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In Vitro Micropropagation of 'Yukiwariso' (*Hepatica nobilis* Schreber var. *japonica* f. *magna*) by Leaf Segment Culture

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Summary

This study was conducted to establish a system of in vitro micropropagation of *Hepatica nobilis*. Leaf segments, excised from commercial plants, formed calli at high frequency in MS medium that contained 0.1 - 10 mg • liter^{-1} NAA and BA. The highest percentage of bud formation was observed to be about 20% in the medium supplemented with 0.5 or 1 mg • liter^{-1} NAA and 5 mg • liter^{-1} BA. Embryoid formation was stimulated on the medium containing 0.1 mg • liter^{-1} NAA and 0.5 mg • liter^{-1} BA, or 1 mg • liter^{-1} NAA and BA. When 1 to 2 mm long adventitious buds were excised from leaf segments and recultured every 8 weeks on MS medium supplemented with BA, GA_3 or both for 24 weeks, they developed into shoots (referred to as the primary shoots in this text). The survival rates of the primary shoots were higher in the medium containing both BA and GA_3 than in the medium with BA alone. When primary shoots were cultured on MS medium containing 5 mg • liter^{-1} BA and 10 mg • liter^{-1} GA_3, they formed the highest number of new shoots, referred to as secondary shoots in the text. When excised secondary shoots were cultured in MS medium containing 10 mg • liter^{-1} NAA or IBA in the dark for a week and then transplanted into MS - medium without any hormones, they developed roots at a high frequency, whereas shoots transplanted to MS - medium with NAA or IBA formed only callus at the base. Following acclimatization, rooted plants that were transplanted into pots and grown in a glasshouse, flowered within 2 years.

Key Words: adventitious bud, flowering, rooting, shoot.

Introduction

Hepatica nobilis var. *japonica* f. *magna* Nakai, 'Misumiso' or 'Yukiwariso', is a member of the family Ranunculaceae. 'Yukiwariso' that has been propagated by sexual and asexual methods has recently become a popular potting plant in Japan. The main disadvantage to seed propagation, besides genetic segregation in heterozygous plants, is its long juvenile period of 4 to 5 years to flower (Nagashima, 1992). On the other hand, the asexual propagation rate by plant division is much lower than that of seedlings. For these reasons, in vitro propagation by tissue culture has been conducted to improve the multiplication rate in *Hepatica nobilis*. Micropropagation generally involves four steps: (a) initiation of aseptic culture, (b) shoot multiplication, (c) rooting of in vitro formed shoots and (d) transplantation of plants to a potting mix (Murashige, 1974; Bhojwani and Radzan, 1983). For the first and second steps of in vitro propagation of *H. nobilis*, Murayama and Mii (1981) and Tsuyuki et al. (1989) reported that embryoids and adventitious buds were initiated from leaf segments, whereas Shimada (1994, 1996) and Hoshi et al. (2001) observed adventitious buds developing on young leaves. Furthermore, Tsuyuki et al. (1989), Shimada (1994) and Hoshi et al. (2001) successfully regenerated plantlets from embryoids or buds through step c, but Shimada (1994) and Hoshi et al. (2001) failed to achieve step d or commercially marketable plants.

In this paper, the methods for regeneration of adventitious buds and embryoids from leaf segments of *H. nobilis*, the multiplication of shoots, the formation of roots, and the successful production of the commercial plants are described.

Materials and Methods

1. Leaf segment culture

Commercial plants of *H. nobilis* var. *japonica*, raised from seedlings (meaning that all plants used in the present study are not entirely homogeneous), were purchased from the Kitazawa plantation and cultivated in a polyethylene house at the experimental field in Niigata University until utilized.

Fully expanded leaves that were collected from the

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plants from late April to early May were surface-sterilized with 70% ethanol for 10 sec, followed by sodium hypochlorite solution with 1% active chlorine for 10 min, and rinsed three times with sterile distilled water. Leaves were then cut into 5 × 5 mm segments; three segments were placed in a culture vessel (59 × 90 × 40 mm; bottom diameter × height × neck diameter) (Iwaki Glass) with the abaxial side of each segment in contact with a medium. Each treatment that consisted of 12 segments was triplicated.

