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Tissue Culture of Aloe arborescens Miller var. natalensis Berger

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Running title: Examination of Culture Condition on Tissues of **Aloe Arborescens**

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ABSTRACT

We examined the culture conditions for callus induction in the tissue of **Aloe arborescens.** When incubated in the dark at 25℃, for about 30 days callus was induced. The frequency of callus formation was in the order of superior stalk > middle stalk > inferior stalk. No callus was formed in the leaf. When the Murashige-Skoog (MS) basal agar medium was supplemented with 3% sucrose, 0.1-0.5μM kinetin(a plant growth regulator) and 10-50μM alphanaphthaleneacetic acid, callus formation occurred at an incidence of 20-52% (mean: 39%). When the MS basal agar medium was supplemented with 3% glucose and 10% Alpha Modification of Eagle's Medium instead of 3% sucrose, callus formation was promoted, but the protein and aloin concentrations, and carboxypeptidase activity in the aloe callus decreased compared to those observed in the sucrose-containing medium.

INTRODUCTION

Aloe is a very important plant in the field of phytotherapy. The genus Aloe constitutes about 600 species and **Aloe ferex** and **Aloe barbadensis** are the species which are used not only for the phytotherapy but also for the investigation. We have been studying about **Aloe arborescens** for the past 20 years. The reason we have chosen this species is that we can cultivate it outside, in our country. Concerning the medically effective compounds, however, **Aloe arborescens** does not necessarily contain much more than other species.

To overcome this point we wanted to apply the biotechnology including tissue culture techniques and gene engineering. As has been proved in many plants, when small pieces of plant tissues keeping the capacity of proliferation are cultured in an adequate condition, they can grow to form amorphous cell clusters called callus. Moreover, when callus cells are incubated in presence of the specific growth regulators, regeneration of whole plants can be induced. It is also possible to introduce exogenous genetic material into callus cells by transfection of genes or fusion of cells (protoplasts) derived from different species.

Therefore, we wanted to make callus from **Aloe arborescens** and using this as the starting material we planned two projects; first, to find out the culture condition in which callus of **Aloe arborescens** can synthesize active components of Aloe such as carboxypeptidase or aloin more effectively; second, to get a more useful hybrid plant by the technique of cell fusion and regeneration of plants.

Regarding tissue culture of plants belonging to the Aloe genus of **Liliaceae**. Yagi **et al.** reported callus formation from the root of **Aloe saponaria** in 1983, Konishi **et al.** reported tissue culture of the stamen of **Aloe bellatura** in 1985, and Sanchez **et al.** reported the tissue culture of the leaf of **Aloe barbadensis** in 1988. There have not been so many descriptions about Aloe tissue culture. Especially, there have been no reports of tissue culture of **Aloe arborescens** (Kidachi aloe).

In this report we will describe culture conditions to get a good yield of callus from **Aloe arborescens.**

MATERIALS AND METHODS

Sterilization of stalks and leaves of Kidachi aloe

Axillary buds of 4- to 5-year-old Kidachi aloe, cultivated outdoors, was used. The bud used had about 6-7 leaves with a weight of about 5g/leaf. From this bud, a stalk and a leaf were separated, and used for experiments (Fig. 1). Then, each organ was sterilized by washing with distilled

water, immersing in 0.5% Tween 80 for 20 min, washing with distilled water, immersing in 2% sodium hypochloride for 50 min, washing with sterile water and then immersing in 70% ethyl alcohol for 30 seconds.

Fig 1. Axillary bud, stalk and leaf of Aloe arborescens

Thereafter, each organ was washed well with sterile water on a clean bench and cut into about 5 x 5 x 3mm^3 pieces (with a weight of about 18 mg for the stalk piece and about 20 mg for the leaf piece). Then, these clean materials were seeded in culture bottles containing Murashige-Skoog (MS) basal agar medium. For the sterilizing and cutting of the tissue of Kidachi aloe we referred to the published methods (Tsunewaki, 1984; Mantell **et al.**, 1985; Yamada, 1986; Takeuchi, 1987; Yamada, 1988; Dixon, 1991).

Preparation of media

The MS basal medium (Flow Laboratories, Table 1) was supplemented with 0.8% agar, 0-50 μM alpha-naphthaleneacetic acid (NAA; a plant growth regulator) and 0-5 μM kinetin. As a source of carbon, 3% sucrose or glucose was added to the medium. In addition, an Alpha Modification of Eagle's Medium (AMEM; Flow Laboratories, Table 1), which has been developed for animal cell culture and contains amino acids and vitamins, was added at 10%.

