A STUDY ON APPLICATION OF PLANT HAIRY ROOTS TO USEFUL METABOLITES AND ARTIFICIAL SEED PRODUCTION

植物毛状根を用いた有用代謝産物及び人工種子生産に関する研究

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. 1. Useful metabolite production using plant biotechnology

Plants synthesize a broad repertoire of complex organic molecules, and are an important source of fine chemicals that have found expensive use in the pharmaceutical industry. Among the most widely prescribed plant products are steroids, analgesics, agents active on the cardiovascular system and anticholinergic compounds: specially, diosgenin-derived steroids, tropane alkaloids such as atropine and scopolamine, digitoxin, reserpine and quinidine. In addition, the food industry utilizes several plant-derived flavors, colorings and proteins. A central characteristic of these is that they are typically low-molecular-weight secondary metabolites whose synthesis is tightly regulated by the plant.

At present, a small number of plant compounds are manufactured using suspension culture, in place of whole-plant systems, and there is considerable interest in expending this to many other products. A bioreactor-based process, however, is not necessarily competitive when compared with whole-plant harvesting and extraction: one of the primary limitations of *in vitro* culture is low yield and, consequently, low productivity. The relative few successful examples of the production of fine chemicals from plant-cell culture have been in instances where selection or media manipulation to generate high yielding cell lines and efficient bioprocessing techniques are combined. The manufacture of shikonine (1), ginseng compounds (2) and berberine (3) are often cited in this regard.

Understanding the dynamics of metabolite production in suspension cultures is a prerequisite to controlling bioreactor productivity. As in whole plants, information on the mechanisms of transport of metabolic intermediates and products is important to this understanding. The ability to release metabolites from intracellular storage compartments should decrease feedback inhibition that might otherwise shut down metabolic pathways. This relief from inhibition, coupled with secretion of released metabolites, would result in greater throughput to the product, and thus higher productivity.

1. 2. Micropropagation techniques of elite plant using plant biotechnology

Another techniques of plant biotechnology is micropropagation, by which

many clonal plant from only one plant can be propagated. This is based on the technique of *in vitro* culture of plants. *In vitro* culture of higher plants has shown a spectacular development since 1975. Methods have been developed for the culture of plants, seeds, embryos, shoot tips, meristems, tissues, cells and plotoplasts on sterile nutrient media, resulting in the production and regeneration of viable individuals of many plant species. Many plant species are propagated through micropropagation now. The culture of shoot tip excised from mature plant which is not usually grown under aseptic condition was used as the seed materials . Contamination by virus inhibit normal growth of plant. Because virus don't usually contaminate shoot tip, shoot tip culture leads to a increase in biomass yield. However, current micropropagation system are labor intensive and more expensive than propagation through seeds.

1. 3. Artificial seed system

Murashige proposed the artificial seed to supply micropropagated plantlets in 1978. The conceptual scheme of artificial seed system is shown in Fig. 1–1. Somatic embryo and adventitious shoot obtained though callus has been investigated for use in the production of artificial seeds (4-6). Artificial seed contain of the following processes; screening of elite plant from transformants obtained through conventional breeding and/or genetic manipulation, establishments of cell line of somatic embryogenesis callus, efficient production of somatic embryos, maturation of embryos, coating technique of embryos, preservation of artificial seed for transport to further area, and conversion to mature plant on actual soils. Artificial seed is integrated system of many kinds of biotechnology. One of the most important techniques is mass production of "seed plant" to be introduced into capsule. To propagate clonal elite plant by lower cost, micropropagation through somatic embryos which differentiate from vegetable cells is expected. Use of bioreactor is allowed for the production of somatic embryos. Each somatic embryos can develop independently and grow over mature plant. Complete automation of propagation system can be done. Therefore, many researchers study efficient production of somatic embryos.

1. 4. Production of useful metabolites and artificial seed using plant hairy roots

Recently, so-called plant "hairy roots" have become of interest because of their

indefinite and fairly active proliferation in phytohormone-free media and their capacity capacity to synthesize the products such as enzymes (7-8) and secondary metabolites (9) at levels similar to that of the original plants. It is recognized that root-inducing (Ri) plasmid in the soil pathogenic bacterium *Agrobacterium rhizogenes* causes the transformation of dicotyledonous plant cells by producing the T-DNA region of the plasmid into genomic DNA of the plant cells (Fig. 1-2), and that the transformed plant cells give rise to hairy roots with the phenotype even after bacterium elimination and long-term subcultures.

