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In Vitro Propagation Techniques of Tea Plants

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Abstract

Effective methods, simple and reliable of *in vitro* propagation of tea plants have successfully been established. The growth of shoots in culture of shoot tips and axillary buds was remarkably accelerated when the media were mixed with the combination of BA(0.1-1.0 mg/*l*) + GA_3(5.0 mg/*l*) or BA(1.0, 5.0 mg/l) + GA_3(1.0 mg/*l*). And it was observed that the axillary bud culture provided a more effective method, which was easier, simpler and quicker in securing the growth of shoots, as compared with the shoot tip culture. Differentiation of organs was observed on adventitious embryos (cotyledons used as the explants) and adventitious buds (stem segments used as the explants) cultured on the media containing BA(1.0-5.0 mg/*l*) and IAA(0.01-1.0 mg/*l*) + GA_3(1.0-5.0 mg/*l*), respectively. A small shoot each developed from these differentiated adventitious embryos and buds on the same media. As the number of shoots grown was limited, passage culture by in vitro cutting of nodal segment culture with axillary buds was repeated to propagate them, for which the optimum combinations of hormones were IBA(0.1 mg/*l*) + BA(1.0 mg/*l*) + GA_3(5.0 mg/*l*) or IBA(0.1 mg/*l*) + BA(0.1 mg/*l*) + 2iP(5.0mg/*l*) + GA_3(5.0 mg/*l*). By repeating this technique of nodal segment culture every 2 months, at least 47,000 (i.e. 6^6) plantlets could be multiplied from an original plantlet in a year. And rooting from these shoots was easily induced on 1/2 or 1/4 MS media incorporated with IBA(0.5-1.0 mg/*l*).

Discipline: Tea industry /Biotechnology

Introduction

Propagation practices utilizing an *in vitro* technique are presently employed with many plants. In the case of woody plants such as apple, grape and cherry, the techniques have found commercial uses[4].

The methods of *in vitro* propagation of plants can broadly be classified into two groups. The first is to use shoot apices, shoot tips and axillary buds as the explants. And this is a useful means for rapid clonal propagation, production of virus-free plants and preservation of genetic resources. The second group of *in vitro* propagation is to utilize adventitious embryos or buds formed in callus culture or even directly from somatic tissues. The latter is very useful for rapid mass propagation or in such breeding programs that culture zygotic embryos obtained from incompatible crosses.

With regard to the species belonging to *Camellia*, both of the above methods have been examined in several studies[1-3, 5-19]. These works, however, aim at establishment of primary culture to identify effects of explants[7,13-15], cultural conditions[1,2,6-19] and varietal differences[3,13,14]. The past papers have, however, fallen: (1) to establish a method to prevent shoot tip and axillary bud culture from bacterial contamination brought over by explants[3,5]; (2) to obtain plantlets simply and quickly; and then (3) to secure differentiation of adventitious embryos and buds at high rates[7-9,15].

The present paper attempts to review the results of the studies, which were undertaken by the author for the purpose of establishing practical methods: 1) to stimulate growth of shoots through the culture of shoot tips and axillary buds; 2) to achieve high rates of differentiation of adventitious embryos or buds through culture of cotyledons or young stems. It also includes some results and discussions on; 3) simple and reliable methods of *in vitro*

propagation on subculture using nodal segment culture with axillary buds in shoots that are derived from adventitious embryos; and 4) adequate conditions for the induction of roots from the subcultured shoots (Plate 1).

Results and discussions

1) Growth of shoot

(1) Shoot tip culture

When shoot tip explants were cultured, only 40–60% of the explants were found to be free from bacterial contamination as have been reported elsewhere[3,5]. This problem, however, was overcome through the selection of adequate time of culture for the collected shoots and the screening test of uncontaminated explants[12]. The optimum time of shoot tip culture was the beginning of the first flush, i.e. mid April in Shizuoka, Japan, when the air-temperature was relatively low and tea plants were juvenile and vigorous.