Table 1. Compositions of Murashige and Skoog Medium and Alpha Modification of Eagle's Medium

Components	MS[a] (mg/L)	Components	Alpha[b] (mg/L)	Components	Alpha (mg/L)
Major inorganic nutrients:		**Inorganic salts:**		**Amino acid:**	
$Cacl_2.2H_2O$	440.0	$CaCl^2$ (anhyd.)	200.0	L-Alanine	25.00
KH_2PO_4	170.0	KCl	400.0	L-Arginine.HCl	126.4
KNO_3	1,900	$MgSO_4$	97.70	L-Ascorbic acid	50.00
$MgSO_4.7H_2O$	370.0	NaCl	6,800	L-Asparagine.H_2O	50.00
NH_4NO_3	1,650	$NaH_2PO_4.H_2O$	140.0	L-Aspartic acid	30.00
				L-Cysteine.HCl.H_2O	100.0
Trace elements:		**Vitamins:**		L-Cystine.Na_2	24.00
$CoCl_2.6H_2O$	0.025	D-Ca pantothenate	1.00	L-Glutamine	292.0
$CuSO_4.5H_2O$	0.025	Choline chloride	1.00	L-Glutamic acid	75.00
KI	0.83	Biotin	0.10	Glycine	50.00
H_3BO_3	6.20	Folic acid	1.00	L-Histidine.HCl.H_2O	41.90
$MnSO_4.4H_2O$	22.30	i-Inositol	2.00	L-Isoleucine	52.50
$Na_2MoO_4.2H_2O$	0.25	Nicotinamide	1.00	L-Leucine	52.50
$ZnSO_4.7H_2O$	8.60	Pyridoxal.HCl	1.00	L-Lysine.HCl	73.10
		Riboflavin	0.10	L-Methionine	14.90
Iron source:		Thiamine. HCl	1.00	L-Phenylalanine	33.00
FeNaEDTA.$2H_2O$	36.70	Vitamin B_{12}	1.40	L-Proline	40.00
		Lipoic acid	0.20	L-Serine	25.00
		Na Pyruvate	110.0	L-Threonine	47.60
Organic supplement:				L-Tryptophan	10.20
Inositol	100.0	**Other components:**		L-Tyrosine	36.20
Nicotinic acid	0.50	Dextrose	1,000	L-Valine	46.90
Pyridoxine.HCl	0.50	Phenol red	10.00		
Thiamine.HCl	0.10				
Glycine	2.00				
Carbon source:					
Sucrose or Glucose	3,000				

[a]: Murashige and Skoog Medium, [b]: Alpha Modification of Eagle's Medium.

The pH of the medium was adjusted to 5.7. In the experiments to get preferable culture conditions, for media were prepared: The A medium is MS medium +3% sucrose. This is standard medium. The B medium is MS medium +3% sucrose +10% AMEM. The C medium is MS medium +3% glucose, and the D medium is MS medium +3% glucose +10% AMEN. Each medium was autoclaved for 15 min at 121℃ before use.

Culture environment

Culture was carried out in the dark at 25℃ or under 7,500 lux illumination at 25℃.

Measurement of growth

After the incubation for 30 days, we determined the weight of callus, the rate of callus formation. concentration of protein and aloin, and carboxypeptidase activity.

Callus homogenate

For biochemical analysis, 2.5g of callus was homogenized in 20 ml of distilled water and then centrifuged at 17,000 G for 15 min. The supernatant was used for biochemical assays.

Assay for protein

Protein content was measured with a commercially available BCA protein assay reagent (Pierce). This reagent (2.0 ml) was added to 100 µl of callus homogenate solution. The mixture was then incubated at 37℃ for 30 min, followed by cooling and colorimetric analysis at 562 nm.

HPLC analysis of aloe aloin

Aloin is an active component of Aloe and has a laxative effect. It was assayed by high performance liquid chromatography (HPLC) (Fig 2.), using a Radial PAK-C_{18} column (Waters) and employing water and methyl alcohol as mobile phases.

The sample was injected into the column in the presence of H_2O flow. Then, water was replaced with methyl alcohol. The flow rate was set at 0.2 ml/min. A Waters 484 tunable absorbance detector was used at 256 nm (Kawai et al, 1988).

To extract aloin from each callus, cold acetone was added to the homogenate solution. The mixture was stirred well and centrifuged at 17,000 G and 4℃ for 5 min. The residue on evaporation of the supernatant was dissolved in methyl alcohol to serve as a sample for HPLC.