On the other hand, plant regeneration from hairy roots has been reported in several plant species (10-12). Plants regenerated from hairy roots have altered phenotypes such as wrinkled leaves, plagiotropic roots, reduced apical dominance and short internodes, some of which have a potential of agronomic application. In addition, the use of *A. rhizogenes* offers the opportunity of introducing foreign genes into plant genomes at the same time (13). The development of a micropropagation process using elite hairy roots would have an impact on agriculture and biochemical production of useful materials compared with that with somatic embryos and plant callus (Table 1-1).

Therefore, efficient methods for production of useful metabolites and artificial seed using plant hairy roots were investigated in this study. At first, the effect of ion supplementation to medium on Peroxidase excretion, its application on fermenter scale culture and long term culture was tested in chapter 2. Next, effects of auxin supplementation on growth behavior of horseradish hairy root were evaluated, and then the kinetic model for the growth was studied to improve the property of hairy root growth in chapter 3. In chapter 4, the regeneration frequency from horseradish hairy roots is tested by using excision and encapsulation combined with supplementation of growth regulators. In chapter 5 and 6, we tested mechanical fragmentation of hairy roots suitable for efficient production of plantlets, plant growth regulator supplementation and dehydration to increase the plantlet development frequency from encapsulated plantlets.

TABLE 1-1. Comparison of properties of hairy roots and somatic embryo

	Hairy roots	Somatic embryo
Genetic stability	◎	△
Genetic improvement	◎	○
Mass production	○	○
Induction and regeneration ability	△	△
Plantlet development frequency	△	△
Tolerance for Physical stress	○	△

