In Vitro Culture of Forest Tree Calluses and Organs

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In vitro culture of forest tree calluses and organs was mainly developed as a method of forest tree breeding in Japan. It consists of micropropagation in test tubes for replacing cuttings, anther culture to produce homozygous plants, and protoplast techniques such as protoplast isolation and cell fusion for somatic hybridization.

Pine and *Paulownia* calluses were used for biosynthesis study and biochemical analysis of lignin formation pathway. In other case pine and *Cryptomeria* calluses were used to reproduce pine wood nematode (*Bursaphelenchus Xylophilus*) on pine callus tissues.

Cryptomeria callus was cultured to study organ formation from cultured callus.

Root tip culture was performed to study physiology of tree growth.[4,24]

Callus culture

1) *Study of lignin by tissue culture of Pinus strobus (Eastern white pine) and Paulownia tomentosa (Kiri)*

Callus culture of Eastern white pine succeeded in 1960 by using the RW medium* for *Picea glauca* tissue, while Japanese pines could not be cultured by this medium. Using this callus, formation of shikimic acid and lignin from glucose in the cambium tissue was investigated.[2] Glucose-1-^{14}C, shikimic acid-G-^{14}C, sodium acetate-1-^{14}C and sodium acetate-2-^{14}C were administered to the tissue culture of Eastern white pine. Glucose was well incorporated into shikimic acid, showing that the extent of the conversion of glucose to shikimic acid was about 3–5 times greater than that of acetic acid. Shikimic acid was very efficient as a precursor of aromatic nucleus, and glucose was also converted efficiently to lignin. The extent of incorporation of acetic acid, however, was considerably low. A possibility of synthesis of lignin and its precursor from glucose *via* the shikimic acid pathway in the cultured tissue was discussed.

Chemical nature of lignin in cultured tissues of Eastern white pine and *paulownia* tree was studied in another experiment.[3]

2) *Callus culture of Pinus densiflora (Japanese red pine), Pinus thunbergii (Japanese black pine) and Cryptomeria japonica (Sugi) for multiplication of Bursaphelenchus Xylophilus (pine wood nematode) or as a method of long-term preservation of favorable germ plasm*

Pine and Sugi callus cultures were performed with an attempt to propagate the pine wood nematode in a nematological study.[22,23] Cultured callus of Japanese red pine, Japanese black pine and Sugi on the LS medium* supplemented with several organic substances or cultured callus of Japanese black pine on the modified SSS medium* supplemented with yeast extract was suitable for reproduction of pine wood nematodes inoculated to these calluses. Calluses of the three species showed amber color after inoculation of the nematodes. Non-inoculated pine callus also changed to

RW medium*: Reinert & White's medium

LS medium*: Linsmaier and Skoog's medium
SSS medium*: Steinhart, Standifer and Skoog's medium

amber color but only non-inoculated Sugi callus continued growing. The nematodes multiplied up to 4,792 from 140 per tube in Japanese black pine callus after one month, and reached the maximum, 7,960, after 2 months.

Callus culture of Japanese black pine was made by Sato[20] for the purpose of long-term storage of callus tissue as a method to preserve favorable germ plasm to be used for future forest tree breeding. As for the culture medium for Japanese black pine callus, neither WS* nor MS* medium was suitable, but modified SSS medium was suitable. As to growth regulators NAA*, 2,4-D*, BAP*, and KIN* were compared each other in the range of 10^{-4}M–10^{-8}M. Concentration of 10^{-5}M of NAA or 10^{-4}–10^{-5}M of 2,4-D gave vigorous growth of the callus. Neither BAP nor KIN was effective by a single application, but a mixture of 10^{-5}M NAA and 10^{-7}M BAP showed a good result. The tissues were transplanted at intervals of 60 days and transplanting to subculture was repeated up to the sixth generation in a year.

3) Callus formation of Sugi, growth regulators for callus growth, and subculture test

Sugi callus was induced from a culture of immature seeds by Sato.[18] In his experiment growth regulators, i.e., combination of NAA and BAP, or 2,4-D and BAP, were supplemented to the WS medium. Good callus formation was obtained by combined supplement of 10^{-5}M NAA and 10^{-6}M BAP, or single supplement of 10^{-5}M 2,4-D. In this case, the callus formation was greatly influenced by immaturity of seeds. Subculture test was continued up to the tenth generation with good growth, by repeating transplantation every four months over a period of three years. Effects of inorganic elements and growth regulators on somatic callus culture of Sugi were studied by Saito.[12,16]

In Isikawa's experiment, adventitious roots were generated in somatic callus culture of Sugi by combined supplement of 1×10^{-5}M NAA and $1–8 \times 10^{-6}$M BAP to the WS medium[8] (Plate 1). Adventitious bud formation was not observed in this experiment.