Segments were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 36 combination of NAA and BA at 0, 0.1, 0.5, 1, 5 and 10 mg · liter^{-1}. Thirty g · liter^{-1} sucrose was added to each medium and adjusted to pH 5.7 prior to the addition of 2 g · liter^{-1} gellan gum, and then autoclaved at 120 ℃ for 10 min.

Cultures were maintained at 25 ℃ for 16 weeks under continuous illumination with fluorescent lamps at a photon flux density of 20-30 μmols^{-1} · m^{-2} · sec^{-1}.

2. Shoot induction from adventitious buds and histological observation

When adventitious buds regenerated on leaf segments, were about 1-2 mm long, they were excised aseptically and cultured in a test-tube (20 × 90 mm) (Iwaki Glass) with 10 ml MS medium containing 0, 0.5 or 5 mg · liter^{-1} of BA and 0 or 10 mg · liter^{-1} of GA_3. A preliminary experiment showed that adventitious dormant-typed buds were formed when only BA was added to a culture medium. They were subcultured every 8 weeks with fresh medium and maintained as above. The number of newly formed shoots (referred to as a secondary shoots in the present study) was counted 24 weeks after culture.

To observe the initiation and development of secondary shoot, the basal part of the primary shoot, cultured in MS medium supplemented with 5 mg · liter^{-1} BA and 10 mg · liter^{-1} GA_3, was collected after 8 weeks of culture and fixed in a solution of FAA (formalin: acetic acid: 70% alcohol = 5: 5: 90; v/v/v) for 24 hr. The base was embedded in paraffin after dehydration in an alcohol series, and cut into 10 μm sections with a rotary microtome. The sections were stained with Delafield's Hematoxylin and observed under a light microscope.

3. Rooting of secondary shoots and transplant to soil

Secondary shoots (10-13) with 2-3 leaflets about 10 to 20 mm long were excised from primary shoots and cultured in the dark by imbedding the basal ends into the medium to about 5 mm in depth in a 100-ml Erlenmeyer flask that contained 30 ml MS medium with NAA or IBA at concentrations of 5 or 10 mg · liter^{-1} (referred to as the first phase of culture in the text). After a week, the shoots were subcultured for 8 weeks in a test-tube (22 × 90 mm) with fresh medium with or without hormones under continuous illumination as above (referred to as the second phase of culture in the text). Any shoot with roots more than 3-mm long was considered a rooted shoot. The number of shoots with roots, the number of roots per shoot, and the average length of roots (longer than 3 mm) per shoot were recorded. Each treatment that consisted of 10 shoots was triplicated.

Plantlets were removed from the tubes after 8 weeks, fully washed with tap water, planted into polyethylene pots that contained a mixture of Akadama: Kanuma soils (8:2, v/v), and cultivated at 25 ± 3 ℃ under a high humid condition for one month. Afterwards, the potted plants that were shaded with a layer of cheesecloth and raised in a glasshouse flowered in about 2 years.

Results

Effect of NAA and BA on callus, adventitious bud and embryoid formation in leaf segment culture

The leaf segments began to form calli at the cut surface in about 2 weeks followed by the appearance of adventitious buds (Fig. 1A) and embryoids (Fig. 1B) after 7 to 9 weeks of culture. The formation of calli, buds and embryoids 16 weeks after culture (Table 1) indicates that callus formation was stimulated in the medium supplemented with both NAA and BA, more so than in a medium containing NAA or BA alone. Although calli (5 mm diam.) were subcultured on MS medium, supplemented with 8 combination of 2 or 4 mg · liter^{-1} NAA that contained 1, 2, 4 or 8 mg · liter^{-1} BA, respectively, no organs primordium was initiated (data not shown). Vegetative buds, which were morphologically similar to the dwarf shoots observed on commercially grown *H. nobilis* plants in winter, differentiated at a relatively high frequency on medium with 0.1-1 mg · liter^{-1} NAA in combination with 0.5-5 mg · liter^{-1} BA; the highest percentage, 21%, was observed in the medium containing 0.5 mg · liter^{-1} NAA and 5.0 mg · liter^{-1} BA. Embryoids also developed at the cut surface of leaf segments cultured on medium, containing NAA of 0.1-0.5 mg · liter^{-1} in combination with BA of 0.5-5 mg · liter^{-1}. The largest number of embryoids was formed on medium containing NAA and BA at a concentration of 1 mg · liter^{-1}, but no embryoids developed into plantlets.