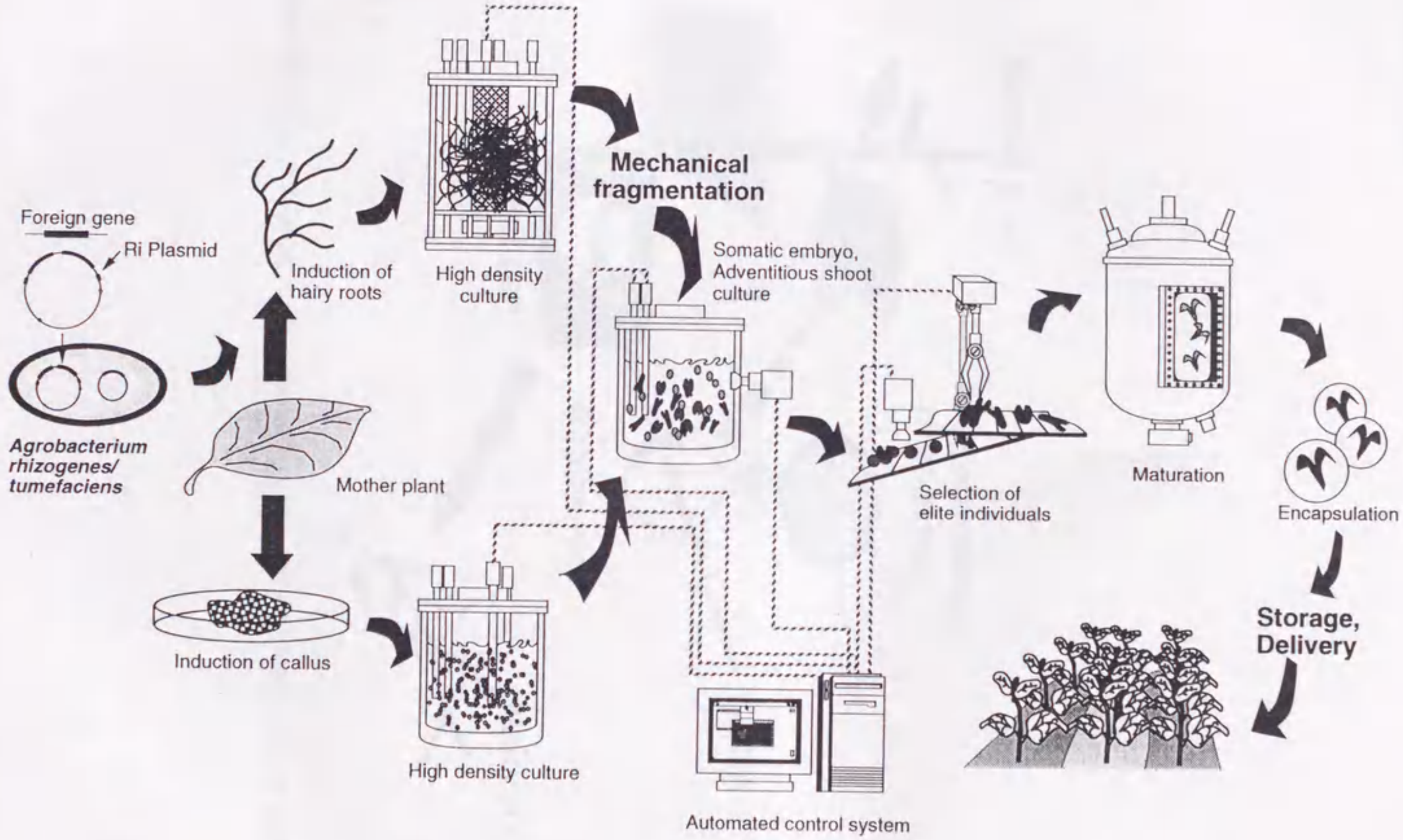

FIG. 1-1. The conceptual scheme of artificial seed system using somatic embryos or hairy roots

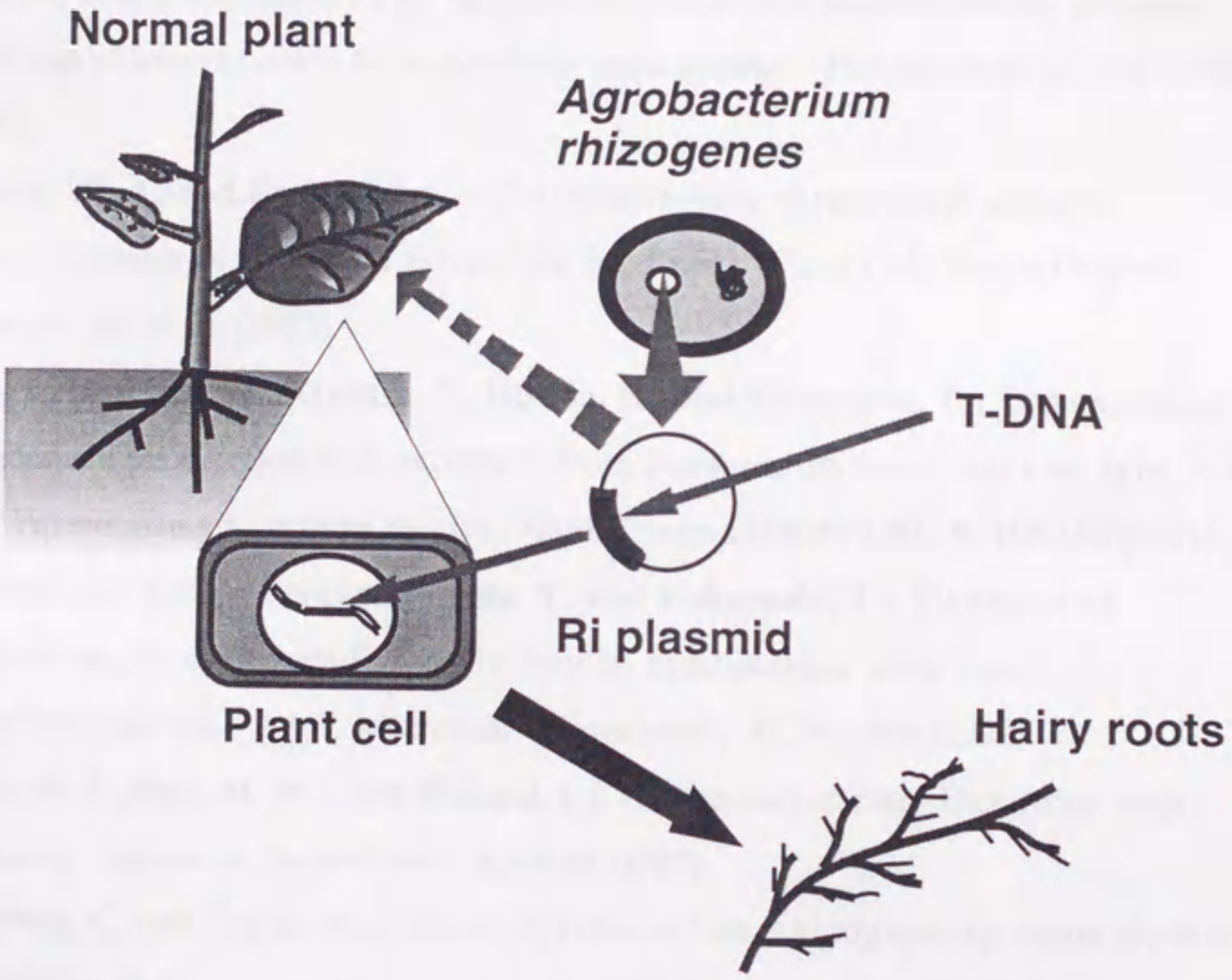

FIG. 1-2. Introduction of T-DNA in Ri plasmid to plant cell and induction of hairy roots