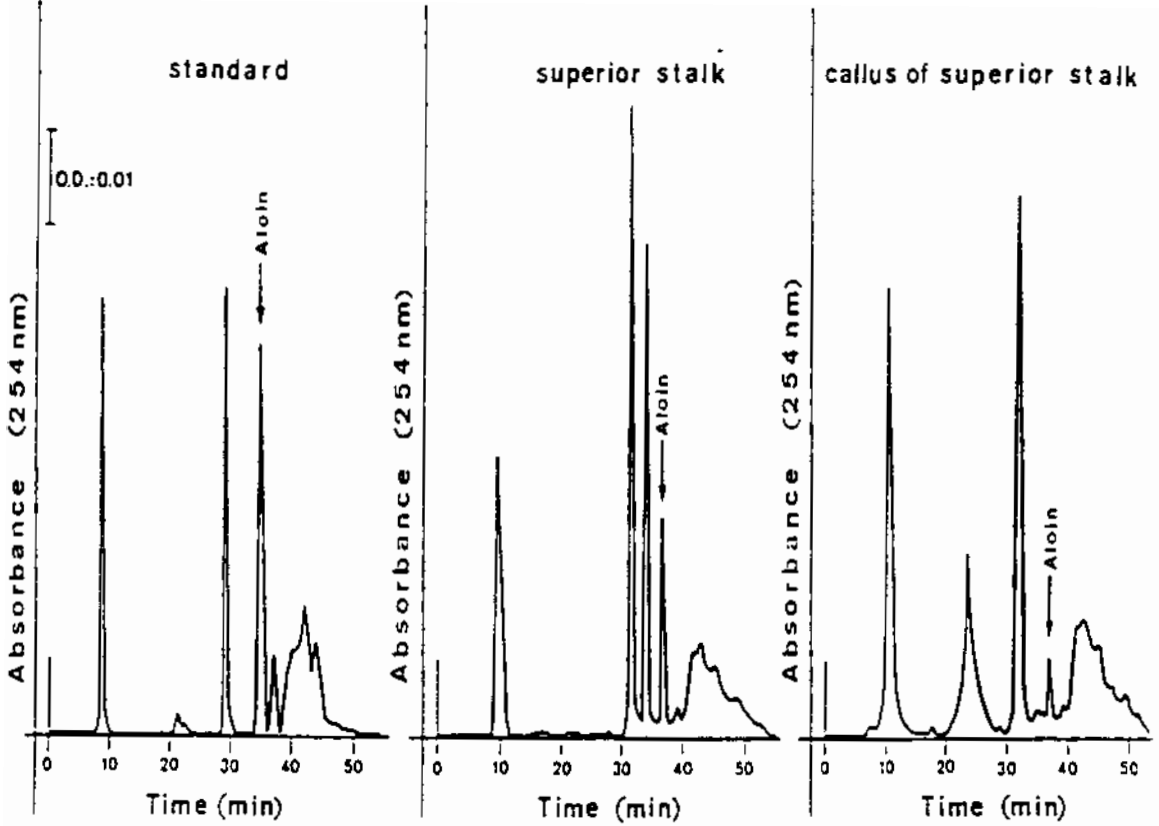

Fig 2. HPLC analysis of aloe aloin

COLUMN:	**Radial PAK-C^{18} (8mm ϕ x 10cm) and Radial Compression Separation System RCM-100**
MOBILE PHASE:	**H_2O and Methyl alcohol**
FLOW RATE:	**0.2ml/min**
DETECTOR:	**Waters 484 Tunable Absorbance Detector, UV 254nm**
CHART SPEED:	**2.5mm/min**

Assay for carboxypeptidase activity

Carboxypeptidase seems to be an anti-inflammatory factor (Fujita et al., 1975, 1979), was determined using carbobenzoxy - L - phenylalanyl - L - tyrosine (PEPTIDE INSTITUTE, INC.) as a substrate. This substrate and 50 mM sodium acetate buffer (pH 5.0) were added to the liquid homogenate of each callus. The mixture was stirred well and then incubated at 37℃ for one hour. Then, the mixture was boiled for 5 min to stop the reaction. After the mixture was cold, Ninhydrin reagent was added to induce reaction with free tyrosine. Calorimetry was conducted at 570 nm.

Comparison of the Culture environment for the callus formation

Fig. 3 shows components of culture medium, tissue culture conditions, and incidences of callus formation. Callus formation was observed when tissue pieces were incubated in the dark at 25℃. Under this condition, the incidence of callus formation was 40% for the superior stalk, 16% for the middle stalk and 12% for the inferior stalk.

