTERISTICS OF *ALLIUM VICTORIALIS* L. SSP. *PLATYPHYLLUM* HULT.

IV. Plantlet regeneration through bud-multiplying body formation from apical-meristem tissues cultured *in vitro*.

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Introduction

Allium victorialis L. ssp. *platyphyllum* Hult. (Gyoja-nin-niku in Japanese) belongs to Allium species, and is widely distributed throughout Japan, especially in Hokkaido and Tohoku district, in the northern parts of Japan. The plants are perennial and grow in forests of mountainous area. People eat their bulbs, leaf sheaths and leaf blades as a pickles or a condiment vegetable. *A. victorialis* L. ssp. *platyphyllum* Hult. may be exterminated, because they are overharvested as a wild vegetable. Recently, necessity of its cultivation has arisen depending upon the increasing demand. *A. victorialis* L. ssp. *platyphyllum* Hult. has two reproductive systems, seed propagation and vegetative propagation. Because of their practical imperfect, a mass propagation method is required to be established through tissue culture.[1,2]

Materials and Methods

Expt. 1. Effects of BA and NAA on morphogenesis of apical-meristem tissues cultured in vitro. In May, 1986, an apical-meristem tissue (about 2 mm in length) with leaf primordia and a basal plate tissue (1 mm in thickness) was excised from a bulb of *Allium victorialis* L. ssp. *platyphyllum* Hult. (Gyoja-nin-niku in Japanese) mature plants collected from Mukawa, Hidaka, Hokkaido, Japan. Surface sterilization of the bulbs were made with 70% ethanol (1-min immersion) and sodium hypochlorite solution (available chlorine of 1%, with tween 20, 10-min immersion), followed by 3-time rinses with sterile distilled water. The apical meristems excised were cultured on a solidified culture medium (contained in a 10 -ml test tube) under the conditions of 25 °C and 4,000 lx (16-hour day length). Number of the explants cultured was 8-10 per treatment (Fig. 1). The culture medium contained MS medium,[3] 20 g/l sucrose, 7 g/l agar and growth regulators (0, 4.4×10^{-8}, 4.4×10^{-7} and 4.4×10^{-6} M of BA ; 0, 5.4×10^{-8}, 5.4×10^{-7} and 5.4×10^{-6} M of NAA). pH of the medium was adjusted uniformly to 5.7. After 16 weeks of culture, final observations were made on percentage of bud-multiplying body

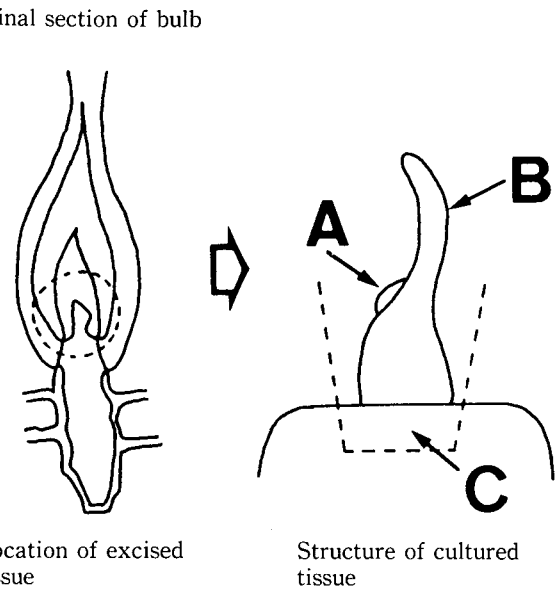

Fig. 1. Illustrations by longitudinal sections of an explant excised and cultured, which contains apical meristem, leaf primordium and basal plate tissue.
(A) Apical meristem. (B) Leaf primordium.
(C) Basal plate tissue.

(BMB) formation, average number of plumules, average length of leaves, percentage of root formation, average number of roots, average length of roots, and callus formation.

Expt. 2. Effects of BA and NAA on bud-multiplying body formation of apical-meristem tissues cultured in vitro. *A. victorialis* L. ssp. *platyphyllum* Hult. plants were collected from Mukawa, Hokkaido, in Oct. 1986. Growth regulators used were BA (0, 10^{-5}, 10^{-4} M) and NAA (0, 10^{-5}, 10^{-4} M). The growth regulator concentrations were higher than those of previous experiments described above. Number of cultured explants was 20 per treatment. Other culture conditions followed those of Expt. 1.