Shoot multiplication

About 50% of the adventitious buds, cultured on a BA + GA_3 medium, grew well and formed secondary shoots consisted of a shoot apex and two leaf primordia, at the base of the shoot (Fig. 2A, B, C). The highest number of shoots being obtained in the medium containing 5 mg · liter^{-1} BA and 10 mg · liter^{-1} GA_3 (Table 2). In contrast, the shoots cultured on medium without any hormones did not develop, but gradually turned brown and finally wilted (Table 2). About 30-40% of the buds, cultured on the medium with only BA, survived, and new adventitious buds were formed at the shoot base, although these newly formed buds remained small (Fig.

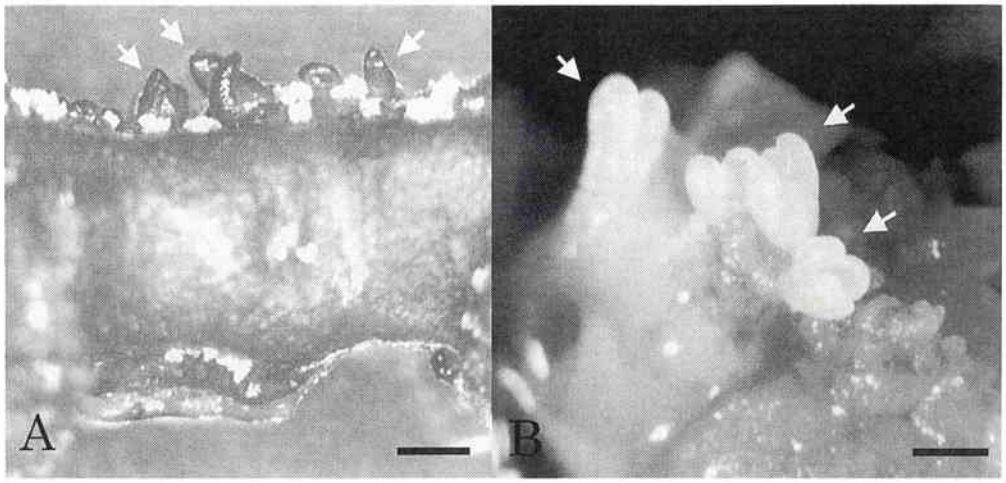

Fig. 1. Buds and embryoids formed on a leaf segment. (A) Adventitious buds (arrows) formed on a leaf segment cultured on MS medium with 1 mg・liter^{-1} NAA and 5 mg・liter^{-1} BA. Bar = 1 mm. (B) Embryoids (arrows) formed on the leaf segment cultured on MS medium with 1 mg・liter^{-1} NAA and 1 mg・liter^{-1} BA. Bar = 1 mm.

Table 1. Effects of NAA and BA on formation of calli, adventitious buds and embryoids on leaf segments in *H. nobilis* after 16 weeks of culture.

BA (mg・liter^{-1})	Callus						Bud						Embryoid					
	NAA (mg・liter^{-1})						NAA (mg・liter^{-1})						NAA (mg・liter^{-1})					
	0	0.1	0.5	1	5	10	0	0.1	0.5	1	5	10	0	0.1	0.5	1	5	10
0	0	18.3	63.0	58.5	75.0	92.8	0	0	0	0	0	0	0	0	0	0	0	0
0.1	0	85.9	97.8	100	97.8	91.7	0	0	0	0	0	0	0	0	0	0	0	0
0.5	13.3	100	100	100	94.4	95.5	0	13.7	19.2	4.5	0	0	0	9.3	8.9	0	0	0
1	0	97.8	97.8	97.8	100	88.9	0	15.6	15.6	15.6	13.7	0	0	2.2	4.5	10.0	6	0
5	0	78.5	88.0	88.9	93.3	88.9	0	6.3	21.3	20.0	6.7	0	0	0	2.8	6.7	2.2	0
10	0	78.5	100	97.8	100	84.4	0	17.0	11.1	9.3	9.6	0	0	0	0	0	0	0
Significance																		
NAA (A)			**						**						**			
BA (B)			**						**						**			
A × B			**						**						**			

** Significant at P=0.01, using 2-way analysis of variance.

Table 2. Effect of BA and/or GA_3 on survival and multiplication rates of adventitious buds in *H. nobilis* after 24 weeks of culture.