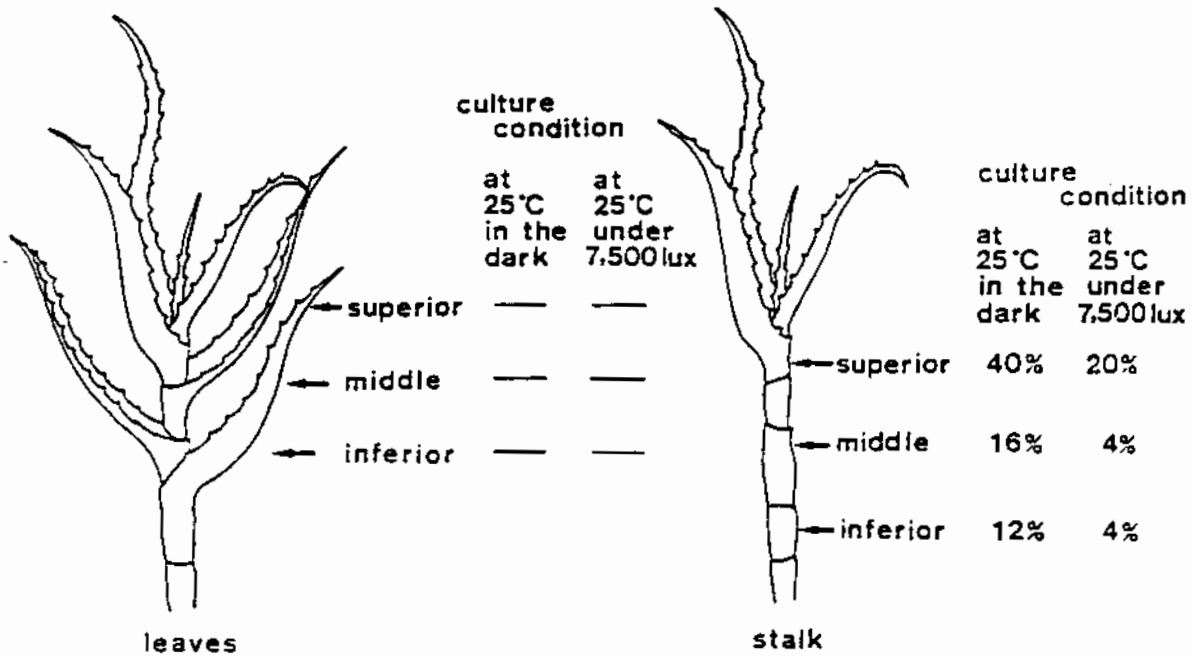

Fig 3. Callus formation in the dark or under illumination
Medium: Murashige and Skoog Medium, pH 5.7 containing 3% sucrose, 0.8% agar power, 0-5μm kinetin, 0-50μm α-naphthaleneacetic acid

When incubated at 25℃ under 7,500 lux illumination, the incidence of callus formation was 20% for the superior stalk, 4% for the middle stalk and 4% for the inferior stalk. A higher callus incidence was observed in superior stalk and more callus was obtained in the dark rather than under illumination.

Histology of stalk tissues

We examined tissue of stalks histologically. The tissue of stalk was made into paraffin sections and stained with Hematoxylin & Eosin for observation of nuclear morphology and cell

distribution. Fig. 4 (a) shows tissue of superior stalk, and Fig. 4 (b) shows tissue of middle stalk, and Fig. 4 (c) shows tissue of inferior stalk. The tissue of superior stalk was rich in nuclei.

