of *Trifolium repens* genotypes. Physiol. Plant. 52:187–190.
2. Dale, P.J. 1980. A method for in vitro storage of *Lolium multiflorum*. Ann. Bot. 45:497–502.
3. Lundergan, C. and J. Janick. 1979. Low temperature storage of *in vitro* apple shoots. HortScience 14:514.
4. Miedema, P. 1982. A tissue culture technique for vegetative propagation and low temperature preservation of *Beta vulgaris*. Euphytica 31:635–643.
5. Morel, G. 1975. Meristem culture techniques for the long-term storage of cultivated plants, p. 327–332. In: O.H. Frankel and J.G. Hawkes (eds.). Crop genetic resources for today and tomorrow. Cambridge Univ. Press, London.
6. Mullin, R.H. and D.E. Schlegel. 1976. Cold storage maintenance of strawberry meristem plantlets. HortScience 11:100–101.
7. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
8. Standardi, A. 1982. Effetti di subcolture ripetute in germogli di Actinidia chinensis (Pl.) coltivati in "vitro". Riv. Ortoflorofrutt. It. 66:419–429.
9. Westcott, R.J., G.G. Henshaw, B.W.W. Grout, and W.M. Roca. 1977. Tissue culture methods and germplasm storage in potato. Acta Hort. 78:45–49.

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In Vitro Propagation of Japanese Persimmon

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Additional index words. dormant bud, shoot tip, culture establishment, shoot proliferation, cytokinin, Diospyros kaki

Abstract. **Dormant bud explants taken from mature trees of Japanese persimmon cv. Hiratanenashi were established successfully on modified Murashige and Skoog's medium with nitrate reduced to half-strength [MS (½NO_3)] or woody plant medium, both supplemented with 22.2 μM (5 mg·liter^{-1}) BA. Shoot proliferation in subcultures also was best at 22.2 μM (5 mg·liter^{-1}) BA in MS (½NO_3) medium, but growth was of the rosette type. Shoot elongation, however, was stimulated the most in the same medium supplemented with 24.6 μM (5 mg·liter^{-1}) 2iP instead of BA. Rooting of the proliferated shoots was enhanced by the treatment with IBA at 1.23 mM (250 mg·liter^{-1}). Chemical names used: *N*-(phenylmethyl)-1*H*-purin-6-amine (BA), *N*-(3-methyl-2-butenyl)-1*H*-purin-6-amine (2iP), 1*H*-indole-3-butanoic acid (IBA).**

Japanese persimmon is one of the difficult-to-root fruit species. Commercial cultivars in Japan have been propagated by grafting mostly onto their seedling roots. This fruit species tends to grow to a large tree and growers often encounter difficulties in orchard management, especially when trees are grown on hilly slopes. Recently, some efforts have been directed toward seeking dwarfing rootstocks as done with other species (7). If surveyed extensively throughout the country, some promising dwarfs might be found among local cultivars and their seedling trees. In concert with this effort, a useful propagation method should be developed for candidate rootstocks or scion cultivars. Root cuttings seem to have some promise for propagation, but are not very efficient (8).

In vitro propagation has become a practical means for rapid and large-scale multiplication of some fruit species (6), but seldom with Japanese persimmon. Yokoyama and Takeuchi reported plantlet formation from calli derived from immature embryos of Japanese persimmon (9). They also induced roots and bud initials on calli isolated from cambial tissues of twigs of certain cultivars (10).

In our preliminary trials, shoot tip explants from several week-old seedlings or mature trees of various Japanese persimmon cultivars were cultured in Murashige and Skoog's medium (3). Rooted plantlets could be produced readily from young seedlings but rarely from mature trees. This study explored the optimal culture medium for explants from mature Japanese persimmon trees.