It is generally recognized that an addition of a cytokinin is necessary in shoot tip culture of woody plants[4]. This is also true with tea plants, which may require it for the growth of shoot tips. The growth of shoots cultured from the shoot tips of a matured tea plant, a cultivar of Yabukita, was accelerated on 1/2 MS when BA(0.1–3.0 mg/*l*) was added, as shown in Fig. 1. The largest number of leaves was obtained with BA(3.0 mg/*l*). In shoot tip culture of *Camellia japonica*, the appropriate concentration

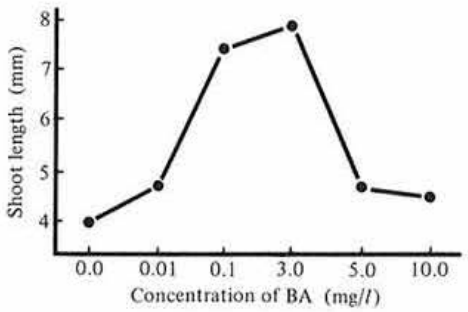


Fig. 1. Effects of BA on the growth of shoot tips
The medium consisted of 1/2 MS, 3% of sucrose and 0.75% of agar.
The explants were incubated in a growth room at 26–28°C under the dark condition.
The results obtained after incubation of ca. 2 weeks.

of BA was 0.5–1.0 mg/*l*[6,16,18], and in *C. sinensis* it was 0.5–3.0 mg/*l* of BA[1–3,12]. It appears, therefore, that the suitable strength of BA ranges 0.5–3.0 mg/*l* in the shoot tip culture of *Camellia* spp.

To promote growth of shoot tips, BA combined with auxins or gibberellin at various concentrations was applied. The combination of BA and auxins induced callus formation at the base of tips, while the growth was inhibited. The combination of BA(0.1–1.0 mg/*l*) and GA_3(5.0 mg/*l*), however, stimulated the growth of shoot tips to a greater extent than the other combinations examined (Table 1).

(2) Axillary bud culture

The axillary bud culture was examined so that plantlets could be obtained more easily and quickly than the aforementioned shoot tip culture.

The rate of plantlets free from bacterial contamination was significantly higher in the axillary bud culture as compared with that of the shoot tip culture. When axillary buds were cultured on the 1/2 MS medium without hormone, the buds incubated under the illuminated condition (16-hr lighting, 3,000–5,000 lx) grew three times faster than those under the dark condition. Effects of same combination of BA and GA_3 that greatly stimulated the growth of the tips in the above shoot tip culture were also examined. It was observed that all the combinations were scarcely effective when illuminated. In the dark condition, however, the addition of either BA(1.0, 5.0 mg/*l*)+GA_3(0.0, 1.0 mg/*l*) or BA

Table 1. Effects of combinations of BA and GA_3 on the growth of shoot tips[a)]

Concentration of BA(mg/*l*)	Concentration of GA_3(mg/*l*)		
	0.0	1.0	5.0
Shoot length (mm)			
0.01	6.5	12.2	9.2
0.1	6.0	9.3	21.2
1.0	12.4	10.8	22.8
5.0	6.8	4.7	10.3
lsd 0.05 = 5.9			
No. of leaves			
0.01	2.9	3.3	2.9
0.1	2.4	3.4	3.4
1.0	3.9	3.6	3.4
5.0	3.4	2.4	3.2
lsd 0.05 = N.S.			

a): The medium and explants were the same as Fig. 1. The results obtained after incubation of ca. 10 weeks.

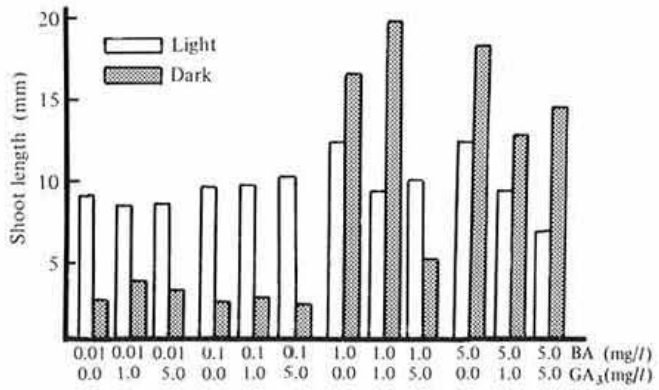


Fig. 2. Effects of combination of BA and GA_3 on the growth of axillary buds
The medium consisted of 1/2 MS, 3% of sucrose and 0.75% of agar.
The results obtained after incubation of ca. 40 days.
Light: 16-hr illumination, Dark: no illumination.