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CHAPTER 2

Excretion of Peroxidase from Horseradish Hairy Root in Combination with Ion Supplementation

2. 1. INTRODUCTION

Plant represents a vast source of untapped chemicals. A wide variety of proteins and secondary products have been isolated from plant tissue culture. The desire to acquire these substances has generated great interest in biotechnology fields because their complicated structures are hard to be synthesized in chemical industry. Many reports have published about suspension culture using dedifferentiated cell callus. Since callus is more susceptible to mutation and often contains lower amount of commercially important products than organized tissue, hairy root culture which is transgenic plant induced by *Agrobacterium rhizogenes*-mediated gene transfer has been studied (1).

Various properties of plant cells and technological limitation are responsible for the limited industrial success of plant cell cultures as a source of biochemicals. A major problem in producing these natural substances which are produced intracellularly has been the difficulty of obtaining high yield of the target substance. Only shikonine production is known to be a successful example in industrial production (2) .

To overcome the difficulty, several approaches have been examined from industrial point of view. One approach is to develop a high density culture of plant cells. Suitable design of bioreactor and optimum control of oxygen concentration and nutrient supplementation are needed (3 -7). Another approach is to excrete the product of interest from the cell. Plant cells often store desirable products intracellularly, which makes a continuous operation of a process impossible. Several excretion methods (8, 9) have significant potential to improve the feasibility of processes for producing favorable products from plant cell tissue culture. These involve pH cycling (10), use of permeabilizing agents (9) and ultrasound (11). Such methods successfully enhanced product release although the permeabilization mechanism was unknown, and they are not generally used for any products in diverse plant cells.

Horseradish Peroxidase (EC 1.11.1.7) is widely used in the colorimetric analysis of biological materials, and consists of isozymes which are localized at various compartments in cell. For Peroxidase excretion, we have studied the NaCl addition to medium in combination with light and adsorption *in situ* (12). In this chapter, we studied the effect of ion supplementation to medium on Peroxidase excretion, its application on fermenter scale culture and long term culture.

2. 2. MATERIALS AND METHODS

Plant cell culture and culture medium Horseradish (*Armoracia rusticana*) hairy root induced by the leaf-disc method (5, 13) was used, and maintained by subculture in the dark every 3 weeks on hormone-free Murashige and Skoog (MS) (14) medium supplemented with 2 % (w/v) sucrose. For the culture experiments, the roots were grown for 14 d at 25°C and then transferred aseptically into 100-ml Erlenmeyer flasks containing 40 ml aliquot of the required medium with inoculum size of 2 g of fresh root (approximately 0.2 g of dry cell) per liter of medium. Fresh hairy roots (2 g) were also inoculated in 1 *l* liquid MS medium containing 2 % sucrose in a turbine-blade reactor (TBR-2, Sakura Seiki Co., Tokyo). The volume of the blade rotation space where the root could not grow was 150 ml. The aeration rate was increased with the proliferation of the root (6 Kondo *et al.* 1989) in order to maintain a dissolved oxygen concentration above the critical value. Temperature during the culture was kept at 25°C. All cultures were performed under light condition where illumination with fluorescent white light (ca. 3500 lux) was 14 h a day. On-line estimation of cell concentration during the culture was based on conductmetry as reported previously (4). At the end of the culture, dry cell mass was measured gravimetrically after drying the root at 60°C for 24 h, which was within 10 % of that estimated conductmetrically.

Peroxidase assay Peroxidase activity was determined at 25°C with *o*-aminophenol as a substrate (15). Extracellular Peroxidase was obtained from the supernatant of the root culture. For the extraction of intracellular Peroxidase, 0.2 g-fresh cell of hairy root was disrupted in 1 ml of phosphate buffer (10 mM, pH 6.0) using a mortar and a pestle on ice. The supernatant was obtained by centrifugation (17,000 x g, 10 min) at 4°C and used as a crude enzyme solution. Peroxidase activity (U/ml) was

calculated using the formula : U /ml = OD_{480} x 0. 48 x df ; OD_{480} = optical density at 480 nm ; 0.48 = molecular extinction coefficient at 480 nm, df = dilution factor of the enzyme solution.