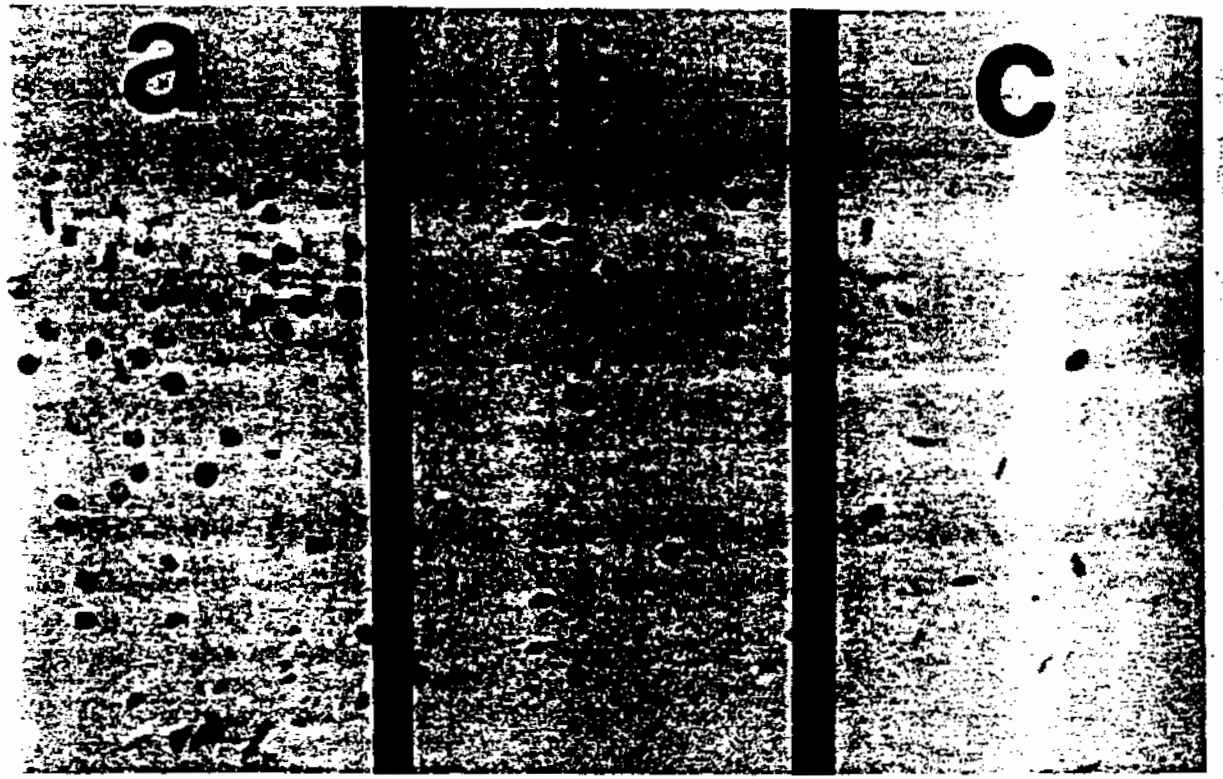

Fig 4. Hematoxylin - and eosin - stained specimen of the stalk, which was superior stalk (a) and middle stalk (b) and inferior stalk (c). x66

Fig. 5 shows the division of nucleus. It was frequently observed in superior stalks, but not in other tissues. These observations suggested that tissues with active cell division would give high incidences of callus. Therefore, we used superior stalks as the main materials.