Expt. 3. Effects of BA and 2, 4-D on bud-multiplying body formation of apical-meristem tissues cultured in vitro. The plants used were collected from Urakawa, Hidaka, Hokkaido, in May and June, 1988. BA (0, 10^{-5}, 10^{-4} M) and 2, 4-D (0, 10^{-7}, 10^{-6}, 10^{-5} M) were added to the media. Number of cultured explants was 15 per treatment. Other conditions were the same as those of Expt. 1.

Expt. 4. Effects of concentrations of sucrose and MS constituents on bud-multiplying body formation of apical-meristem tissues cultured in vitro. According to the treatments, concentrations of basal nutritious compounds (inorganic substances, vitamins, amino acids, etc.) added to the media were prescribed by revising MS medium : 1× (control), 1/2×, 1/4×, 1/8× and 1/16× concentrations

of standard constituents of MS medium. Growth regulators of 10^{-5} M BA and 10^{-7} M 2, 4-D were uniformly added to the media. Number of the explants cultured was 15 per treatment. Other conditions followed those of Expt. 1.

Expt. 5. Effects of temperature and day length on bud-multiplying body formation of apical-meristem tissues cultured in vitro. Culture room air temperatures were adjusted to 10, 15, 20 and 25°C, and the day length to 24-hour illumination per day. Growth regulators used were 10^{-5} M BA and 10^{-7} M 2, 4-D. Other conditions were in accordance with the above procedures.

Expt. 6. Effect of subculture medium composition on morphogenesis of plumules.

In the case of transfer to a medium without growth regulator, after 16 weeks of primary culture, BMB were transferred to media without growth regulators. Other culture conditions followed those of Expt. 1. In the case of transfer to a medium containing BA and 2, 4-D, after 16 weeks of primary culture, BMB induced by 10^{-4} M BA and 10^{-6} M 2, 4-D were transferred to media supplemented with the BA and 2, 4-D that were added separately or in combination. Other conditions were the same as those of the previous experiments.

Results and Discussion

Expt. 1. Effects of BA and NAA on the morphogenesis of apical-meristem tissues cultured in vitro. Primordia-like white projections began to be observed on basal plate tissue after 4 weeks of culture (Fig. 2A), and developed into a bud-multiplying body (BMB) that had a few or tens of adventitious buds of which the tips were green (Fig. 2B, C). Percentage of BMB formation was low (about 10%) in the media without BA. However, the percentage increased with the increase of the BA concentration, and reached approximately 70% especially at 4.4×10^{-7} M, 4.4×10^{-6} M or 5.4×10^{-7} M of NAA (Table 1). In addition, the number of plumules per disc was enlarged by NAA, while the average length of leaves tended to increase slightly when the concentration of NAA was low. The highest percentage (45.9%) of root formation was found in the media without growth regulator, and decreased with the increase of BA and NAA concentrations. The roots were fine on the media without growth regulator, and became big on the growth regulator-added media. The number of roots was higher on the media without NAA than on those with NAA, whereas the average length of roots in the former media was shorter than that in the latter media. Callus was formed vigorously when the concentration of NAA was high and also when 4.4×10^{-7} M BA alone was added. The callus clumps formed consisted of small grains with a smooth surface, and the small grains turned green, subsequently developing into adventitious buds (Fig. 3A).

Expt. 2. Effects of BA and NAA on bud-multiplying body formation of apical-meristem tissues cultured in vitro. An addition of BA over 10^{-5} M could show more than 50% of BMB formation (Table 2). The average number of plumules