(5.0 mg/*l*) + GA_3 (5.0 mg/*l*) accelerated the growth of shoots significantly as shown in Fig. 2. The different light conditions showed different patterns of shoot formation. The shoots grown under illumination had short internodes and broad, green leaves, while those from the dark condition developed long and slender internodes and small, whitish leaves. The latter, when put into the illuminated condition, turned green in a week.

The above results confirmed that the growth of shoots with the shoot tip culture and the axillary bud culture was accelerated in both cases by the supplement of BA and GA_3, and that the axillary bud culture was more effective to secure the growth of shoots easily, simply and quickly, as compared with the shoot tip culture.

2) Differentiation of adventitious embryos or buds

(1) Differentiation of adventitious embryos

The differentiation of adventitious embryos in cotyledon cultures has so far been reported with only a few species of *Camellia*[3,9,13,19]. According to those reports, the differentiation was achieved in the media supplemented by high concentrations of a cytokinin plus a low auxin or a cytokinin only.

In the course of micropropagation through cotyledon culture of tea plant Yabukita, the effects of different concentrations of BA and time of culture on the differentiation of adventitious embryos was examined[13]. When cotyledons were cultured on the MS medium containing agar (0.75%) and sucrose (3.0%), a great number of adventitious embryos were formed directly on the surface of the half split cotyledons. The addition of BA (1.0–5.0 mg/*l*) raised the rate of the differentiation to the level of 30% or higher from 16.7% of that without the hormones. The differentiation of adventitious embryos in a few basal media and a few combinations of either BA + NAA or BA + GA_3 was lower in those rates and more erratic than that of BA (1.0–5.0 mg/*l*) alone. Also, it was observed that the time of culture influenced very significantly the rate of differentiation of adventitious embryos, as shown in Fig. 3. The optimum time of cotyledon culture for achieving the highest differentiation rate of adventitious embryos fell in the period of late September to mid October (Stage 2), when matured seeds were used, in Shizuoka, Japan. The rate of differentiation was lowered when unmatured seeds (Stage 1) or stored ripe seeds (5°C for 2–3 months, Stage 3) were used for the culture.

(2) Differentiation of adventitious buds

Differentiation of adventitious buds in tea plants has been observed only in the calluses developed on the cut surfaces of young stems, young leaves and/or young buds[7,14,15].

In this experiment, young stem segments of matured tea plants Yabukita were cultured on the

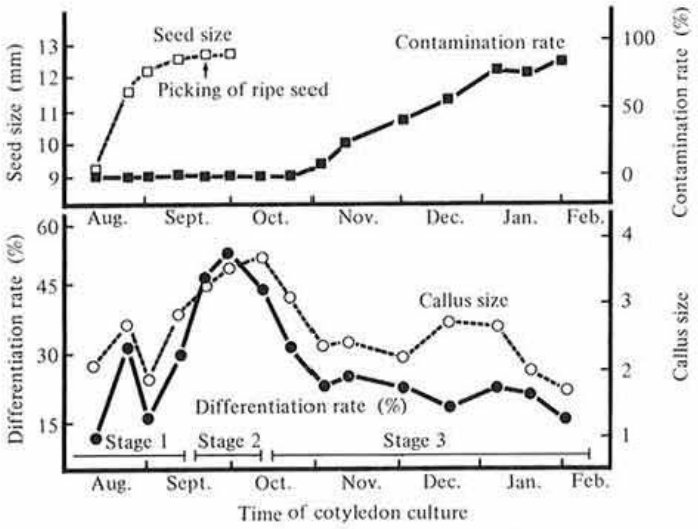


Fig. 3. Effects of the time of cotyledon culture on the differentiation of adventitious embryo

Stage 1: The cotyledons of unripe seeds were picked from plants and were incubated immediately.

Stage 2: Ripe seeds were collected at the abscission time and were incubated immediately.

Stage 3: The cotyledons of ripe seeds stored in a polyethylene bag at about 5°C were cultured.

The medium consisted of MS with 3% of sucrose and 0.75% of agar and 3.0 mg/l of BA was added.

The explants were incubated in a growth room at 28°C under the dark condition.