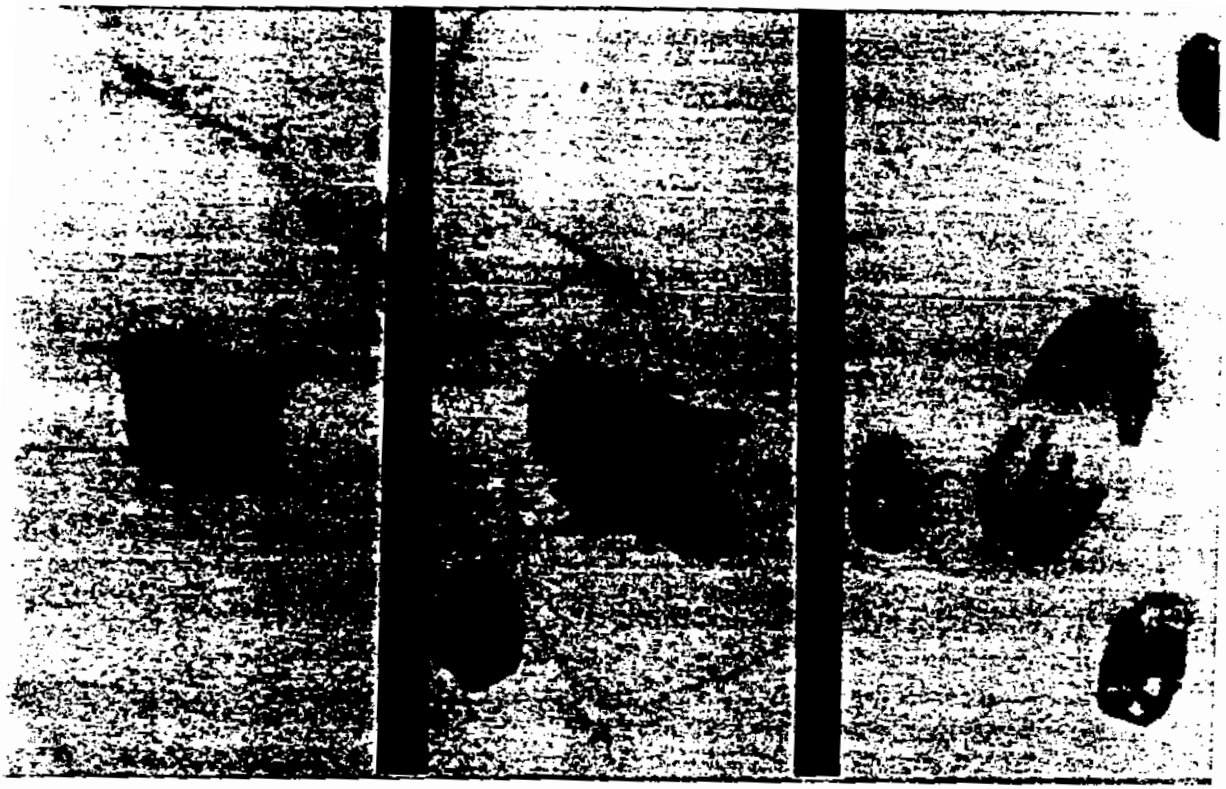

Fig. 5 Hematoxylin-and eosin-stained specimen for the division of nucleus in superior stalk. x330

Influence of the plant growth regulators on callus formation in superior stalk of Aloe arborescens

In order to increase the percentage of callus formation., we tested 2 kinds of growth regulators and their concentration. Table 2. shows the summary of series of experiments. The basal culture condition was described under the table. When no growth regulators were added, no callus was formed. When NAA was added alone, about 20% callus formation was observed. However, when kinetin was added alone, only a few callus formation was observed. When both of NAA and kinetin were combined, a remarkable increase in callus formation was obtained. We used both growth regulators in the standard condition. When the MS basal agar medium was supplemented with 0.1-5 μM kinetin and 10-50 μM alpha-naphthaleneacetic acid, the incidence of callus formation ranged from 20 to 52% (mean: 39%) when the superior stalk was used as a starting material and cultured in the dark. Fig. 6 shows the starting material and the callus formed at 30 days.

Table 2. **Influence of the plant growth regulators on callus formation in superior stalk of Aloe arborescens**

Concentration of kinetin	Concentration of α -naphthaleneacetic acid (NAA)				
	50μM	**10μM**	**5 μM**	**1μM**	**0μM**
5 μM	48%[a] (10:1)[b]	24% (2:1)	4% (1:1)	4% (0.2:1)	0% (0:5)
1 μM	52% (50:1)	40% (10:1)	28% (5:1)	12% (1:1)	4% (0:1)
0.5 μM	44% (100:1)	44% (20:1)	32% (2:1)	8% (2:1)	8% (0:0.5)
0.1 μM	40% (500:1)	20% (100:1)	32% (50:1)	12% (10:1)	0% (0:0.1)
0 μM	20% (50:0)	24% (10:0)	4% (5:0)	8% (1:0)	0% (0:0)

[a]: % of Callus formation. [b]: NAA and kinetin ratio.
Medium: Murashige and Skoog Medium + 3% sucrose + 0.8% agar powder
Incubation was carried out for one month at 25℃ in the dark.