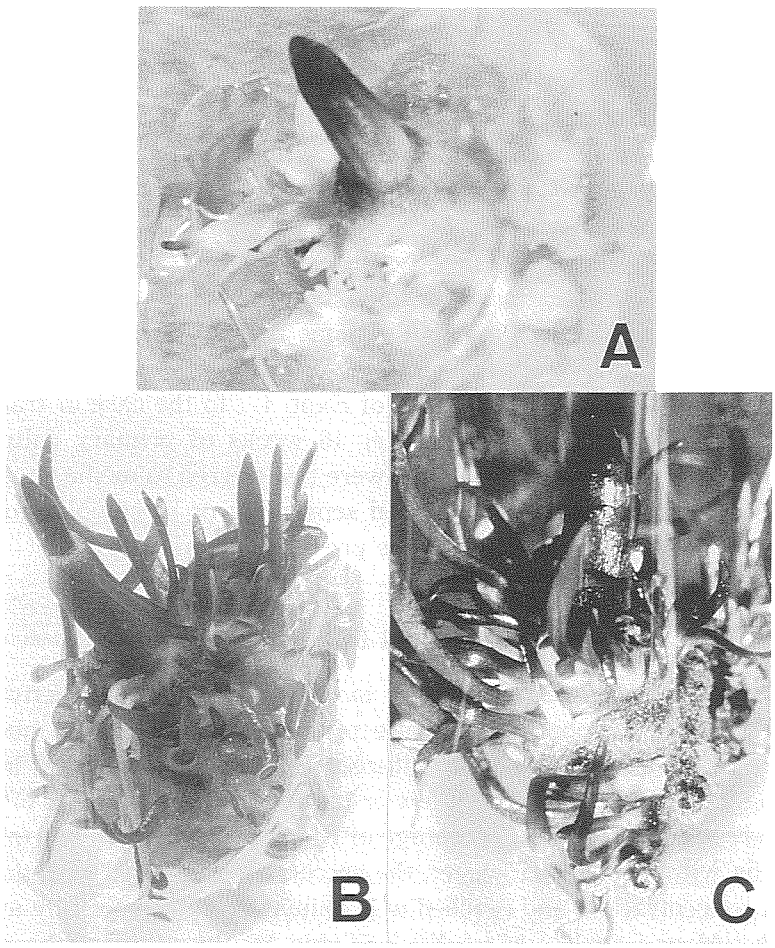

Fig. 2. Bud-multiplying body formation from a central, internal tissue of a bulb in tissue culture of *A. victorialis* L. ssp. *platyphyllum* Hult..
(A) Primary formation.
(B), (C) Further development of a multiple shoot.

was larger with 10^{-4} M BA than with 10^{-5} M BA, and relatively larger (24 plumules) especially with the combined addition of 10^{-4} M BA and 10^{-5} M NAA. Consequently, it was found that the higher concentrations of BA were effective to increase number of plumules. Percentage of root formation was lower at 10^{-5} M of BA, and finally no root was formed at 10^{-4} M of BA. The growth regulator promoted callus formation, but the callus partially failed to grow on the media with both 10^{-4} M BA and 10^{-4} M NAA. Therefore, for mass propagation, it may be a practical and efficient way to first promote the BMB emergence vigorously, and subsequently form a plumule which can make a rooting by transfer and subculture on the no-growth regulator media.[4)-7)]

Table 1. Effects of BA and NAA on morphogenesis of apical-meristem tissues cultured *in vitro* in *A. victorialis* L. ssp. *platyphyllum* Hult.[z].

B A (M)	NAA (M)	Average length of leaf buds (mm)	Percentage of bud-multiplying body formation (%)	No.of plumules	Percentage of root formation (%)	No.of roots	Average length of roots (cm)	Percentage of callus formation (%)
0	0	9	0	–	45.9	1.0	1.1	0
0	5.4×10^{-8}	9	0	–	28.6	2.0	0.9	12.5
0	5.4×10^{-7}	7	12.5	1.0	28.6	3.0	0.3	37.5
4.4×10^{-8}	0	12	0	–	25.0	2.0	2.3	0
4.4×10^{-8}	5.4×10^{-8}	11	10.0	1.5	12.5	1.0	1.6	0
4.4×10^{-8}	5.4×10^{-7}	7	25.5	5.0	37.5	2.0	1.1	30.0
4.4×10^{-7}	0	8	22.2	2.0	11.1	1.0	0.8	10.0
4.4×10^{-7}	5.4×10^{-8}	8	22.2	7.0	35.0	2.5	1.5	20.0
4.4×10^{-7}	5.4×10^{-7}	9	71.4	4.4	16.7	3.0	0.8	33.4
4.4×10^{-6}	0	10	37.5	1.0	0	–	–	0
4.4×10^{-6}	5.4×10^{-8}	11	37.5	2.8	0	–	–	36.7
4.4×10^{-6}	5.4×10^{-7}	8	77.8	6.5	12.5	2.0	0.5	73.4

[z] After 16 weeks of culture. The media contain MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators shown in the table. Culture was carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

Table 2. Effects of BA and NAA on bud-multiplying body formation of apical-meristem tissues cultured in vitro *in vitro* in *A. victorialis* L. ssp. *platyphyllum* Hult.[z].