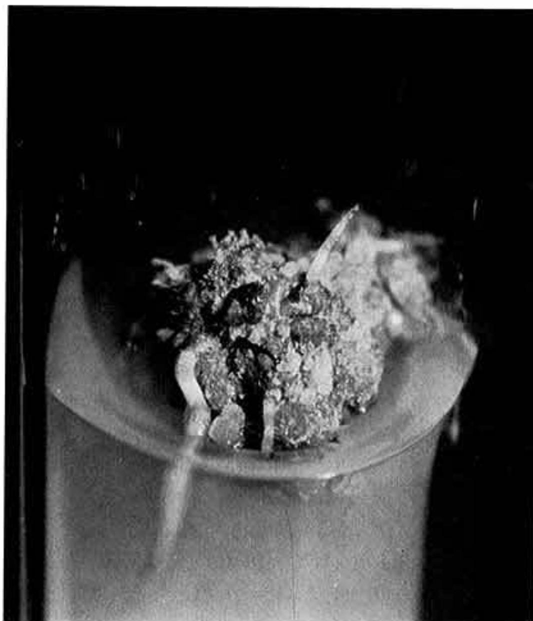

Plate 1. Adventitious root formation of *Cryptomeria* callus

4) Callus induction from small pieces of cambium attached to cortex, and shoot or embryoid formation from the calluses

Poplar Y-102 (*Populus sieboldii* × *P. grandidentata*) callus induction from a small piece of cortex with cambium of a new shoot was tested.[21] The poplar callus was cultured in the MS medium supplemented with 2,4-D and BAP. The subculture was repeated four times. After that, effect of NH_4NO_3 on shoot formation from the callus in the WS medium was examined at the concentration of 676 ppm or 1,637 ppm, and 50 ppm, a standard concentration of NH_4NO_3. Concentration of 676 ppm NH_4NO_3 and 3.16×10^{-5}M and 10^{-6}M zeatin showed better shoot formation after three

WS medium*: Wolter and Skoog's medium
MS medium*: Murashige and Skoog's medium
NAA*: Naphthalene-1-acetic acid
2,4-D*: 2,4-Dichloro-phenoxy acetic acid
BAP*: 6-Benzyl aminopurine
KIN*: Kinetin

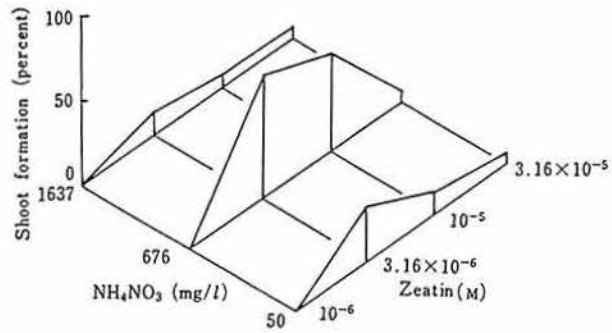

Fig. 1. Effect of NH_4NO_3 and zeatin on shoot formation of callus of a poplar hybrid