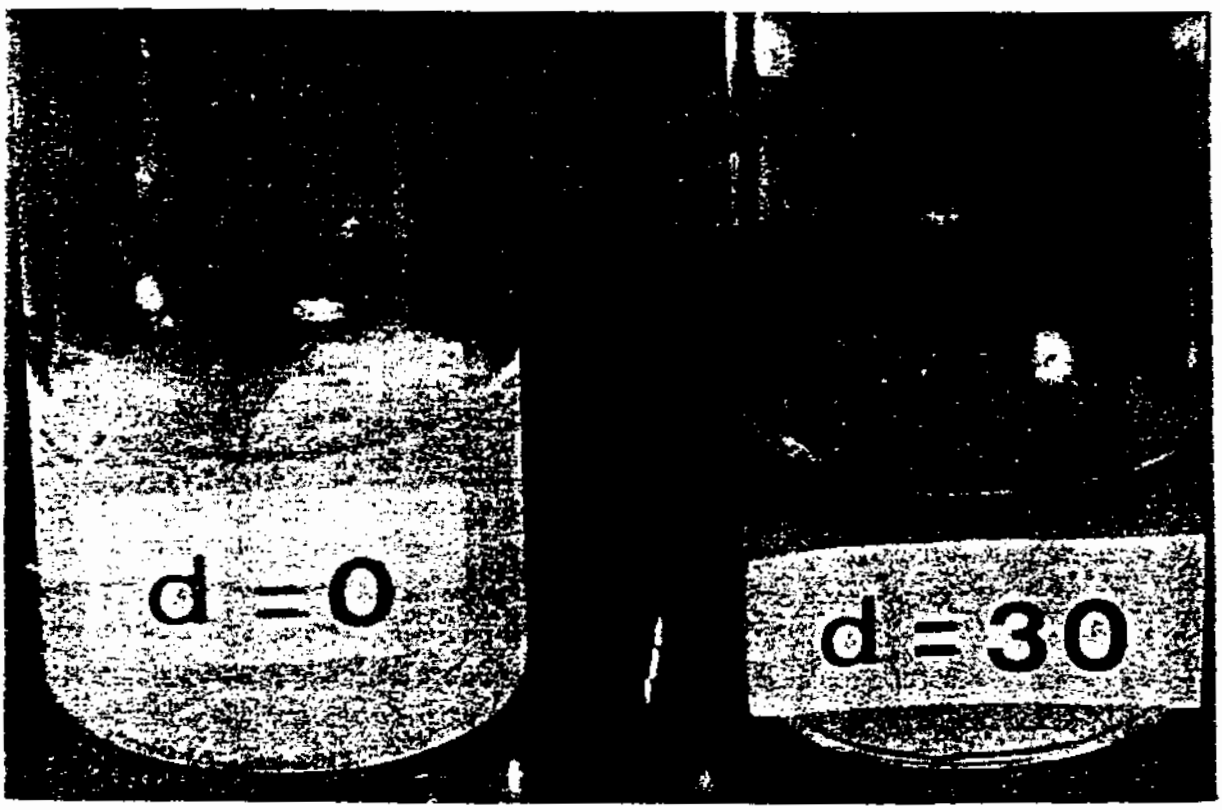

Fig 6. The starting material and after 30 days of the superior stalk when incubated at 25℃ in the dark. (a) starting material (b) at 30 days.

Effects of various medium conditions on the weight of callus and the frequencies of callus formation

We tested the influence of medium on callus formation and the results are shown in Table 3. The weight of callus formed from about 18 mg superior stalk tissue in 1 month was 382 mg in the A medium, 517 mg in the C medium, 224 mg in the B medium, and 288 mg in the D medium. Thus, the largest callus was formed in the C medium.

Table 3. Effects of various medium conditions on weight, formation rate, protein and aloin concentrations, and carboxypeptidase activity of aloe callus

	Weight (mg)	Callus formation (%)	Protein (mg/g)[a]	Aloin (μg/g)[a]	Carboxypeptidase (Units/g)[a]
Starting material (tissue of superior stalk)	18	—	7.5	794.0	19.11
MS medium[b] +3% sucrose (A medium)	382	39	2.7	6.3	7.80
MS medium +3% sucrose +10% α medium[c] (B medium)	224	56	0.8	1.9	2.22
MS medium +3% glucose (C medium)	517	83	2.4	1.3	1.74
MS medium +3% glucose +10% α medium (D medium)	288	58	0.7	4.3	1.23

a: wet weight of callus.

b: Murashige and Skoog Medium, containing 0.1-5㎛ kinetin and 10-50㎛ α -naphthaleneacetic acid.

c: Alpha Modification of Eagle's Medium, which has been developed for animal cell culture and contains amino acids and vitamins.

The frequency of callus formation in various media was examined. The callus formation was 39% in the A medium, 83% in the C medium, 56% in the B medium, and 58% in the D medium.

Effects of various medium conditions on the protein and aloin concentration and carboxypeptidase of aloe callus

Protein concentration of aloe callus (mg/g wet weight) was 2.7 in the A medium, 2.4 in the C medium, 0.8 in the B medium and 0.7 in the D medium. Thus, protein level was highest in the callus formed in the A medium.

Aloin concentration of aloe callus (μg/g wet weight) was 6.3 in the A medium, 1.3 in the C medium, 1.9 in the B medium and 4.3 in the D medium. Like protein, aloin concentrations were highest in aloe callus formed in the A medium.

Carboxypeptidase activity of aloe callus (units/g wet weight) was 7.8 in the A medium, 1.7 in the C medium, 2.2 in the B medium and 1.2 in the D medium. Thus, carboxypeptidase activity was highest in the aloe callus formed in the A medium.

The incidence of callus formation was evidently promoted by the addition of glucose in place of sucrose. However, the content of both aloin and carboxypeptidase decreased in this medium. When we compared these values with those of starting materials, carboxypeptidase was synthesized not so much as expected. The larger the growth rate became, the less the carboxypeptidase activity. It would be possible that the enzyme activity would increase if the growth of callus was rather inhibited. Therefore, the next purpose is to find out the callus culture condition that would increase the contents of effective components of Aloe.

DISCUSSION

A standard culture conditions to induce callus formation from Kidachi aloe tissue was established. A high incidence of callus formation was observed in the dark than under illumination. The site of aloe tissue allowing the best induction of callus formation was the superior stalk, and callus formation was not induced from the leaves.