B A (M)	NAA (M)	Average length of leaf buds (mm)	Percentage of bud-multiplying body formation (%)	No.of plumules	Percentage of root formation (%)	No.of roots	Average length of roots (cm)	Percentage of callus formation (%)
0	0	17.9	0	–	62.5	4.0	1.9	0
0	10^{-5}	14.6	7.7	2.0	53.8	8.9	0.5	63.6
0	10^{-4}	13.8	11.1	5.0	0	–	–	90.9
10^{-5}	0	21.4	52.6	7.7	10.0	4.5	1.9	20.0
10^{-5}	10^{-5}	15.7	50.0	8.8	27.3	2.0	0.7	44.4
10^{-5}	10^{-4}	10.7	57.1	1.8	7.7	2.0	0.3	76.9
10^{-4}	0	19.7	50.0	13.4	0	–	–	50.0
10^{-4}	10^{-5}	17.1	33.3	24.0	0	–	–	70.0
10^{-4}	10^{-4}	5.3	66.7	8.5	0	–	–	57.1

[z] After 16 weeks of culture. The media contain MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators shown in the table. Culture was carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

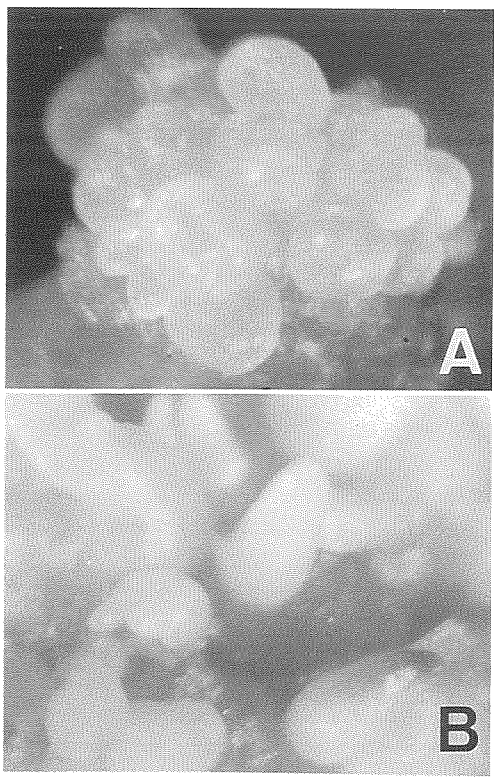

Fig. 3. Callus and plumule formation from *in vitro*-cultured apical-meristem tissues with small basal plate tissue.
(A) callus formation.
(B) plumule formation.

Expt. 3. Effects of BA and 2, 4-D on bud-multiplying body formation of apical-meristem tissues cultured in vitro. BMB formation occurred after 4-5 weeks of culture, and reached the peak after 8 weeks (Fig. 4). Percentage of BMB formation was higher with 10^{-4} M BA than with 10^{-5} M BA (Table 3). A concentration of 2, 4-D added to the medium should be carefully defined, because too high concentrations of it inhibit the BMB formation. Percentage of BMB formation was the highest (91.7%) with 10^{-4} M BA and 10^{-7} M 2, 4-D. Average number of plumules per explant was more than 20 in both the 10^{-5} M BA combined with 10^{-6}-10^{-5} M 2, 4-D and 10^{-4} M BA combined with 10^{-6} M 2, 4-D, and the largest number of plumules (44.3) per explant was obtained with 10^{-4} M BA and 10^{-6} M 2, 4-D. In the other case, the percentage of callus formation was less than 40%. The highest percentage of root formation was 58.3% with the media without growth regulator. Additions of 10^{-4} M BA or of the 10^{-4} M BA combined with 10^{-7} M 2, 4-D were suitable for the BMB formation because of less callusing and higher plumule formation rates. In addition, it was clarified that 2, 4-D induced less callus formation, and more efficiently enhanced the BMB formation than NAA.

Expt. 4. Effects of concentrations of sucrose and MS constituents on bud-multiplying body formation of apical-meristem tissues cultured in vitro. Macroscopically, frequency of BMB formation decreased with the increase of sucrose concentration, and was slightly lowered with 1/4× to 1× concentrations of standard constituents of MS medium (Fig. 5). Moreover, 1/8× to 1/16× conc. of those considerably decreased the BMB formation rate and number of plumules (Fig. 6). It was clearly shown that 10-20 g/l sucrose and the 1/4× to 1× conc. of MS effectively promoted BMB formation.

Expt. 5. Effects of temperature and day length on bud-multiplying body formation of apical-meristem tissues cultured in vitro. Temperatures of 20-25 ℃ enhanced growth of BMB more efficiently than 15 ℃, and plumules in the bodies showed