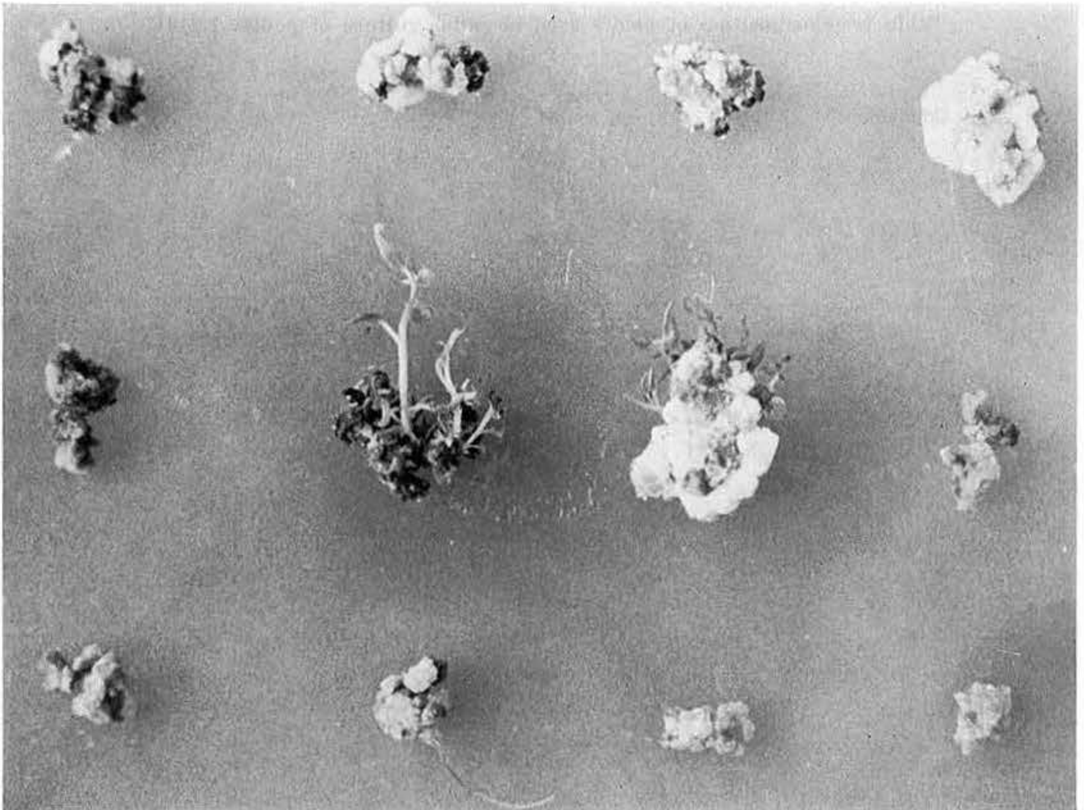

Plate 2. Shoot formation on poplar hybrid calluses
Top: WS standard medium (NH_4NO_3 50 mg/l)
Middle: WS medium N (NH_4NO_3 676 mg/l)
Bottom: WS abundant N (NH_4NO_3 1,637 mg/l)
Columns from left to right: 10^{-6}, 3.16×10^{-6}, 10^{-5}, and 3.16×10^{-5}M zeatin

months (Fig. 1, Plate 2). The culture was continued in a culture room at the condition of 24°C, 14 hr illumination of 1,200 lux in a day and 40–60% air humidity. Modified MS medium was tested for other poplar I-214 (*P. euramericana* cv. I-214) by Saito.[13] Different concentration of NH_4NO_3, NaH_2PO_4 and KCl in the medium were tested. The result showed that the best shoot formation from the callus was obtained by 2,370 ppm NH_4NO_3, 45 ppm NaH_2PO_4, and 1,087 ppm KCl. Formulae of the media for better callus and shoot formation were presented in Table 1.[13] It was clarified that poplar can give fairly better callus formation and shoot formation from the callus than other trees.

Callus culture of red horse chestnut (*Aesculus carnea*) was performed in the medium A (Table 2), and embryoid formation was observed in the medium B (Table 3, Plate 3).[14]

In other report on growth of callus tissue from poplar (cv. I-214) cambium *in vitro* by Oota et al.[10], effect of initial pH and concentration of 2,4-D, NAA and sucrose in modified SSS medium on the growth was studied. The best growth was obtained with the medium containing 0.05–0.1 ppm 2,4-D, 2,5 ppm NAA and 3% sucrose. Fresh weight of the tissue increased about six times after 50 days of incubation. The optimum pH was between 5 and 6.

Table 1. Composition of media used in callus culture of poplar I-214[13]

Component	Basal medium* (ppm)	Medium for shoot formation (ppm)		
		(L)	(M)	(H)
NH_4NO_3	1,650	88	568	2,370
$NaH_2PO_4 \cdot 2H_2O$	—	9	45	170
KCl	—	—	229	1,087
KH_2PO_4	170		—	
KNO_3	1,900		78.7	
KI	0.83		0.83	
$CaCl_2 \cdot 2H_2O$	440		642	
$MgSO_4 \cdot 7H_2O$	370		1,562	
$Na_2 \cdot EDTA$**	37.3		37.3	
$FeSO_4 \cdot 7H_2O$	27.8		27.8	
H_3BO_3	6.2		6.2	
$MnSO_4 \cdot 4H_2O$	22.3		22.3	
$ZnSO_4 \cdot 7H_2O$	8.6		8.6	
$Na_2MoO_4 \cdot 2H_2O$	0.25		0.25	
$CuSO_4 \cdot 5H_2O$	0.025		0.025	
$CoCl_2 \cdot 6H_2O$	0.025		0.025	
Thiamine HCl	0.4		0.4	
Inositol	40		40	
Niacin	1.5		1.5	
Pyridoxine HCl	0.4		0.4	
L-glycine	0.2		0.2	
L-tyrosine	—		32	
2,4-dichlorophenoxyacetic acid (2,4-D)	0.04		0.04	
6-benzylaminopurine (BAP)	0.20		0.50	
Biotin	—		0.5	
Sucrose	30,000		30,000	
Agar	10,000		10,000	