Sanchez **et al.** (1983) reported tissue culture of the leaves of **Aloe barbadensis** at 27℃ and 3, 400 lux for 30-45 days. In that study, the Murashige-Skoog medium was used, like in the present study. The medium was supplemented with 0.25mg/l of 2,4-dichlorophenoxyacetic acid and 1. 0mg/l of kinetin (plant growth regulators) as well as with p-aminobenzoic acid (0.1mg/l) and tyrosine (100mg/l). pH was adjusted to 5.7. Yagi **et al.** (1983) attempted callus induction by incubating the root of **Aloe saponaria** in the MS medium supplemented with 2,4-dichlorophenoxyacetic acid (1 ppm) and kinetin (2 ppm) for 3 months at 25℃ and 2,500 lux. Our results differs from these reports in that we incubated the superior stalk of Kidachi aloe in the dark at 25℃. In addition the kind of Aloe used in the present study is different from that in the previous studies.

Another important factor for induction of callus is the components of the medium. Auxins, growth regulators, are most important for inducting callus and for maintaining its proliferation. Auxins include 3-indoleacetic acid (IAA), alpha-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Cytokinins, which are also plant growth regulators, play important roles in promoting cell division. They include kinetin, 6-benzyladenine and zeatin

riboside. When the levels of auxins were elevated, differentiation of roots occurred, while an elevation in the cytokinin level resulted in differentiation of stalks and roots (Mohr, 1978).

Also in the present study, callus induction was observed in the 3% sucrose-added MS agar medium treated with kinetin (0.1-5 μM) and alpha-naphthaleneacetic acid (10-50 μM). These results indicate That the presence of NAA, IAA and kinetin are required to induce cell division during incubation of Kidachi aloe tissue. In future experiments, these two classes of plant growth regulators have to be combined at various concentrations.

We additionally examined carbon sources in the medium. Conventionally, the MS medium was supplemented with 3% sucrose. When sucrose was replaced with glucose in the present study, callus formation was promoted. However, protein, aloin and carboxypeptidase levels in callus were lower in the glucose-added medium than in the sucrose-added medium. These results indicate that the addition of glucose to the medium accelerates the formation of callus as compared to the sucrose-added medium, but that the formation of callus contents does not simultaneously occur in the glucose-added medium.

The influence of amino acids and vitamins, which are not present in the MS medium, on the callus formation was examined using an Alpha Modification of Eagle's Medium (AMEM). Callus formation and the levels of callus components were higher in the AMEM - free medium. This result suggests that the early stage of callus formation is not affected by amino acids or vitamins.

The callus obtained in the present study is now being incubated in the agar media which are known to promote the induction of leaves and roots.

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REFERENCES

Dixon, R., A. (1991). Plant cell culture. IRL Press Limited.

Fujita, K., Teradaira, R., Nagatsu, T. (1975). Bradikininase activity of aloe extract. Biochemical Pharmacology, 25.205.

Fujita, K., Ito, S., Teradaira, R. and Beppu, H. (1979). Properties of a Carboxypeptidase fr Aloe. Biochemical Pharmacology. 28.1261-1262.

Kawai, K., Beppu, H., Takahashi, H., Ishiguro, I. and Fujita, K. (1988). Determination of Al Alion by High-performance Liquid Chromatography. Bulletin of the Fujita-Gakuen Medi Society. 12. 159.

Konishi, T. and Amano, M. (1985). Callus Formation in Stamens of **Aloe bellatura**. Annals the Tsukuba Botanical Garden. 3.19-26.

Mantell, S., H., Matthews, J. A. and Mckee, R.A. (1985). Principles of Plant Biotechnolo Blackwell Scientific Publications.

Mohr, H. and Schopfer, P. (1978). Lehrbuch der Pflanzenphjsiologie. p. 387. Springer Verlag.

Sanchez, I.C., Natali, L. and Cavallini, A. (1988). In vitro Culture of **Aloe barbadensis** Mi Morphogenetic Ability and Nuclear DNA content. Plant Science. 55.53-59.

Takeuchi, M. (1987). Plant Tissue Culture. Shokabo.

Tsunewaki, K. (1984). Experimentation of plant genetics. Kyoritsushuppan.

Yagi, A., Shoyama, S. and Nichioka, I. (1983). Formation of Tetrahydroanthrachen Glucosic by Callus. Phytochemistry. 22. 1483-1484.

Yamada, Y. (1986). Manual of plant cell culture. Kodansha.

Yamada, Y. and Okada, Y. (1988). Principles of Plant Biotechnology. Tokyo kagaku Dojin.