The results obtained after incubation of ca. 3 months.

Callus sizes were expressed by the indices of 0 (living without callus) to 5 (large callus covering the whole of segment).

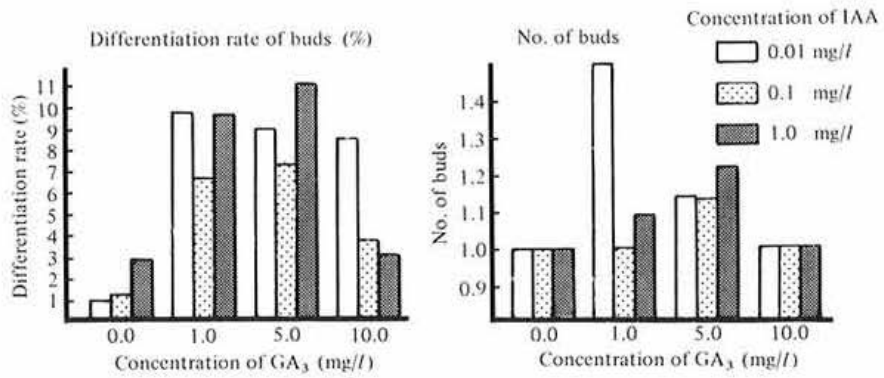


Fig. 4. Effects of combination of IAA and GA_3 on the differentiation of buds

The medium consisted of 1/2 MS, 3% of sucrose and 0.75% of agar, and culture condition was at 26–28°C under the dark.

The explants were cut by knife to 2–3 mm long of second or third internode of 5-leaf-stage-shoots.

The results obtained after incubation of ca. 3 months.

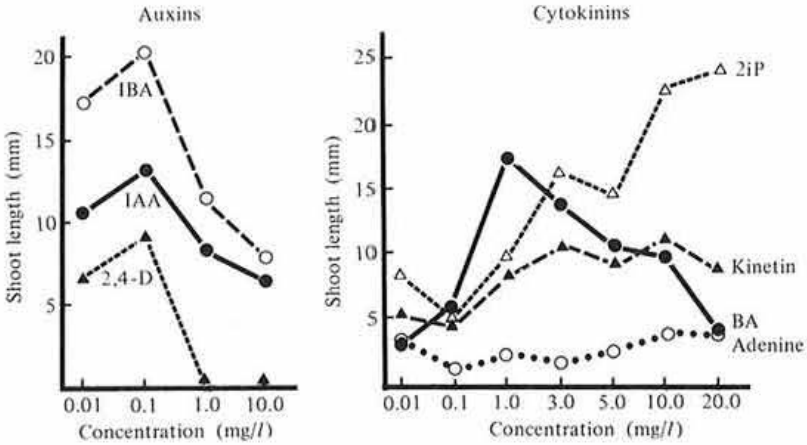


Fig. 5. Effects of auxins and cytokinins on the growth of axillary buds
The medium consisted of 1/2 MS, 3% of sucrose and 0.75% of agar, and culture condition was at 26–28°C under the 16-hr illumination.
The explants were cut into 4–7 mm-long nodal segments with an axillary bud from the shoots that were 5 cm-long.
The results obtained after incubation of ca. 2 months.

Table 2. Effects of explant shape on callus size and differentiation of adventitious bud[a)]

Shape of explants	Callus size[c)]	Differentiation of buds	
		Differentiation rate of bud (%)	No. of buds
Stem cutting[b)]	1.07	7.6	1.0
Half stem cutting	1.12	21.2	1.1

a): The medium consisted of MS with IAA(0.1 mg/*l*) + GA_3(5.0 mg/*l*) and culture condition was at 25–28°C under the dark.
The results obtained after incubation of ca. 75 days.
b): Stem cutting explants were cut by knife to 2–3 mm-long of second or third internode of 5-leaf-stage shoots, while the half stem cutting explants were the longitudinally bisected stem cut pieces.
c): Callus sizes were expressed by the indices of 0 (living without callus) to 5 (large callus covering the whole of segment).