Note: pH was adjusted to 5.8 with NaOH or HCl prior to addition of agar and autoclaving.
* Modified from Murashige and Skoog's medium (1962)
** $Na_2 \cdot EDTA$: disodium ethylenediaminetetraacetate, dihydrate

Table 2. Composition of nutrient medium A used to induce *Aesculus* callus and for its subculture

Component	ppm	Component	ppm
KCl	1, 500	Inositol	10
$NaH_2PO_4 \cdot 2H_2O$	39	Thiamine HCl	0. 6
$MgSO_4 \cdot 7H_2O$	370	Adenine sulfate	50
KI	1. 6	Folic acid	0. 1
$MnSO_4 \cdot 4H_2O$	17	Choline chloride	1
$Na_2MoO_4 \cdot 2H_2O$	0. 25	L-glycine	1
$CoCl_2 \cdot 6H_2O$	0. 025	L-serine	1
$CaCl_2 \cdot 2H_2O$	440	Vitamin B_{12} (VB_{12})	0. 0015
Fe · EDTA*	5. 5	L-cysteine	3
Na_2SO_4	425	Vitamin C (VC)	1
KNO_3	100	Guanosine	10
L-arginine	87	Glucronic acid	100
Formate	3	3-indolebutyric acid (IBA)	10
Calcium pantothenic acid	0. 1	α-naphthylacetic acid (NAA)	0. 06
Niacin	0. 8	6-benzylaminopurine (BAP)	0. 40
Biotin	0. 1	Sucrose	20, 000
L-glutamic acid	5	Agar	8, 000
Pyridoxine HCl	0. 1		

Notes: pH was adjusted to 5.8 with HCl or NaOH before adding the agar.
* Fe·EDTA: Ferric monosodiumethylenediaminetetraacetate

Table 3. Composition of medium B for inducing shoots from *Aesculus* callus

Component	ppm	Component	ppm
KCl	1, 200	Inositol	100
$NaH_2PO_4 \cdot 2H_2O$	40	Thiamine HCl	0. 6
$MgSO_4 \cdot 7H_2O$	410	Adenine sulfate	20
H_3BO_3	3. 2	Folic acid	0. 1
$CoCl_2 \cdot 6H_2O$	0. 025	Choline chloride	0. 5
$CaCl_2 \cdot 2H_2O$	330	L-glycine	3
$Ca(NO_3)_2 \cdot 4H_2O$	530	NADP**	2
Na_2SO_4	426	Sodium *iso*-ascorbate	2
KNO_3	850	Adenosine triphosphate (ATP)	1
sym-diphenylurea	3	Guanosine	10
L-arginine	70	L-aspartic acid	10
Fe · EDTA*	5. 5	L-phenylalanine	1
NH_4NO_3	130	Gibberellin (GA_3)	0. 5
$NiCl_2 \cdot 6H_2O$	0. 03	IBA*	1
NH_4Cl	35	3-indoleacetic acid (IAA)	0. 20
Sodium citrate	4	BAP*	0. 40
Calcium pantothenic acid	0. 1	Sucrose	10, 000
Niacin	0. 8	Sorbitol	5, 700
L-glutamic acid	10	Agar	8, 000
Pyridoxine HCl	0. 1		

pH was adjusted to 5.8 with HCl or KOH before adding the agar.
* cf. Table 1
** NADP: Nicotineamide-adenine dinucleotide phosphate