Isoelectric focusing gel electrophoresis of Peroxidase isozyme Isoelectric focusing gel electrophoresis was carried out as described by Hoyle (16) with some modifications (Shinmyo, Osaka Univ., Japan, personal communication). A 10 % polyacrylamide gel was made with 6 ml of distilled and deionized water, 12 mg of riboflavin, 0.6 ml of 40 % Ampholine (pH 3.5-10) purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Sample solution, which consists of 66 µl of culture supernatant or the crude intracellular enzyme solution described above and 33 µl of 3 x stock solution (1 x stock solution = 2 % of Ampholine, 30 % glycerol in water) was applied on the gel using a syringe. Electrophoresis was carried out at 4 ℃ for 2 h at 100 V followed by for 4 h at 200 V using anolyte solution (0.1 M HCl) and catholyte solution (0.5 M NaOH). The pI of proteins were determined from pI markers purchased from Oriental Yeast Co., Tokyo. To detect Peroxidase isozyme, the gel was stained at room temperature in 50 ml of freshly prepared solution containing 10 mg of 3,3' diaminobenzidine tetra-HCl and 1 mM H_2O_2 for 30 min.

Chemicals Antifoam was purchased from Shin-etsukagaku Kogyo Co., Tokyo. All other reagents were of reagent grade.

2. 3. RESULTS AND DISCUSSION

Effect of osmotic pressure on Peroxidase excretion In the previous report, NaCl was found to stimulate Peroxidase excretion from horseradish hairy root (12). To test whether the osmotic effect of the ion supplementation in the medium had any influence in Peroxidase excretion, various concentrations of mannitol were supplemented to the medium as shown in Fig. 2-1. It is known that mannitol is not metabolized and does not support plant tissue growth. Osmotic pressures of mannitol at 99 mM and 198 mM corresponded to those of NaCl at 50 mM and 100 mM, respectively. Cell growth pattern of the culture supplemented with mannitol was similar to that with NaCl. At day 30, extracellular Peroxidase activity of the root treated with mannitol was lower than that of the non-treated root. This indicated that

the osmotic pressure has no influence on Peroxidase excretion from horseradish hairy root.

Effect of various ions on cell growth and Peroxidase excretion We have studied the dependency of the strength of various ions for Peroxidase excretion. Peroxidase production of horseradish hairy roots cultured in media with various cations for 40 d is presented in Table 2-1. Ferric ion inhibited the cell growth completely. Na^+, K^+, Mg^{2+} and Ca^{2+} ions acted as excreting agent, and inhibition of the growth by these ions was relatively small. Extracellular Peroxidase activity increased by the addition of Mg^{2+} and Ca^{2+} ions. The treatment of 50 mM divalent cations exhibited a marked increase in Peroxidase activity in the medium; approximately 3-fold increase compared with that of monovalent cations. The morphological change that occurred on the root surface treated with various ions was similar to the microscopic examination of root surface treated with NaCl which was previously reported (12). Figure 2-2 shows the root surface of hairy root growing in the presence of Ca^{2+} ion. The detachment of root epidermis except apical meristem was observed. It seems that Peroxidase excretion increased in proportion to the area of the detached surface.

Table 2-2 shows the effect of anion on Peroxidase production in the medium and cell mass. Supplementation of Cl^-, NO_3^-, SO_4^{2-} and CO_3^{2-} ions did not affect the cell mass except for Na_2-EDTA which inhibited the cell growth completely. Supplementation of CO_3^{2-} and Cl^- ions increased extracellular Peroxidase activity per cell compared with non-supplemented culture, but NO_3^- and SO_4^{2-} ion supplementations were less effective for Peroxidase excretion.

The intracellular Peroxidase activity per dry cell did not differ in various ion supplementations in contrast to that of extracellular one (Tables 2-1 and 2-2). There seems to be a homeostatic system which keeps the intracellular Peroxidase at a constant level by synthesizing Peroxidase in the cell. The ions supported the Peroxidase excretion but did not affect the cell viability extensively except for Fe^{3+} ion and EDTA.

To determine which ions should be added to the medium for Peroxidase excretion, extracellular Peroxidase activity per dry cell was plotted against ionic

strength. Extracellular Peroxidase activity per dry cell was proportional to ionic strength of all cations tested (Fig. 2-3), but was not dependent on the type of cation. Experiments with higher KCl concentration than 50 mM revealed that the extracellular Peroxidase activity per dry cell and the hairy root decreased (data not shown), which may also be observed for other ions. On the other hand, the extracellular Peroxidase activity per dry cell was dependent on the type of anion. These results suggested that 50 mM $CaCl_2$ and 50 mM $MgCl_2$ are suitable for the Peroxidase excretion than any other ion combinations. Therefore, 50 mM $CaCl_2$ was used in all subsequent works on the ion supplementation to the medium.