Plant materials were taken from old 'Hiratanenashi' trees (probably >50 years old) grown in the orchard of Kyoto Univ. We used shoot tips collected about 20 days after bud burst and dormant axillary buds collected in December and stored at 0°C for several weeks. Both were sterilized in sodium hypochlorite solution (containing 1% of available chlorine plus 0.1% of Tween 20) for 10 min and rinsed 4 times with sterile water. The shoot tips were trimmed aseptically to about 3 mm, and dormant buds were dissected and apical portions (about 2 mm) removed under a binocular microscope. These explants were placed singly in test tubes (2.3 × 15 cm) containing 20 ml of solidified medium. For culture establishment stage, both the types of basal media and cytokinins were tested. The following 5 mineral salts media were examined with an inclusion of 22.2 μM (5 mg·liter^{-1}) BA or 24.6 μM (5 mg·liter^{-1}) 2iP: Murashige and Skoog's salts (MS) (3), MS salts with nitrate reduced to half strength [MS(½NO_3)], Nitsch and Nitsch's salts (4), Lepoivre's salts (5), and woody plant medium salts (WPM) (1). All of these basal media were supplemented with the same composition of vitamins, amino acid, and Fe as with MS medium. To optimize the cyto-

Table 1. Comparison of 5 basal media supplemented with 22.2 μM (5 mg·liter^{-1}) BA or 24.6 μM (5 mg·liter^{-1}) 2iP on culture establishment of shoot tip and dormant bud explants. Twenty to 25 explants per treatment.

		Shoot tip explants[z]			Dormant bud explants[z]		
Basal medium	Cytokinin	Death rate (%)	Callusing[y] rate (%)	No. shoots per explant	Death rate (%)	Callusing[y] rate (%)	No. shoots per explant
MS	BA	8	0	1.3 ± 0.1[x]	12	0	1.3 ± 0.3[x]
	2iP	8	4	1.1 ± 0.1	100	0	0
MS (½NO_3)	BA	8	0	1.7 ± 0.2	0	0	3.4 ± 0.2
	2iP	0	58	0.4 ± 0.1	0	20	1.3 ± 0.2
Nitsch & Nitsch	BA	12	0	1.3 ± 0.2	13	13	2.0 ± 0.3
	2iP	17	50	0.5 ± 0.2	8	8	1.6 ± 0.3
Lepoivre	BA	9	0	1.2 ± 0.2	29	29	1.0 ± 0.2
	2iP	0	92	0.1 ± 0.1	16	16	0.2 ± 0.1
WPM	Ba	4	0	1.5 ± 0.1	0	0	3.1 ± 0.4
	2iP	4	96	0	0	0	1.8 ± 0.3

[z]Data taken after 40 days in culture.
[y]Callusing rate indicates the percentage of explants that converted to callus masses.
[x]Mean ± SE.

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Table 2. Effect of different concentrations of BA and 2iP in MS (½NO_3) medium on culture establishment of shoot tip and dormant bud explants. Eighteen to 20 explants per treatment.

Cytokinin	Concn (μM)	Concn (mg·liter^{-1})	Shoot tip explants[z] Death rate (%)	Shoot tip explants[z] Callusing[y] rate (%)	Shoot tip explants[z] No. shoots per explant	Dormant bud explants[z] Death rate (%)	Dormant bud explants[z] Callusing[y] rate (%)	Dormant bud explants[z] No. shoots per explant
BA	4.4	(1)	70	0	0.3 ± 0.1[x]	100	0	0[x]
	8.9	(2)	20	0	1.0 ± 0.1	65	0	0.6 ± 0.2
	22.2	(5)	0	0	1.8 ± 0.1	0	0	3.0 ± 0.5
	44.4	(10)	0	0	1.9 ± 0.2	0	0	2.3 ± 0.2
	88.9	(20)	5	0	1.3 ± 0.1	20	0	1.5 ± 0.3
2iP	4.9	(1)	45	45	0.1 ± 0.1	100	0	0
	9.8	(2)	15	70	0.2 ± 0.1	95	0	0.1 ± 0.1
	24.6	(5)	0	90	0.1 ± 0.1	0	20	1.3 ± 0.2
	49.3	(10)	0	83	0.2 ± 0.1	0	0	2.1 ± 0.2
	98.5	(20)	0	100	0	0	0	2.1 ± 0.2

[z]Data taken after 40 days in culture.
[y]Callusing rate indicates the percentage of explants that converted to callus masses.
[x]Mean ± SE.