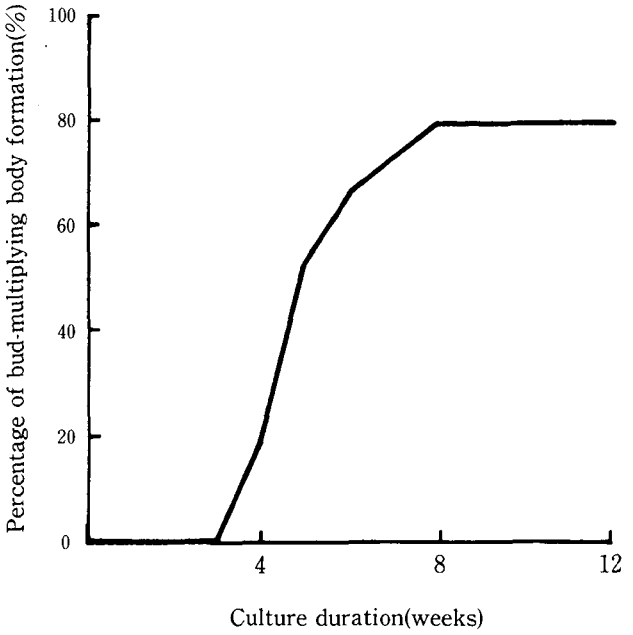

Fig. 4. Time course changes in the rate of bud-multiplying body formed from *in vitro*-cultured apical-meristem tissues with a few primodia and small basal plate tissue.

Table 3. Effects of BA and 2,4-D on bud-multiplying body formation of apical-meristem tissues cultured *in vitro* in *A. victorialis* L.ssp.*platyphyllum* Hult.[z].

B A (M)	2,4-D (M)	Percentage of bud-multiplying body formation (%)	No.of plumules	Percentage of root formation (%)	Percentage of callus formation (%)
0	0	0	–	58.3	0
10^{-5}	0	33.3	8.0	33.3	0
10^{-5}	10^{-7}	57.1	7.5	50.0	0
10^{-5}	10^{-6}	42.9	22.7	28.6	0
10^{-5}	10^{-5}	30.8	20.0	7.7	23.1
10^{-4}	0	73.3	23.2	0	0
10^{-4}	10^{-7}	91.7	28.3	8.3	0
10^{-4}	10^{-6}	50.0	44.3	0	35.7
10^{-4}	10^{-5}	7.7	10.0	0	38.5

[z] After 12 weeks of culture. The media contain MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators shown in the table. Culture was carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

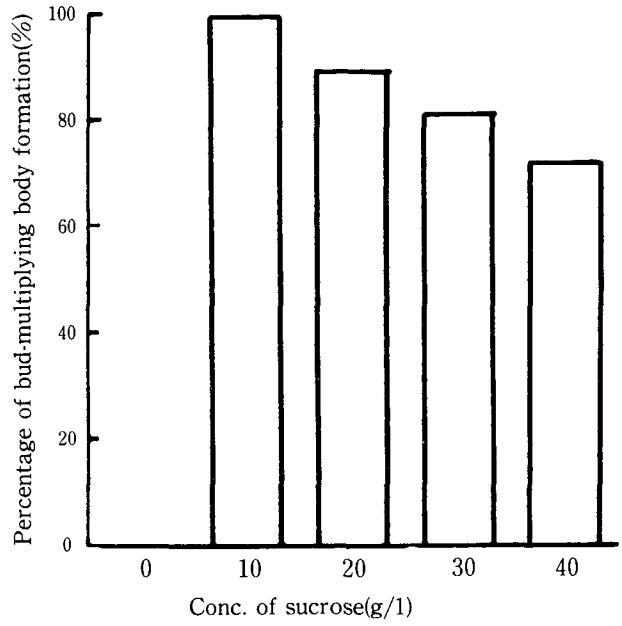

Fig. 5. Effect of sucrose concentration on bud-multiplying body formation of *in vitro*-cultured apical-meristem tissues with a few leaf primodia and small basal plate tissue. Media uniformly contain MS medium, 20 g/l sucrose and 10^{-5} M BA combined with 10^{-7} M 2, 4-D.