Hormones		No. of adventitious buds cultured	No. of buds surviving	Mean no. of secondary shoots per survival shoot
BA	GA_3			
0	0	7	0	0
0.5	0	7	2 (27)[z]	10.0 ± 2.0[y]
5	0	7	3 (43)	16.3 ± 1.8
0.5	10	10	5 (50)	14.8 ± 2.6
5	10	10	5 (50)	18.8 ± 3.3

[z] Parenthesized figures represent % of buds that survived.
[y] Mean ± SE.

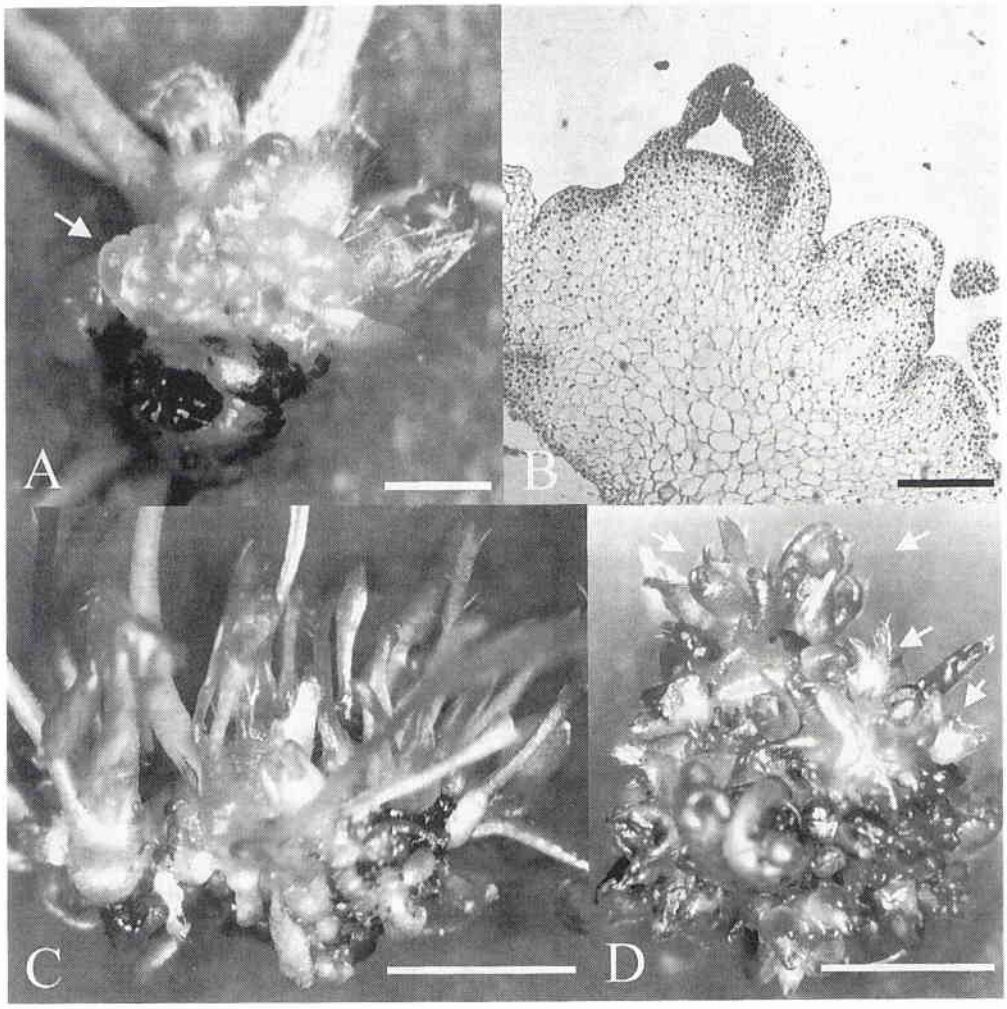

Fig. 2. Shoot multiplication and a cross-section of secondary shoots. (A) Development of secondary shoots (arrow) at the base of primary shoots cultured on MS medium with 1 mg • liter^{-1} BA and 10 mg • liter^{-1} GA_3 8 weeks after culture. Bar = 1 mm. (B) A histological section of secondary shoots with two shoots having leaf primordia. Bar = 0.5 mm. (C) Secondary shoots developed at the base of primary shoots cultured for 24 weeks on MS medium with 5 mg • liter^{-1} BA and 10 mg • liter^{-1} GA_3. (D) Buds remaining small in size (arrows) developed at the base of adventitious buds cultured for 24 weeks on MS medium with 5 mg • liter^{-1} BA. Bar = 5 mm.