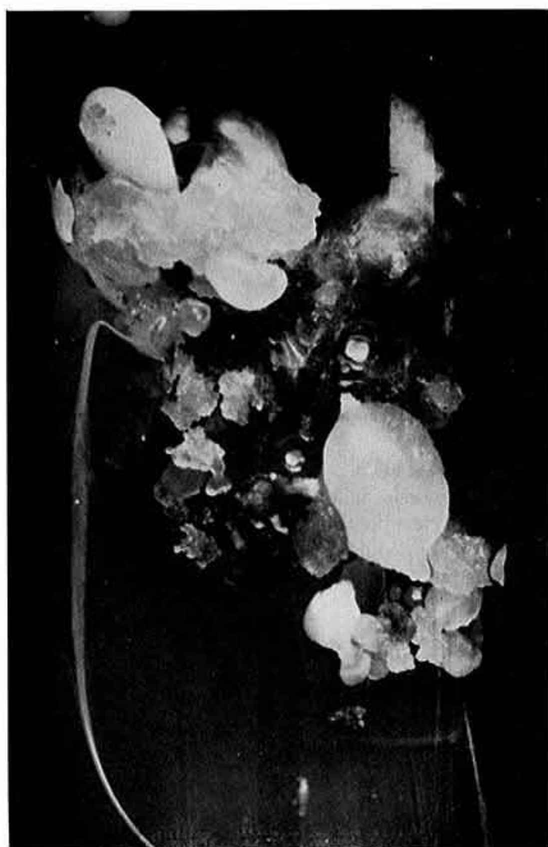

Plate 3. Embryoid formation observed in cultured callus of *Aesculus carnea*

Isolation of protoplast from *Paulownia* and *Populus*, and cell fusion of *Populus* protoplasts

Callus cultures and plantlet generation from calluses of *Poplar* and *Paulownia* were previously reported by Wolter,[26] Winton[25] and Fan & Hu.[1] These species have a vigorous power of regeneration from callus among forest trees. *Paulownia fortunei* and *Populus euramericana* cv. I-45/51 were used as materials to isolate protoplasts from mesophyll cells.[11] Protoplasts were obtained from expanding young leaves of them grown in open air. A method to induce fusion of the isolated protoplasts was developed.[15] It was found out that polyethylene glycol (PEG) had the effect of causing aggregation at the ratio of one to one in a mixture of protoplasts isolated from two different species, and also from different individual cells in various strains within the same species. Complete fusion of cells between different strains of poplar or different individuals of paulownia was observed (Plate 4). However a specific phenomenon was found out in the fusion of cells between *Populus* and *Paulownia*. Cytoplasm in a protoplast of poplar was transferred into a protoplast of *Paulownia* in a moment, but cell membrance of *Populus* was left behind indefinitely.

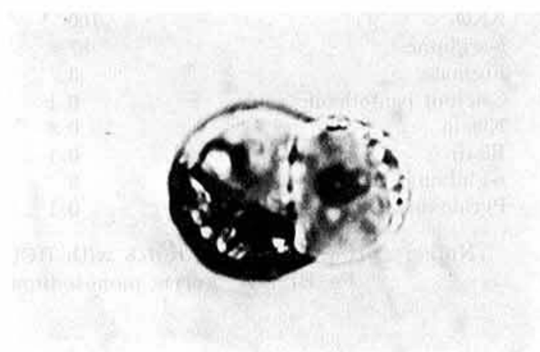

Plate 4. Somatic cell fusion: *Populus* to *Populus*

Further proliferation of these cells and generation of plantlets were not observed. Anyway, at the present stage of investigation methods to harvest fused cells from mixture of cells in the fusing medium should be developed.

Organ culture of forest trees

1) Anther culture

Experiments were carried out to establish a method of anther culture of poplars (*Populus sieboldii* × *P. grandidentata*, *P. maximowiczii* and *P. deltoides*).[17] Effects of auxins (NAA, IBA, 2,4-D), and cytokinin (BAP) on callus induction and organogenesis were tested by combining a wide range of concentrations of auxins and BAP added to WS and M* medium. The results are summarized as follows:

(1) Effects of auxins and BAP combined

M* medium: Miller's medium

at various concentrations on callus induction; Callus of aspen was induced only on WS medium with 2×10^{-7}M NAA combined with BAP, and small shoots were often generated on the callus, but no effect was obtained by IBA and 2,4-D. *P. maximowiczii* responded to WS medium supplemented with 2,4-D at all concentrations tested and with NAA at high concentrations, and to M medium supplemented with 2,4-D at high concentrations. In *P. deltoides*, callus was raised only on WS medium supplemented with 2,4-D and NAA at high concentrations.

(2) Root and shoot formation from callus of aspen; In the experiment (1), calluses having generated shoots, 2 mm or longer in length, and calluses without generated shoots were produced as stated above. Then, the former was transferred to WS medium supplemented with NAA alone, and the latter to the medium supplemented with BAP and NAA in combination.