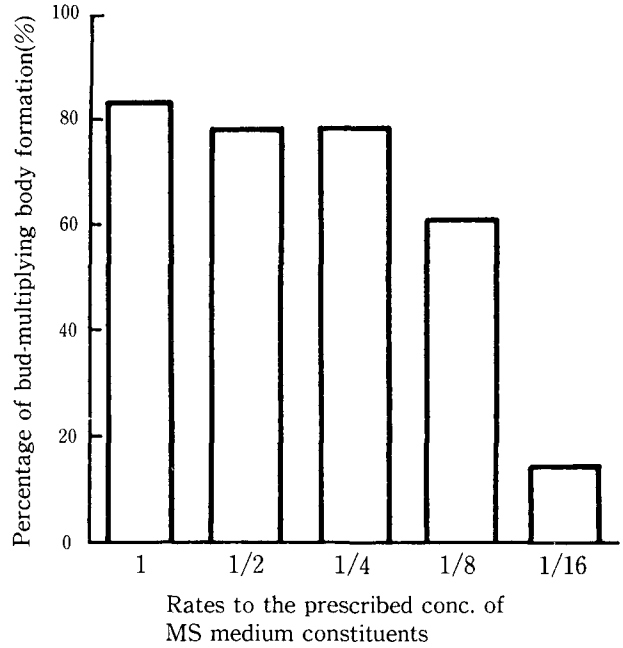

Fig. 6. Effect of the prescribed concentration of MS constituents on bud-multiplying body formation of *in vitro*-cultured apical meristem tissue with a few leaf primodia and small basal plate tissue. Media uniformly contain MS medium, 20 g/l sucrose and 10^{-5} M BA combined with 10^{-7} M 2, 4-D.

better growth at 20-25 °C (Fig. 7, Fig. 8). Therefore, it was clarified that 20-25 °C greatly promote the growth of plumules. In BMB formation, 8-hour day length gave the highest percentage but the lowest growth-enhancing effect, while in callus formation, 8- to 16-hour day lengths were optimal.

Expt. 6. Effect of subculture medium composition on morphogenesis of plumules.

When transferred to a medium without growth regulator, plumules, which were formed on a growth regulator-containing medium, elongated leaves, developed roots, and increased the number and length of the roots (Table 4, Fig. 9). Growth of subcultured plumules was greatly affected by constituents of the medium before the subculture. Media containing more than 4.4×10^{-7} M of BA differentiated a large and brittle root. Consequently, it was indicated that plumules transferred and subcultured on a medium without growth regulator could form roots and developed into plantlets.

When transferred to a medium containing BA and 2, 4-D, BMB which was subcultured on the media containing 10^{-5} M BA and 10^{-7} M 2, 4-D successfully formed plumules (Table 5). The average number of plumules per BMB was nearly more than 7, and partially exceeded 10 particularly with a combined addition of 10^{-4} M BA and 10^{-6}-10^{-5} M 2, 4-D. The rate of callus formation was about 20%, excepting that a high percentage (71.4%) was obtained by an addition of 10^{-5} M BA with 10^{-5} M 2, 4-D. In the case of transfer-subculture system, rooting rates were high in a subculture medium containing no growth regulator. On the other hand, the fact that a low rooting rate (42.9%) appeared in a treatment without growth regulator indicated that a growth stage of plumules derived and transferred and a composition of primary culture media played an important role in the system.[8),9)]

In the experiment, new plumules could emerge out of BMB transferred and subcultured, and subsequently developed into plantlets through 2nd subculture using a medium suitable for *A. victorialis* L. ssp. *platyphyllum* Hult. plantlet regeneration (Fig. 10). Because *A. victorialis* L. ssp. *platyphyllum* Hult. requires a long time (2-3 years) to grow into a mature plant, further studies are considered to be necessary to establish a mass propagation method and shorten the growth period.

Summary

The purpose of this studies was to establish a mass propagation method of *Allium victorialis* L. ssp. *platyphyllum* Hult. (Gyoja-nin-niku in Japanese) through tissue culture.

Shoot apices with a few leaf primordia and small basal plate tissue were cultured on media containing MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators (NAA, 2, 4-D, BA) added separately or in combination. The cultures were carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

The explants cultured developed into a bud-multiplying body (BMB, cultures

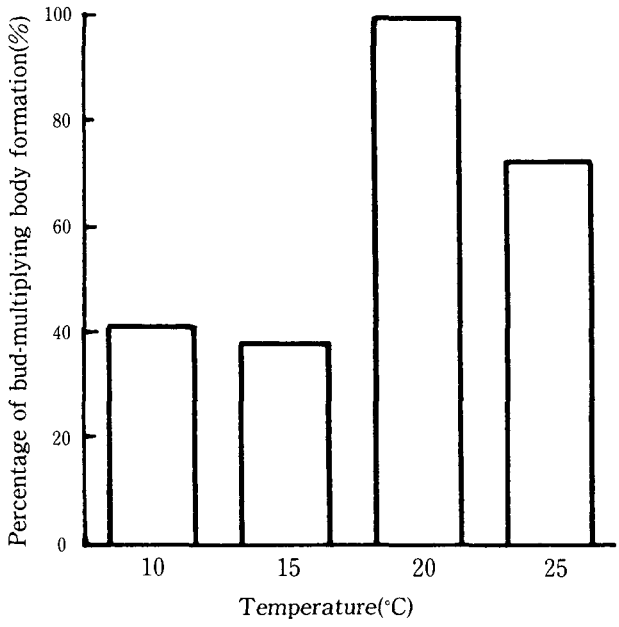

Fig. 7. Effect of temperature on bud-multiplying body formation of *in vitro*-cultured apical-meristem tissues with a few leaf primodia and small basal plate tissue. Media uniformly contain MS medium, 20 g/l sucrose and 10^{-5} M BA combined with 10^{-6} M 2, 4-D.