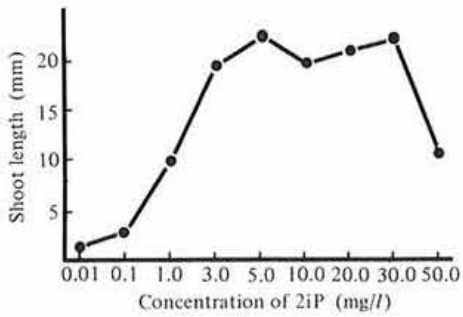


Fig. 6. Effects of 2iP on the growth of axillary buds
The medium consisted of 1/2 MS, 3% of sucrose and 0.75% of agar.
The explants and culture conditions were the same as Fig. 5.
The results obtained after incubation of ca. 2 months.

MS media supplemented with various hormones with the purpose of determining the medium composition suitable for differentiation of adventitious buds. Among the four kinds of auxins tested, adventitious buds were differentiated in the presence of IAA (0.01–1.0 mg/*l*)[14].

To raise a differentiation rate of the adventitious buds, IAA combined with BA, kinetin or GA_3 each at various concentrations was applied. Differentiation of the adventitious bud was observed only with the combinations of GA_3. The differentiation rate of adventitious bud in the combination of IAA (0.01–1.0 mg/*l*) + GA_3(1.0–5.0 mg/*l*) was higher than that of IAA alone, as shown in Fig. 4. The combination of IAA + BA or kinetin however did not induce any adventitious bud, while only large calluses

were developed on the explants. These adventitious buds differentiated only from the small lumps of calluses developing on the cut surface of the explants. In case of longitudinal slices of ordinary cross sections, the cut surface areas were enlarged and the differentiation rate of adventitious bud was raised to the level of 21.2% from 7.6% of the cross section, as shown in Table 2.

From the above results, it is concluded that adventitious embryos and buds could be successfully differentiated on the MS medium with the addition of either BA or IAA + GA_3. In this study, however, the differentiation of adventitious embryos was achieved only when cotyledons were used as explants. It would be required to use part of the tissues of selected cultivars as explants for clonal propagation in the future.

3) Subculture of the shoot

The simple and reliable method of micropropagation on subculture is *in vitro* cutting of nodal segments[18]. The shoots grown from shoot tips, axillary buds, adventitious embryos or adventitious buds could be used as the explants in subculture.

In this experiment, the shoot was used as an explant derived from an adventitious embryo. When the nodal segments with axillary bud of this shoot were cultured in the 1/2 MS medium supplemented with three kinds of auxins, the bud sprouting was lower and the differentiation rates descended as the concentration of auxin increased. The shoot elongation was higher in order of IBA > IAA > 2,4-D when each auxin was added at the optimum concentration of 0.1 mg/*l*.

The activities of cytokinins for the growth of axillary buds were different with each other among the four kinds of BA, kinetin, adenine and 2iP, and varied with the concentration of each one. The shoot elongation was accelerated by the addition of BA(1.0 mg/*l*), 2iP(3.0–30.0 mg/*l*) or kinetin(1.0–20.0 mg/*l*), whereas the addition of adenine did not affect the growth of axillary buds, as shown in Figs. 5 and 6. To determine the optimum conditions which would accelerate the growth of axillary buds, various combinations of hormones were tested. It was found that the ideal combinations of hormones for shoot elongation were either IBA(0.1 mg/*l*) + BA(1.0 mg/*l*) + GA_3(5.0 mg/*l*) or IBA(0.1 mg/*l*) + BA(0.1 mg/*l*) + 2iP(5.0 mg/*l*) + GA_3(5.0 mg/*l*), as shown in Fig. 7: the shoots grown on these media were 32 mm in length, having 7.4 leaves in average.

For the purpose of obtaining following subcultures, the explants which had about five nodal segments with axillary buds or one shoot tip with young leaves were cut off from the foregoing shoots. By repeating such a nodal segment culture every 2 months