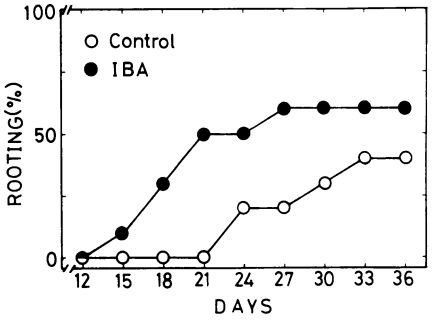

Fig. 2. Effect of IBA treatment at 1.23 μM (250 mg·liter^{-1}) on the rooting process of the shoot cuttings. Twenty cuttings per treatment.

kinin concentration for culture establishment, BA and 2iP were tested in the range of 4.4–88.9 μM (1–20 mg·liter^{-1}) and 4.9–98.5 μM (1–20 mg·liter^{-1}), respectively, using MS (½NO_3) medium. MS (½NO_3) medium, which showed the best results in the culture establishment step, was used for subsequent subcultures. Dormant bud cultures derived from MS (½NO_3) medium supplemented with 22.2 μM (5 mg·liter^{-1}) BA were maintained as mother cultures by subculturing individual shoots in the same fresh medium at 4- to 6-week intervals. The shoots then were used to test cytokinin concentrations for shoot proliferation stage.

Prior to root induction stage, shoots (about 5 mm) from mother cultures were transferred to MS (½NO_3) medium supplemented with 24.6 μM (5 mg·liter^{-1}) 2iP instead of BA to induce shoot elongation. After 4 weeks of culture, uniform shoot cuttings of about 2 cm long were taken and their basal parts immersed in 50% aqueous ethanol with or without 1.23 mM (250 mg·liter^{-1}) IBA for 30 sec. Immediately after drying of the solvent, these cuttings were placed on a half-strength MS (½NO_3) medium without cytokinins.

Throughout the experiment, all the media contained 8 g·liter^{-1} agar (Agar powder; Wako Pure Chemicals Industries, Kyoto, Japan) and 30 g·liter^{-1} sucrose, and the pHs were adjusted to 5.7–5.8 before autoclaving. Culture temperature was 27°C and light intensity at test tubes was 7.5 μmol·s^{-1}·m^{-2} with 24-hr photoperiod, except 16 hr day/8 hr night, for rooting.

Judging from the number of shoots per explant, dormant buds were best established in MS (½NO_3) medium, followed by WPM, when both were supplemented with BA (Table 1). The same media supplemented with 2iP were inferior to those with BA. MS, Nitsch & Nitsch, and Lepoivre salts were less effective. Shoot tip explants did not establish well compared to dormant buds, especially when 2iP was used. 2iP specifically induced excessive callusing of shoot tip explants and inhibited shoot proliferation. This effect was probably due to a synergistic action of 2iP with high endogenous auxin level of the shoot tips. The reason why full-strength MS medium was not successful for the establishment of explants from mature trees might be explained by its high concentrations of N and/or K or of total salts.

The optimum concentrations of BA and 2iP for culture establishment of dormant bud explants in MS (½NO_3) medium were 22.2 μM (5 mg·liter^{-1}) and 49.3–98.5 μM (10–20 mg·liter^{-1}), respectively, but 2iP was less effective than BA (Table 2). At reduced concentrations of either cytokinin, most dormant buds died. Only BA was suitable for shoot tip explants at the range of 22.2–44.4 μM (5–10 mg·liter^{-1}). 2iP promoted callusing of shoot tips with increasing concentrations, contrasting with no callusing when BA was used.