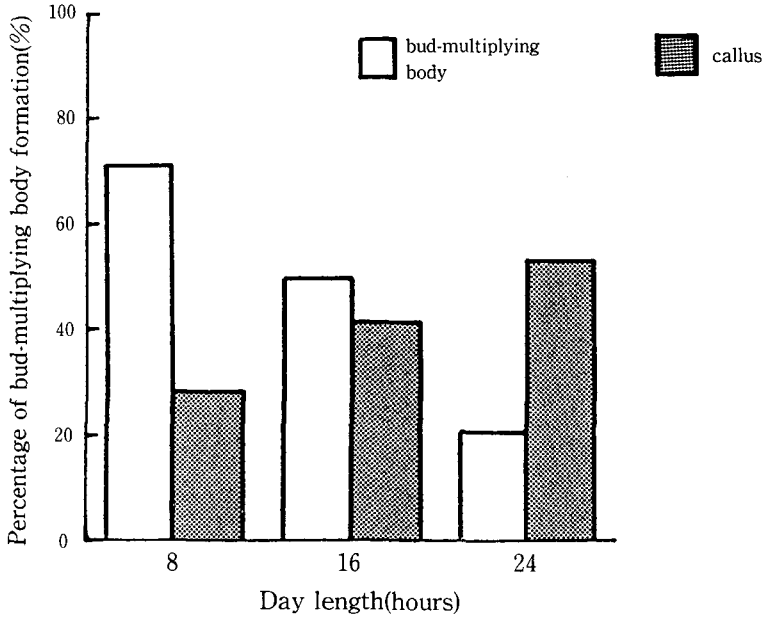

Fig. 8. Effect of day length on bud-multiplying body formation of *in vitro*-cultured apical-meristem tissues with a few leaf primordia and small basal plate tissue. Media uniformly contain MS medium, 20 g/l sucrose and 10^{-5} M BA combined with 10^{-6} M 2, 4-D.

Table 4. Effects of BA and 2,4-D on morphogenesis of plumules which were induced from *A. victorialis* L.ssp.*platyphyllum* Hult. apical-meristem tissues cultured on a medium without growth regulators[z].

Medium before subculture		Average length of leaves	Percentage of root formation (%)	No.of roots	Average length of roots (cm)
B A (M)	NAA (M)				
0	0	76	100	4.3	3.1
0	5.4×10^{-8}	65	100	8.0	2.5
0	5.4×10^{-7}	57	100	6.7	2.5
4.4×10^{-8}	0	37	100	3.5	2.5
4.4×10^{-8}	5.4×10^{-8}	25	100	1.0	0.5
4.4×10^{-8}	5.4×10^{-7}	40	100	6.7	2.2
4.4×10^{-7}	0	59	100	4.5	4.0
4.4×10^{-7}	5.4×10^{-8}	67	100	6.3	2.7
4.4×10^{-7}	5.4×10^{-7}	75	100	6.0	4.0
4.4×10^{-6}	0	–	100	–	–
4.4×10^{-6}	5.4×10^{-8}	57	100	5.7	4.0
4.4×10^{-6}	5.4×10^{-7}	17	100	6.7	3.2

[z] After 16 weeks of culture. The media contain MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators shown in the table. Culture was carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

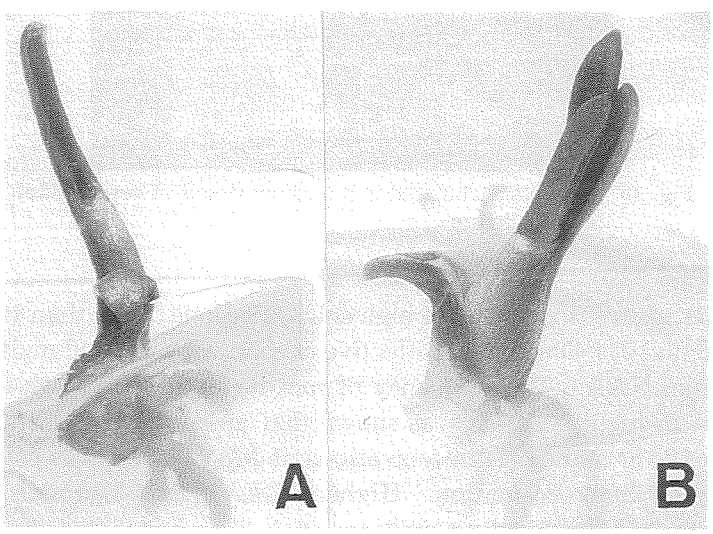

Fig. 9. Root formation of a plumule (A, B).