Intracellular and extracellular Peroxidase profile Figure 2-4 shows the active stain of intracellular and extracellular Peroxidases separated by isoelectric focusing gel electrophoresis. The Peroxidase bands of the root treated with various ions were similar to that of non-treated root. No distinct difference in the number of bands between intracellular Peroxidase and extracellular Peroxidase was observed. The stain showed isozymes of Peroxidase bands reported by Hoyle (16), whose density corresponded with Peroxidase activities in Tables 2-1 and 2-2. The main band of supernatant and intracellular fractions of the root treated with all ions was detected at pI 8.3. The densities of activity bands ranging from pI 9 to 11 of the supernatant from cells treated with ions increased compared with that of the non-treated root. Some peroxidases are located on the cell wall and are considered to be involved in cell elongation through the rigidification of cell wall (17). These peroxidases were consistently exposed to the external environment. Assuming that Peroxidase excretion occurred with retention of cell viability, peroxidases associated with cell wall were released to the medium. Excretion of the peroxidases with pI 9 -11 might contribute to the increase in extracellular Peroxidase activity. Shinmyo and co-workers have cloned the gene for Peroxidase from horseradish (18, 19). The localization of isozyme and the Peroxidase excretion as affected by ion will be assigned on the basis of these information.

Effect of Ca^{2+} ion supplementation timing on Peroxidase excretion It is important to determine the timing of $CaCl_2$ supplementation to the medium since it is considered that the viability of the treated cells was highly influenced by Ca^{2+} ion used.

Extracellular Peroxidase activity as a function of incubation time is shown in Fig. 2-5. The root supplemented with Ca^{2+} ion during various incubation times grew at approximately the same rate as the root supplemented at the beginning of the culture. On the other hand, the root treated with $CaCl_2$ at the beginning of the culture resulted in the highest Peroxidase activity. These result showed Ca^{2+} supplementation at the beginning of culture was the most effective for the production of Peroxidase in medium.

According to the previous study (6, 7), carrot hairy root cultured in a turbine-blade reactor was higher final cell mass than that cultured in a shake flask. There may be a potential for enhancement of cell mass in this reactor if the optimum culture conditions could be determined. Fermenter culture using the turbine-blade reactor was used to produce Peroxidase from horseradish hairy root on the basis of the above results. The result indicated that scaling up from 0.04 *l* to 1 *l* did not lead to any significant loss in Peroxidase production (data not shown). As the root grew, the level of foaming increased. The influence of the addition of antifoam to the medium was also checked. The addition of a few drops of antifoam every five days did not affect to any great extent the cell mass and Peroxidase activity.

Repeated batch culture of horseradish hairy root The previous data suggested that the root at the stationary phase retained the cell viability and had the potential for producing Peroxidase. To facilitate the Peroxidase production from the root, repeated-batch culture was carried out in a shake flask using the medium supplemented with 50 mM $CaCl_2$ (Fig. 2-6). Fresh medium with or without 50 m M $CaCl_2$ was substituted for the culture medium when the decrease in conductivity of the culture medium ceased. The final cell mass at 149 d reached 49.4 g-dry cell */l* in medium supplemented with 50 mM $CaCl_2$ and 66.6 g-dry cell */l* in the medium without $CaCl_2$, respectively. Green portions (shoot formation) on the root surface appeared after 4 cycles and these portions extended to the gas space. Peroxidase activity in medium reached a maximum (80 U/ml) at the 3 rd cycle and then was kept constant at 60 U/ml level. The total extracellular Peroxidase activity in the culture during 149 days reached 12,840 U, whose activity was more than 12-fold as high as that of the batch culture supplemented with $CaCl_2$ during 40 days. Peroxidase productivity in repeated

batch culture reached 0.36 U/ml/day, while the productivity of the non-treated root was 0.16 U/ml/day. This repeated batch culture allowed a continuous retention of cell viability and production of a large amount of the Peroxidase in the medium.

TABLE 2-1. Effect of various cations on cell mass and Peroxidase activity per dry cell after 40 d

Treatment		Dry weight [g/*l*]	Peroxidase activity in medium [U/ml]	Peroxidase activity per 1 g-d.w.	
				Extracellular [U/g-d. w.]	Intracellular [U/g-d. w.]
None		15.8	6.8	430	2180
NaCl	20 m M	14.5	9.9	683	1950
	50 m M	13.8	15.8	1150	2150
KCl	20 m M	15.0	13.2	880	1850
	50 m M	16.0	31.5	1970	2240
$MgCl_2$	20 m M	13.5	17.9	1330	1880
	50 m M	11.8	54.7	4640	2120
$CaCl_2$	20 m M	14.0	28.5	2040	2930
	50 m M	13.8	56.9	4120	2640
$FeCl_3$	20 m M	0.8	—	—	—
	50 m M	0.8	—	—	—

U, units

TABLE 2-2. Effect of various anions on cell mass and Peroxidase activity per dry cell after 40 d