2D).

Rooting of secondary shoots and transplant to soil

No shoots, cultured in hormone-free medium from the beginning to the end of culture, formed roots, and those cultured continuously in medium with auxins only formed a few roots but mostly only calli. Shoots cultured for a week in the medium with auxins in the first phases and then in hormone-free medium in the second phase initiated roots about 4 weeks after subculturing on hormone-free medium (Fig. 3A). Rooting was stimulated more by IBA than NAA (Table 3).

The 50 plantlets that were acclimatized under a high humid condition at 25 ± 3 ℃ for a month (Fig. 3B), and then were cultivated in a polyethylene house under ambient temperature, survived, and flowered within 2 years after transplanting (Fig. 3C).

Discussion

Adventitious bud formation, directly from excised organs, is generally considered a better approach than regeneration through the callus method for in vitro micropropagation of plant species, because callus cells often show the genetic instability (Bhojwani and Razdan, 1983). In vitro shoots, regenerated directly from leaf segments, are seemingly genetically stable at ploidy levels but also maintain the capability of forming shoots for a long time, as in *Haplopappus gracilis* (Tanaka and Ikeda, 1983), *Eleusine coracana* (Wakizuka and Yamaguchi, 1987) and *Cucumis melo* (Ezura et al., 1997). Our study showed that a greater number of the adventitious buds than embryos were formed directly from leaf segments, although calli also developed at a high frequency, suggesting that it is preferable to use adven-

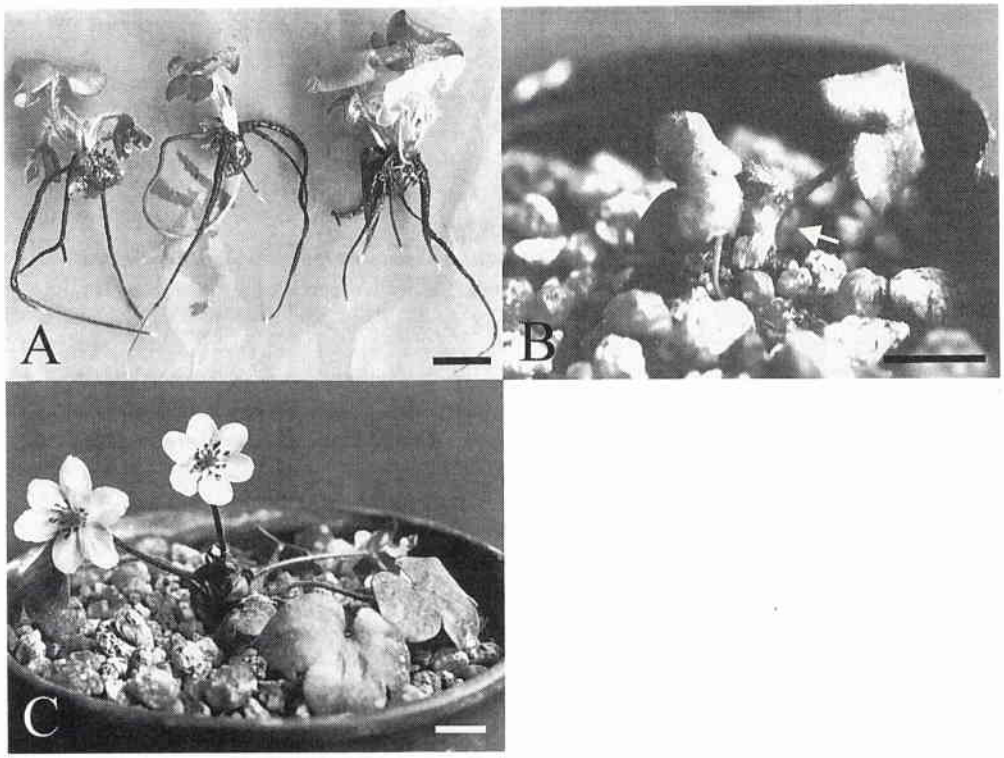

Fig. 3. In vitro propagated plantlets and a flowering *H. nobilis* plant. (A) Plantlets obtained in MS medium containing IBA 10 mg・liter^{-1}. (B) A plant (arrow) growing in a green house 1 month after transplanting. (C) A flowering plant 2 years after transplanting. Bar = 10 mm.