In the former, root initiation occurred within 5–15 days on the WS medium with NAA alone and the rooted shoots grew into normal plants. In the latter, new shoots were produced from the surface of the calluses when the medium contained 1×10^{-6}M BAP (combined with 3.16×10^{-7} or 1×10^{-6}M NAA) and 3.16×10^{-6}M BAP (combined with 3.16×10^{-7}M NAA), while morphogenesis was inhibited by these substances in higher concentrations.

(3) Chromosome number of plants obtained through anther culture; Diploid chromosomes were observed in root tips of generated aspen plantlets, but haploid plants have not yet been recognized.

2) Hypocotyl culture

Experiments were carried out to establish a method of hypocotyl culture of coniferous trees and to obtain adventitious shoots from hypocotyl or hypocotyl callus, as a basic study of micropropagation to acquire adventitious multiple shoots from hypocotyl.[5,6,8,9] Adventitious buds were generated on the WS medium in the culture of hypocotyl cuttings taken from *Cryptomeria*, *Chamaecyparis* and Japanese black pine (Plate 5). The culture medium contained the WS simplified mineral constituents with the following addition: 0.1 mg/*l* of thiamine, 0.5 mg/*l* of nicotinic acid, 100 mg/*l* of meso-inositol, 2 mg/*l* of glycine, 10 g/*l* of agar and 20 g/*l* of sucrose. The pH of the medium showed 5.4 without adjusting after autoclaving. Further addition of BAP, singly

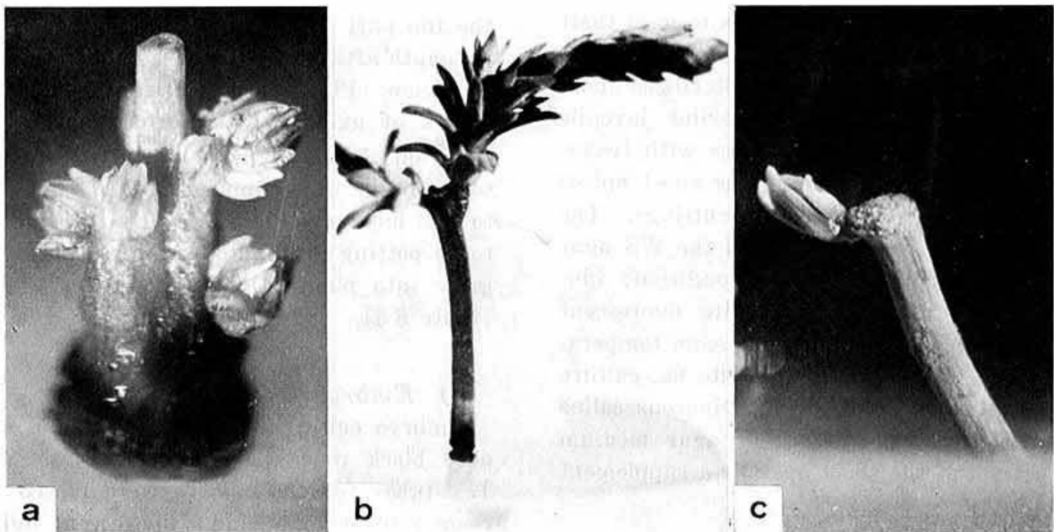

Plate 5. Adventitious bud formation on hypocotyls
a: *Cryptomeria*, b: *Chamaecyparis*, c: Japanese black pine

Table 4. Effects of combined treatments of BAP and CFl on shoot formation in *Cryptomeria* hypocotyl segments in four months of culture

BAP (mg/l)	0	2.0	2.0	2.0	2.0
CFl (mg/l)	0	0	0.5	1.0	2.5
Bud generation (%)	71	60	57	63	18
Average number of buds per segment	1.00	2.33*	2.50**	1.20	1.00
Shape of buds	Normal	Normal	Abnormal	Abnormal	Abnormal

The number of segments used per treatment ranged from 7 to 11.
*, **: Difference from the plot without BAP and CFl was statistically significant at 5% and 1% level, respectively, on the basis of analysis of variance.