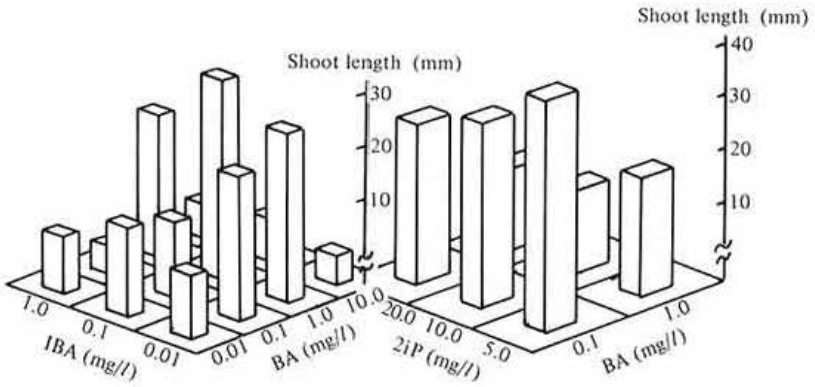


Fig. 7. Effects of combination of IBA and BA, 2iP and BA on the growth of axillary buds
In case of combinations of IBA and BA, the medium consisted of 1/2 MS, sucrose (3%) and agar (0.75%) that was added with GA_3(5.0 mg/*l*).
In case of combinations of 2iP and BA, the medium consisted of 1/2 MS that was added with IBA(0.1 mg/*l*) + GA_3(5.0 mg/*l*).
The explants and culture conditions were the same as Fig. 5.
The results obtained after incubation of ca. 2 months.

Table 3. Effects of different shoot length and IBA concentrations on rooting[a)]

Length of cultured shoots[b)]	Concentration of IBA (mg/*l*)	Rooting rate (%)	No. of roots	Length of the longest root (mm)	Callus formation rate (%)
Short	0.1	20.0	0.3	5.7	30.0
	0.5	30.0	1.4	8.2	20.0
	1.0	40.0	0.9	7.2	60.0
	3.0	10.0	0.4	2.6	60.0
	lsd 0.05		N.S.	N.S.	
Long	0.1	42.9	0.9	3.6	0.0
	0.5	71.4	3.4	11.1	0.0
	1.0	85.7	4.7	12.6	14.3
	3.0	100.0	3.4	16.3	57.1
	lsd 0.05		N.S.	8.7	

a): The medium consisted of 1/2 MS, 3% of sucrose on paper bridge method, and culture conditions were at 26–28°C under the 16-hr illumination.
The results obtained after incubation of ca. 7 weeks.
b): Short shoots were about 10 mm-long, and long shoots were about 20 mm-long.

Table 4. Effects of different shoot length and concentrations of MS macronutrients on rooting[a)]

Length of cultured shoots[b)]	Concentration of MS	Rooting rate (%)	No. of roots	Length of the longest root (mm)	Callus formation rate (%)
Short	1/4	50.0	1.2	8.8	40.0
	1/2	30.0	1.4	8.2	20.0
	1/1	0.0	0.0	0.0	40.0
	lsd 0.05		N.S.	N.S.	
Long	1/4	100.0	4.9	13.0	0.0
	1/2	71.4	3.4	11.1	0.0
	1/1	42.8	0.9	4.6	0.0
	lsd 0.05		2.6	N.S.	

a): Basic medium was MS, 3% of sucrose with 0.5 mg/*l* of IBA on paper bridge method, and other conditions were the same as Table 3.
The results obtained after incubation of ca. 7 weeks.
b): Shoot length: Refer to Table 3.

in the 1/2 MS medium supplemented with IBA (0.1 mg/*l*) + BA(1.0 mg/*l*) + GA_3(5.0 mg/*l*), approximately 47,000 (i.e. 6^6) of shoots could be obtained from one shoot in a year.

4) Rooting from shoot

In tea plants, rooting of main cultivars is easily obtained by the technique of cutting in nursery beds. Rooting from shoots *in vitro* is also rather easy[3,7,11,17]. However, it is influenced by the conditions of explants and culture. The ideal condition of rooting from the shoots obtained from adventitious embryo of cotyledon of Yabukita was examined in a liquid medium on a filter paper bridge. When the shoots with different length were incubated,

Table 5. Effects of temperature on rooting[a)]

Temperature (°C)	Rooting rate (%)	No. of roots	Length of the longest root (mm)
8.0	0.0	0.0	0.0
10.0	0.0	0.0	0.0
15.0	53.0	2.9	6.6
20.0	73.0	3.3	16.9
27.5	100.0	4.2	19.2
30.0	93.0	4.7	15.5

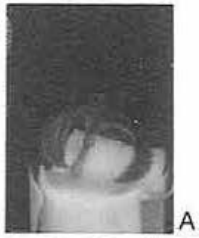
a): The medium was 1/2 MS, 3% of sucrose with 3.0 mg/*l* of IBA on paper bridge method, and culture condition was under the dark.
The explant was cut into 10 mm-long shoot with a bud and two leaves each.
The results obtained after incubation of ca. 2 months.