Table 3. Effect of auxins on rooting of adventitious shoots in *H. nobilis*.

Auxins	Concentration of auxin (mg・liter^{-1})[z]		Percentage of rooted shoots	Root numbers per shoot	Average length of roots (mm)	Percentage of shoots forming callus
	First phase	Second phase				
Control	0	-	0 d[y]	0	0	0 d
NAA	5	0	53.3 b	2.4 ± 0.4[x]	6.9 ± 0.9	36.7 bc
	5	5	0 d	0	0	100 a
	10	0	16.7 c	1.2 ± 0.2	6.6 ± 1.8	50 b
	10	5	0 d	0	0	100 a
IBA	5	0	60.0 b	3.8 ± 0.8	9.3 ± 0.7	3.3 cd
	5	5	10.0 cd	1.7 ± 0.7	4.4 ± 0.8	96.7 a
	10	0	83.3 a	4.1 ± 0.6	6.4 ± 0.4	0 d
	10	10	3.3 cd	1.0 ± 0	4.0 ± 0	100 a

[z] Thirty secondary shoots were cultured for a week in the first phase in darkness and then transferred to fresh medium with or without axins in the second phase under continuous illumination condition. Data were recorded 8 weeks after culturing shoots in the second phase.

[y] Values represent the mean of 3 independent experiments, each of which consists of 10 shoots. Values followed by the same letter are not significantly different according to the LSD test at $P = 0.05$.

[x] Mean ± SE.

titious buds for shoot multiplication. Formation of adventitious buds and embryos from leaf segments was achieved only in combinations of 0.1 - 1 mg・liter^{-1} NAA and 0.5 - 5 mg・liter^{-1} BA. Our data support the results of Murayama and Mii (1981) and Shimada (1994, 1996) in that leaf segments of *H. nobilis* formed adventitious buds and embryos in the medium containing NAA and BA at low concentrations.

That rapid shoot multiplication is critical in in vitro micropropagation was demonstrated in *H. nobilis* by Tsuyuki et al. (1989) who was able to induce an average of 6 shoot-buds from adventitious embryos cultured in

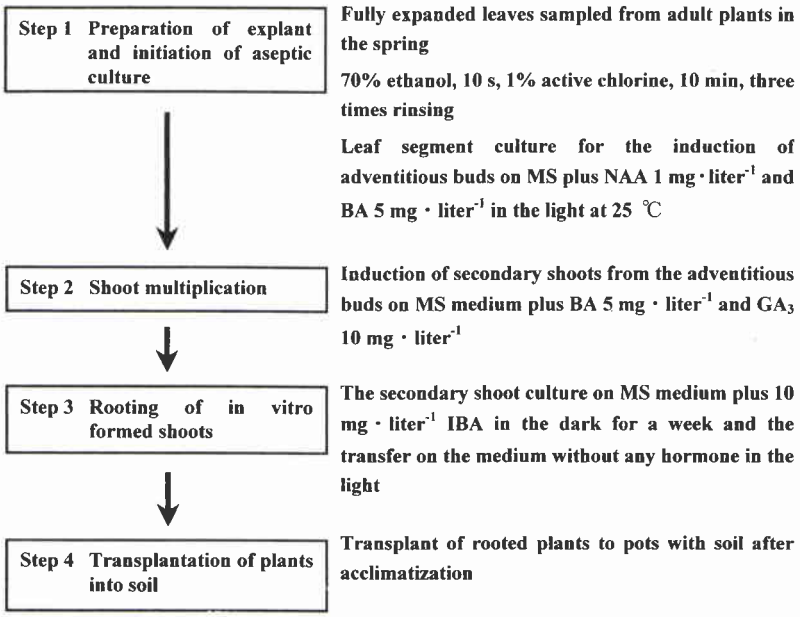

Fig. 4. Scheme of micropropagation in *H. nobilis*.

a liquid medium. Hoshi et al. (2001) showed that adventitious embryos excised from the base of adventitious buds grew into full transplantable plantlets when they were cultured in a medium with GA_3 and then in hormone-free medium. In our study an efficient secondary shoot formation was achieved by subculturing adventitious buds in a medium with BA and GA_3 (Table 2), and that the multiplication rate seems to be enhanced by repeatedly subculturing individual shoots. Furthermore, although repetition of in vitro multiplication cycle resulted in an increase of phenotypic variation in regenerants of *Begonia* × *hiemalis* (Westerhof et al., 1984), no apparent variant phenotype was detected in 50 plantlets regenerated in this trial.