Table 5. Effects of combined treatment of BAP and CFI on shoot formation in *Chamaecyparts* hypocotyl segments in four months of culture

BAP (mg/l)	0	2.0	2.0	2.0	2.0
CFl (mg/l)	0	0	0.5	1.0	2.5
Bud generation (%)	25	88*	86*	88*	57
Average number of buds per segment	2.00	5.14	4.33	2.86	1.50
Shape of buds	Normal	Normal	Abnormal	Abnormal	Abnormal

The number of segments used per treatment ranged from 7 to 8.
*: Significant at 5% level, based on chi-square test.

or in combination with CFl* promoted bud formation (Tables 4 and 5).

3) In vitro culture of shoot apices and axillary buds

Shoot apices and axillary buds of mature trees were cultured in test-tubes to gain their ramets,[7,8] using shoot tips of famous wide-spread cultivars as materials. Sterilized shoot apices were exposed by removing juvenile needles surrounding shoot apices with tweezers, and small tips including the shoot apices were cut off from heads of cuttings. The tips thus prepared were put on the WS agar medium under the following condition: illumination of 1,900 lux of white fluorescent light, day length of 14 hr, and room temperature of 25°C. As shown in Plate 6a, culture of a shoot apex resulted in a vigorous callus formation between surface of agar medium and bottom of the shoot apex by a supplement of 0.1–1.0 mg/l IBA or 0.1–10.0 mg/l 2,4-D (Plate 6 a). No root formation was observed in the shoot apex culture.

In the next attempt, sterilized cuttings of 3 cm in length were put in the same medium. A few axillary buds elongated at axillae of the top part of cuttings, and reached 2–3 cm in length after 8 to 10 months under the same condition (Plate 6 b). Cuttings taken from shoots of axillary buds formed adventitious roots on the basal parts of cuttings after 1 to 3 months by a supplement of 0.3 mg/l IBA to this medium (Plate 6 c). By transplanting to a potting medium from test-tubes, they grew into planting materials after 2 years (Plate 6 d).

4) Embryo culture

Embryo culture of *Cryptomeria* and Japanese black pine was performed by Sato.[19] Test-tubes for the culture were placed in a room kept at 25°C, under fluorescent light of 1,200 lux, and 14 hr illumination per day. *Cryptomeria* embryos were generally easier to

CFl*: Chlorflurenol (Methyl-2-chloro-9-hydroxyfluorene-(9)-carboxylate, Na-salt), one of morphactins.

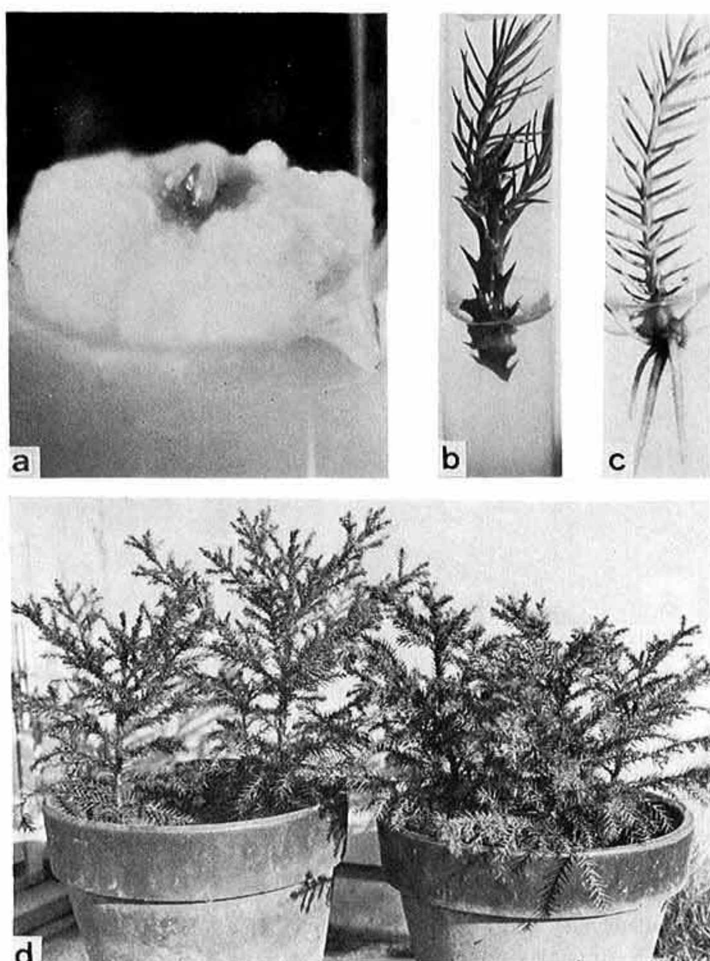

Plate 6. Shoot apex culture of *Cryptomeria*
a: Callus formation of shoot apex, b: Axillary buds formation of branchlet cutting *in vitro*, c: Root formation of transplanted axillary bud cultured in test-tube, d: Planting materials transplanted to potted medium from test-tubes.