Treatment		Dry weight [g/*l*]	Peroxidase activity in medium [U/ml]	Peroxidase activity per 1 g-d.w.	
				Extracellular [U/g-d.w.]	Intracellular [U/g-d.w.]
None		15.4	5.4	351	2540
NaCl	20 m M	14.5	9.8	678	2080
	50 m M	13.0	18.3	1410	2640
$NaNO_3$	20 m M	15.3	5.7	373	2520
	50 m M	14.3	8.5	594	2800
Na_2SO_4	20 m M	15.0	4.7	313	3460
	50 m M	14.0	6.2	443	3080
Na_2CO_3	20 m M	12.8	11.6	906	2460
	50 m M	12.0	24.3	2030	2320
Na_2-EDTA	20 m M	0.3	—	—	—
	50 m M	0.3	—	—	—

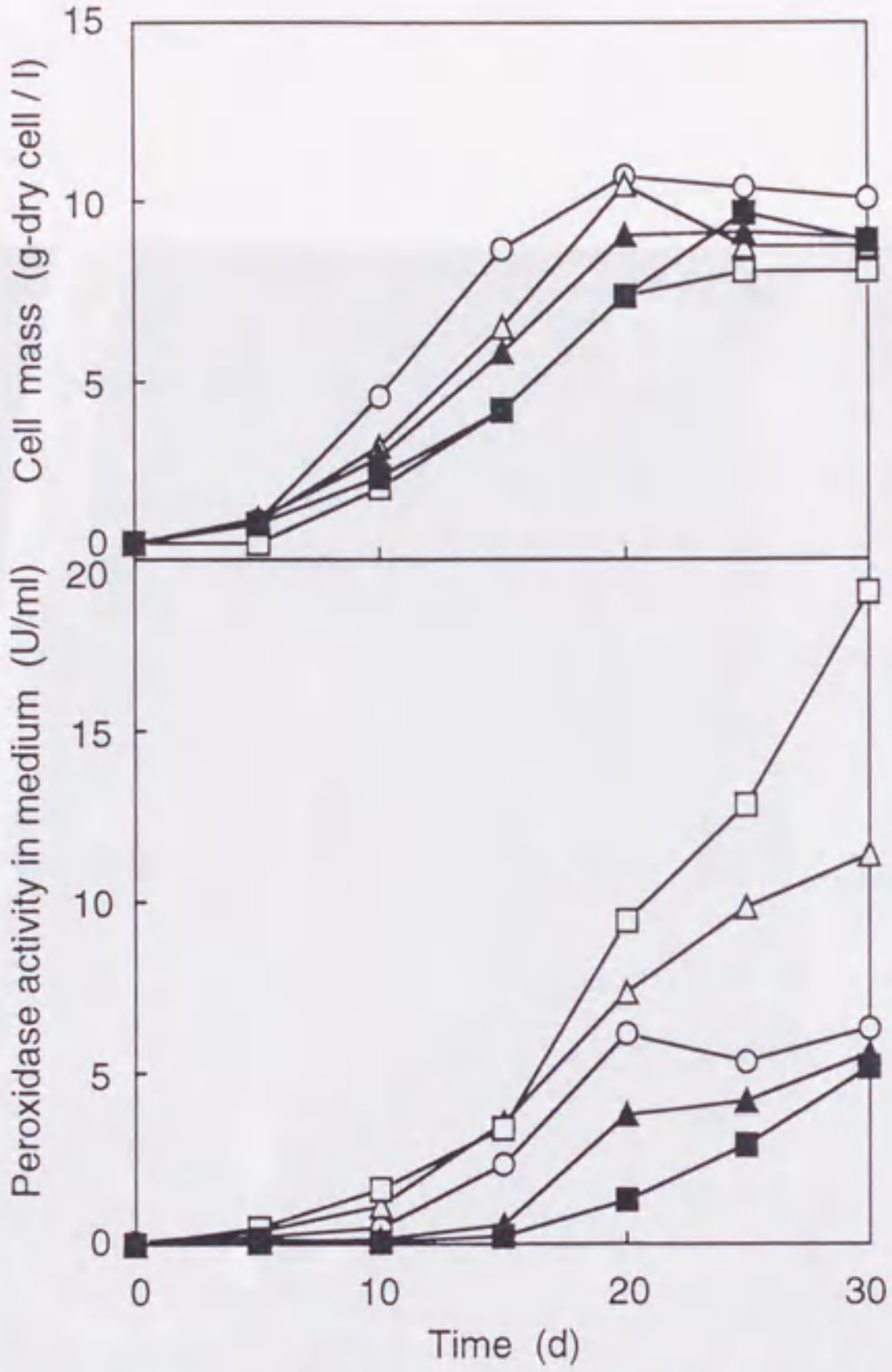

FIG. 2-1. Effect of osmotic pressure in medium on horseradish hairy root growth (upper) and Peroxidase excretion (lower). Cell mass was monitored by conductivity of medium. Final cell mass at 30 days was measured by dry weight (g /*l*). Non-supplementation (○), 50 mM NaCl (△), 100mM NaCl (□), 99 mM mannitol (▲), 198 mM mannitol (■)