At shoot proliferation stage, BA was best at 22.2 μM (5 mg·liter^{-1}), but 2iP was less effective at any concentration (Fig. 1A). Shoot elongation, on the other hand, was greatly promoted by 2iP at 24.6 μM (5 mg·liter^{-1}), while BA retained the regenerated shoots in rosette form (Fig. 1B). These results suggest that the explants can be established and proliferated efficiently on MS (½NO_3) medium supplemented with BA and then transferred to the same medium but replacing BA with 2iP to ensure shoot elongation. Then they

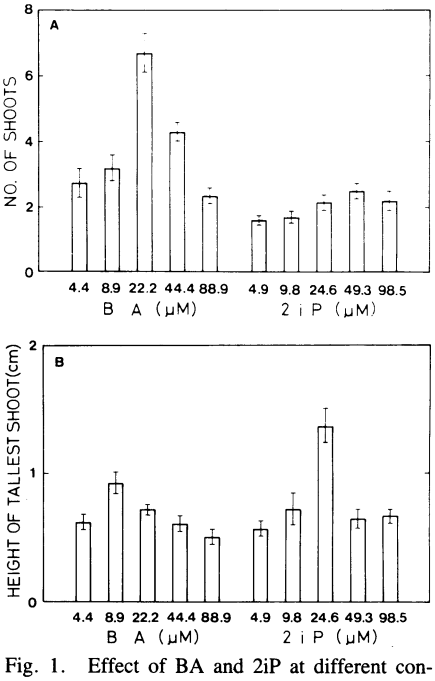

Fig. 1. Effect of BA and 2iP at different concentrations in MS (½NO_3) medium on shoot proliferation (**A**) and elongation (**B**) at shoot proliferation stage. Data taken after 30 days in culture. Vertical bars indicate SE. Twenty cuttings per treatment.

Fig. 3. 'Hiratanenashi' plantlets 70 days after transferring to a pot.

can be placed on rooting medium. The same response was reported with apples (2); BA was superior to 2iP for proliferation but produced a rosette type of growth. The dwarfed shoots were transferred to medium with 2iP to restore normal growth before subjecting them to rooting medium.

At root initiation stage, IBA treatment advanced rooting about 10 days compared to ethanol controls, and the final rate of rooted plantlets was enhanced (Fig. 2). During the rooting process, no shoot growth occurred, and some shoots even weakened. Rooted plantlets were transferred to a mixture of vermiculite and perlite (1:1, v/v) and gradually acclimatized for potting. After transplant to the soil, some of the plantlets began to grow well (Fig. 3).

This work mainly focused on culture establishment and shoot proliferation. Further experiments will be needed to increase rates of rooting and subsequent growth.

Literature Cited

1. Lloyd, G. and B. McCown. 1981. Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Comb. Proc. Intl. Plant Prop. Soc. 30:421–427.
2. Lundergan, C.A. and J. Janick. 1980. Regulation of apple shoot proliferation and growth in vitro. Hort. Res. 20:19–24.
3. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
4. Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Science 163:85–87.
5. Quoirin, M., P. Lepoivre, and P. Boxus. 1977. Un premier bilan de 10 annees de recherches sur les cultures de meristemes et la multiplication "in vitro" de fruitieres ligneux (in French). C.R. Recherches, Gemboux, Belgium.
6. Styer, D.J. and C.K. Chin. 1983. Meristem and shoot-tip culture for propagation, pathogen elimination, and germplasm preservation. Hort. Rev. 5:221–277.
7. Westwood, M.N. 1978. Temperate-zone pomology. Freeman, San Francisco.
8. Yamada, M., T. Sumi, and A. Kurihara. 1984. Studies on root cuttings of Japanese persimmons (in Japanese). Abstr. Jpn. Soc. Hort. Sci. Autumn Meeting p. 150–153.
9. Yokoyama, T. and M. Takeuchi. 1976. Organ and plantlet formation from callus in Japanese persimmon (*Diospyros kaki*). Phytomorphology 26:273–275.
10. Yokoyama, T. and M. Takeuchi. 1981. The induction and formation of organs in callus cultures from twigs of mature Japanese persimmon (*Diospyros kaki* Thunb.). J. Jpn. Soc. Hort. Sci. 49:557–562.