Explant

Shoot apex
Axillary bud
Cotyledon
Leaf
Stem
Others

1) Growth of shoot

(1) Shoot tip culture



A

BA (0.1-1.0 mg/*l*)+GA_3 (5.0 mg/*l*)

(2) Axillary bud culture

B

C

Dark condition

BA (1.0, 5.0 mg/*l*)+GA_3 (0.0, 1.0 mg/*l*)

or BA (5.0 mg/*l*)+GA_3 (5.0 mg/*l*)

2) Differentiation of adventitious embryos or buds

(1) Differentiation of adventitious embryos

D

E

BA (1.0-5.0 mg/*l*)

F

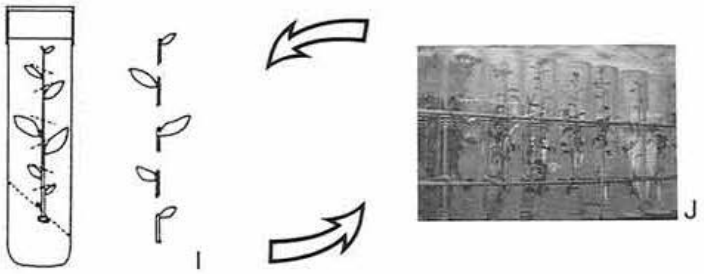
(2) Differentiation of adventitious buds

G

H

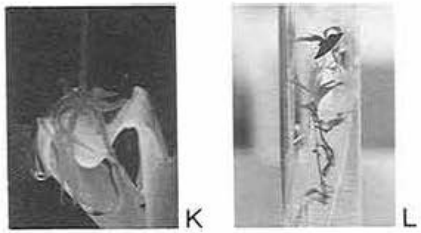
IAA (0.01-1.0 mg/*l*)+GA_3 (1.0-5.0 mg/*l*)

3) Subculture of the shoot



IBA (0.1 mg/*l*)+BA (1.0 mg/*l*)+GA_3 (5.0 mg/*l*) or
IBA (0.1 mg/*l*)+BA (0.1 mg/*l*)+2iP (5.0 mg/*l*)+GA_3 (5.0 mg/*l*)

4) Rooting from shoot



IBA (0.5-3.0 mg/*l*)

Plantlet

A: Growth of shoot.
B: Growth of axillary bud under the dark condition.
C: Growth of axillary bud under the illuminated condition.
D: Longitudinal section of differentiation of embryos from cotyledon surface.
E: Development of adventitious embryos.
F: Growth of adventitious embryo on cotyledon surface.
G: Longitudinal section of differentiation of bud from stem cutting.
H: Differentiation of bud from stem cutting.
I: The preparation of explants.
J: Micropropagation on subculture in *in vitro* cutting of nodal segment.
K: Rooting from shoot on a filter paper bridge.
L: Rooting from shoot on agar medium.
M: Transplantation of plants formed *in vitro* to vermiculite.

Plate 1. The scheme for *in vitro* propagation with a nodal segment culture in tea plants

longer shoots differentiated root more vigorously than the shorter ones did. Among the various concentrations of IBA, 0.5–1.0 mg/l and 0.5–3.0 mg/l provided higher rooting rates for short and long shoots, respectively, as shown in Table 3.

It was observed that rooting was significantly affected by the concentration of MS macronutrients. The basal concentration of MS macronutrients failed to induce root differentiation with short shoots. The rooting rate was 30–50%, when the concentration of macronutrients was reduced to a half or a quarter. On the other hand, long shoots produced more roots at the every concentration of macronutrients given: the very high rooting rate of 100% was observed with one quarter strength, as shown in Table 4.

The rooting rate was greatly influenced by incubation temperature. Although rooting took place at temperatures higher than 15.0°C, the ideal temperature for rooting was 27.5°C in the dark condition (Table 5).

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