grow than pine embryos in various media. WS, 1/3 concentration of WS and 1/3 concentration of HG media are suitable for culture of *Cryptomeria* embryos. NAA drastically reduced mortality of *Cryptomeria* embryos, but it significantly stimulated development of calluses; in some cases every part of an embryo became callus. On the other hand, no media were sufficiently suitable for culture of pine embryos, but WS + NAA, HG* and S* media were rather better for culture than others (Plate 7).

5) *Vascular differentiation within callus by grafting bud to callus*

A morphological observation on vascular differentiation in *in vitro* callus of *Cryptomeria* was made.[27] Xylem core segments of current shoots were cultured for 100 days on

HG* medium: Harvey & Grasham's medium
S* medium: Slankis's medium

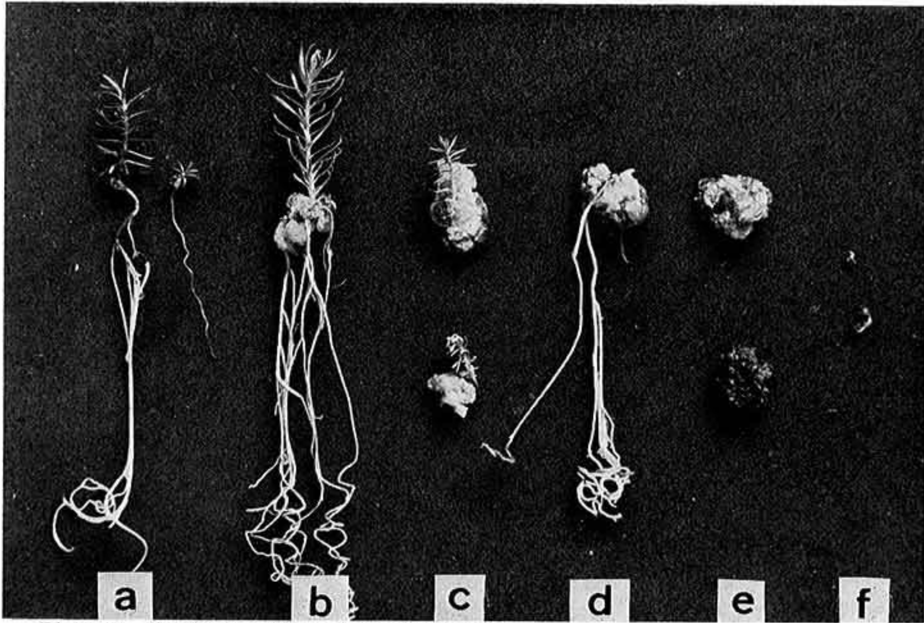

Plate 7. Embryo culture of *Cryptomeria*
a: Normal growth in the WS medium, b: Normal growth with callus formation in the MS medium, c: Callus formation in the MS medium, d: Callus formation, e: Callus formation on whole embryo in the HG medium, f: Dead embryos.

a modified WS medium containing 0.1 mg/*l* NAA and 2% sucrose. In the callus around the xylem surface, two types of vascular differentiation were observed. One was the cylindrical type which appeared around the original xylem and the other was nodular type. Furthermore, buds grafted onto xylem calluses caused another vascular differentiation.

6) *Culture of root nodule tissue of Alnus sieboldiana (Alder)*

This kind of research has been very rare, but will become valuable in future. Alder tissue was well cultured on the MS medium supplemented with 0.2 ppm KIN and 0.2 ppm 2,4-D. By devising complicated means of sterilization, Yambe[28] succeeded in obtaining root nodules with sterilized surface at the ratio of 10%, and by transplanting them to the culture medium, nodule tissue callus (germ-free root nodules) was produced at the ratio of 3%.

Vista to the future investigation

In the near future it is expected that cybrid plants will be generated from fused cells of forest trees on improved culture media. Furthermore, techniques for plant cell transformation by the use of vectors carrying useful genes of the same or other plant species will make a considerable progress.

On the other hand, improvements of micropropagation techniques will make possible mass propagation of candidate trees resistant to diseases and climatic damages or of other useful cultivars. These clones will be used not only for research, but also for afforestation in some cases.

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