Rooting in shoots of *Hepatica* was achieved by pretreatment with IBA and NAA (Table 3), provided they are cultured continuously in the medium with auxins from the beginning to the end of culture (Sudha and Seeni, 1996; Roy et al., 1988) or they are precultured temporarily in a medium with auxins to initiate root primordia and then transferred to a hormone-free medium (Afaque et al., 1996; Sudha and Seeni, 1996). To date, a maximum of 20% of embryos rooted (Shimada, 1994), whereas 83% of the secondary shoots of *H. nobilis* rooted by culturing them on MS medium containing 10 mg · liter^{-1} IBA for 7 days and then in a hormone-free medium (Table 3), indicating that preculturing shoots of *H. nobilis* in auxins promotes rooting, compared with continuously culturing them in an auxin medium. Similar results were reported in the rootstock shoots of apples, in which roots formed well without callus formation when the shoots were treated with a low concentration of IBA for a short time (James and Thurbon, 1979; Snir and Erez, 1980).

The embryo-derived plantlets of *H. nobilis* that were acclimatized well under a high humid condition did not survive the transplantation to soil (Shimada, 1994). However, those derived from shoots flowered within 2 years after transplant with no apparent variation in phenotype. This indicates that vegetative propagation of *Hepatica nobilis* is possible by tissue culture.

In conclusion, this study provides an efficient system for vegetative propagation of *Hepatica nobilis* by using tissue culture techniques. The micropropagation scheme established consisted of the following 4 steps (Fig.4): (1) establishment of infestation-free leaf segments and induction of adventitious buds on MS medium with NAA and BA; (2) stimulation of secondary shoot formation from the adventitious buds on MS medium with BA and GA_3; (3) rooting of the secondary shoots on MS medium with IBA followed by subculturing on a medium without hormone; and (4) transplanting rooted plantlets into pots after acclimatization.

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ユキワリソウ (*Hepatica nobilis* Schreber var. *japonica* f. *magna*) の葉片培養による栄養繁殖

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摘　要

ユキワリソウ (*Hepatica nobilis*) の組織培養法による栄養繁殖法を研究した．開花した植物から当年に成長した葉を採集し，5 × 5 mmの切片を作り培養した．NAAとBAを含むMS培地ではその濃度とは無関係にいずれの培地でも高頻度でカルスを形成した．不定芽の形成率はNAA 0.5 または 1 mg・liter^{-1} とBA 5 mg・liter^{-1} を添加した培地で最も高くなった．一方，胚様体の形成はNAAを0.1 mg・liter^{-1} とBAを0.5 mg・liter^{-1} またはNAAとBAを1 mg・liter^{-1} ずつ添加した培地で促進された．葉切片に形成された長さ1 - 2mmの不定芽(1次不定芽と呼ぶ)を切り取り，BAとGA_3を含むMS培地で8週間毎に継代し，24週間培養した．1次不定芽の生存率はBAを単独で添加したときよりもBAとGA_3を添加した時に高くなった．そして不定芽の成長はBA 5 mg・liter^{-1} とGA_3 10 mg・liter^{-1} を添加した培地で促進され，その基部に新芽(2次不定芽と呼ぶ)を多く形成した．長さ10 - 20 mmに成長した2次不定芽を切り取り，10 mg・liter^{-1} のNAAまたはIBAを含むMS培地，暗黒下で1週間培養し，その後明条件下，ホルモンフリー培地で培養すると，シュートは高頻度で発根した．一方，NAAまたはIBAを含む培地に継代したときは，基部にカルスを形成し，発根しなかった．発根したシュートは約25 ℃の高湿度条件下で約1ヶ月間順化させ，無加温のガラス室に移して栽培すると，移植した苗はほとんどすべて生存し，2年以内に開花した．