Table 5. Effects of BA and 2,4-D on morphogenesis of plumules which were induced from *A. victorialis* L.ssp. *playphyllum* Hult. apical-meristem tissues cultured on a medium without growth regulators[z].

B A (M)	2,4-D (M)	Percentage of bud-multiplying body formation (%)	No.of plumules	Percentage of root formation (%)	Percentage of callus formation (%)
0	0	0	–	42.9	0
10^{-5}	10^{-7}	46.7	8.2	13.3	0
10^{-5}	10^{-6}	13.3	7.1	6.7	6.7
10^{-5}	10^{-5}	0	–	0	71.4
10^{-4}	10^{-7}	20.0	8.3	0	20.0
10^{-4}	10^{-6}	46.7	12.4	0	6.7
10^{-4}	10^{-5}	50.0	14.5	0	21.4

[z] After 12 weeks of culture. The media contain MS medium, 20 g/l sucrose, 7 g/l agar and growth regulators shown in the table. Culture was carried out under conditions of 25°C, 4,000 lx and 16-hour day length.

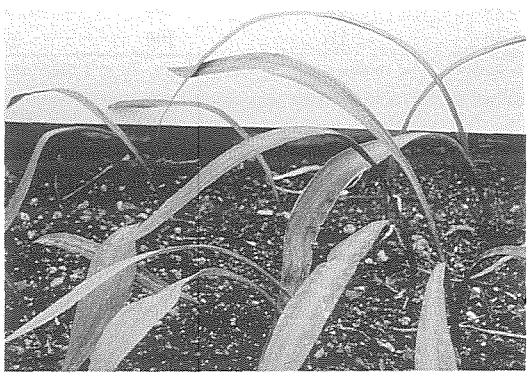

Fig. 10. Plantlets acclimated in a medium (1 soil : 1 vermiculite) in green house.

with multiple adventitious buds) at high concentrations (more than 10^{-5} M) of BA. It was found that 2, 4-D was more effective in enhancing the bud-multiplying body formation than NAA. The frequency of root formation was high in treatments without growth regulators. It was shown that an addition of 10-20 g/l sucrose and the quarter or standard concentrations of MS medium was suitable for the bud-multiplying body formation. Higher rates of the bud-multiplying body formation were obtained under conditions of 20-25°C, and it was clarified that the transfer and subsequent culture of plumules formed on the bodies made the bud-multiplying body emerge more frequently, and that acclimation of the bodies

could regenerate a large number of plantlets.

Literature Cited

1. OOSAWA, K., KURIYAMA, T. and SUGAHARA, Y. : Clonal multiplication of vegetatively propagated crops through tissue culture. I Effective balance of auxin and cytokinin in medium and suitable explant part for mass production of plantlets in strawberry, garlic, scallion, welsh onion, yam and taro. Bull. Veg. & Ornam. Crops Res. Stn. Japan, Ser. A, **9** : 1-46. 1981
2. FUJIEDA, K., ANDO, Y. and FUJITA, Y. : Propagation of welsh onion through shoot tip culture. J. Fac. Agr., Kyushu Univ., **22** : 89-98. 1977
3. MURASHIGE, T. and SKOOG, F. : A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., **15** : 473-497. 1962
4. KEHR, A. E. and SCHAEFFER, G. W. : Tissue culture and differentiation of garlic. HortScience., **11** (4) : 422-423. 1976
5. TANIMOTO, S. : Koutou shokubutsu no kikan bunka. Kagaku to Seibutsu. (in Japanese) **24** (5) : 334-338. 1986
6. FRIDBORG, G : Growth and organogenesis in tissue culture of *Allium cepa* var. *proliferum*. Physiol. Plant., **25** : 436-440. 1971
7. ZEE, S. Y., FUNG, A. and YUE, S. B. : Tissue culture and differentiation of chinese chive. HortScience., **12** (3) : 264. 1977
8. HUSSEY, G. : *In vitro* propagation of the onion *Allium cepa* by axillary and adventitious shoot proliferation. Scientia. Hortic., **9** : 227-236. 1978
9. PHILLIPS, G. C. and LUTEYN, K. J : Effects of picloram and other auxins on onion tissue cultures. J. Amer. Soc. Hort. Sci., **108** (6) : 948-953. 1983