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Cold Hardening of in Vitro Apple and Saskatoon Shoot Cultures

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Additional index words. acclimation, tissue culture, *Malus domestica, Amelanchier alnifolia*

Abstract. **In vitro shoot cultures of MM 106 apple (*Malus domestica* Borkh.) and 'Smoky' saskatoon (*Amelanchier alnifolia* Nutt.) that were subjected to a 10-week, short-photoperiod, low-temperature hardening treatment, including a −3°C exposure followed by 5–7 days at 2°, were 4–8° hardier than untreated shoot cultures. Apple shoot cultures grown on media with elevated sucrose concentrations (3–14%), but not subjected to acclimating conditions, had reduced shoot moisture content and increased up to 6° in hardiness. Apple and saskatoon shoot cultures given a short-photoperiod, low-temperature hardening treatment and apple cultures grown on medium containing a high sucrose level developed red and purple leaf coloration.**

Cold-hardened and/or dehydrated plant materials are known to survive lower temperatures than nonhardened, succulent materials (12, 13, 15). In vitro cultures are often the source of material for low-temperature storage and cryopreservation studies. Normally, in vitro shoot cultures are maintained at warm temperatures of 23–32°C with long photoperiods (11); these conditions do not usually promote development of cold hardiness in woody plants (15). Chen et al. (2) applied short-photoperiod and low-temperature acclimation regimes to stem-cultured plants and leaf callus of *Solanum* species and found that some increased in hardiness by up to 7°. Callus cultures of poplar (9) and chrysanthemum (1) showed an increase in hardiness when acclimated at low temperatures.

A positive relationship between sugar levels and cold hardiness in plant materials has been reported (6). Tumanov et al. (14) found that hardiness of cold-acclimated cherry callus increased when grown on medium containing elevated sucrose levels.

This work was undertaken to investigate methods of cold hardening in vitro shoots as pretreatments for storage of cultures under minimal-growth conditions or cryopreservation of shoots or meristems.

Shoot proliferation cultures of MM 106 apple rootstock and 'Smoky' saskatoon were grown in vitro on medium containing Murashige and Skoog major and minor salts (7), B5 vitamins (3), 3% sucrose, and 0.8% Difco Bacto-agar. Benzyladenine (BA) at 8.9×10^{-6} M (2.0 mg·liter^{-1}) was included in the apple medium, while 1.1×10^{-5} M (2.5 mg·liter^{-1}) BA and 5.4×10^{-7} M (0.1 mg·liter^{-1}) naphthaleneacetic acid were included in the saskatoon medium. The pH of all media was adjusted to 5.8 and all media were sterilized by autoclaving for 20 min at 120°C and 1.46 kg·cm^{-2} (20-lb) pressure. Cultures were grown in 110-ml glass specimen jars containing 25 ml of medium and were incubated at 20° ± 2° under Gro-Lux lights (GTE, Danvers, Mass.) for 16-hr days.

Temperature and photoperiod hardening treatments. Apple and saskatoon shoots, 1 cm long, were excised from proliferating cultures and placed on 25 ml of proliferation medium in 25 × 150 mm test tubes. All cultures were incubated for 2 weeks at 20° ± 2°C under 16 hr days and subsequently subjected to one of 5 low-temperature, short-day (8 hr) hardening treatments (Fig. 1) for 8 weeks. In treatment 3, shoots were given two 48 hr exposures to −3°, while in treatment 4, shoots were given a single 48 hr, −3° exposure. A control group of shoots was incubated at 20° ± 2° under 16-hr days for the entire 10-week period. The lethal temperature for 50% of the shoots (LT_{50}) in each treatment then was determined.

Elevated sucrose levels in medium. Apple shoots, 1 cm long, were excised from proliferating cultures and grown for 2 weeks at 20° ± 2°C under 16-hr days on media containing elevated levels of sucrose (6%, 8%, 10%, 12%, 14%, 20%, or 30%). The LT_{50} and moisture content of shoots from each treatment were then determined (Fig. 2).

Moisture content determinations. Fresh and oven dry (80°C for 24 hr) weights were determined for the shoots and moisture content was expressed as a percentage of the fresh weight.

Determination of LT_{50}. A moist sterile piece of Kimwipe tissue (Kimberly-Clark, Toronto, Ontario, Canada) was placed on top of the apple or saskatoon shoot in each test tube to inoculate the sample with ice. The test tubes were placed in a programmable freezer and held at −3°C for 16 hr. The temperature was then lowered −2° per hour

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