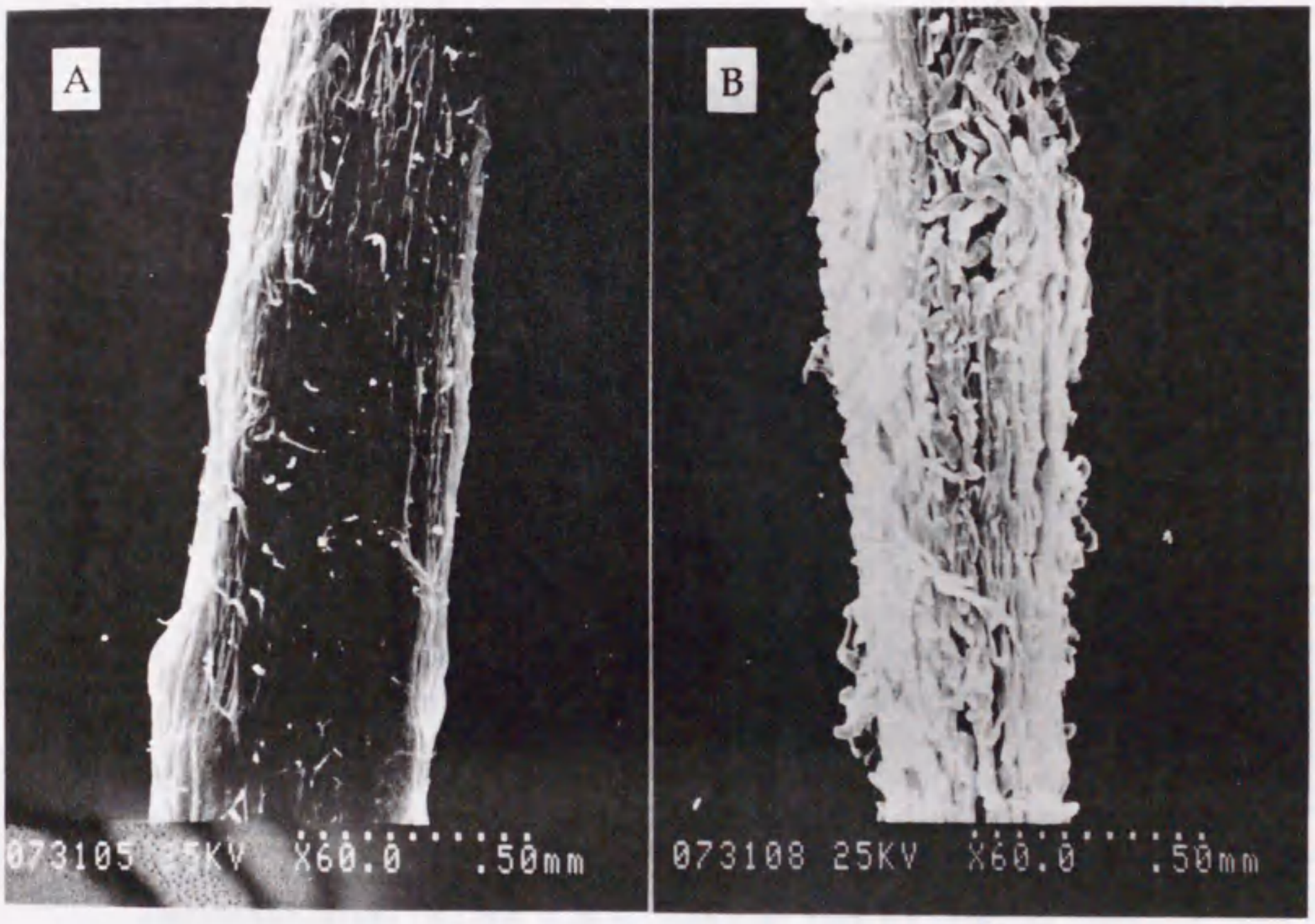

FIG. 2-2. Scanning electron micrographs of the surface of horseradish hairy roots grown for 30 d in non-supplementation (A) and with 50 mM $CaCl^2$ supplementation (B).

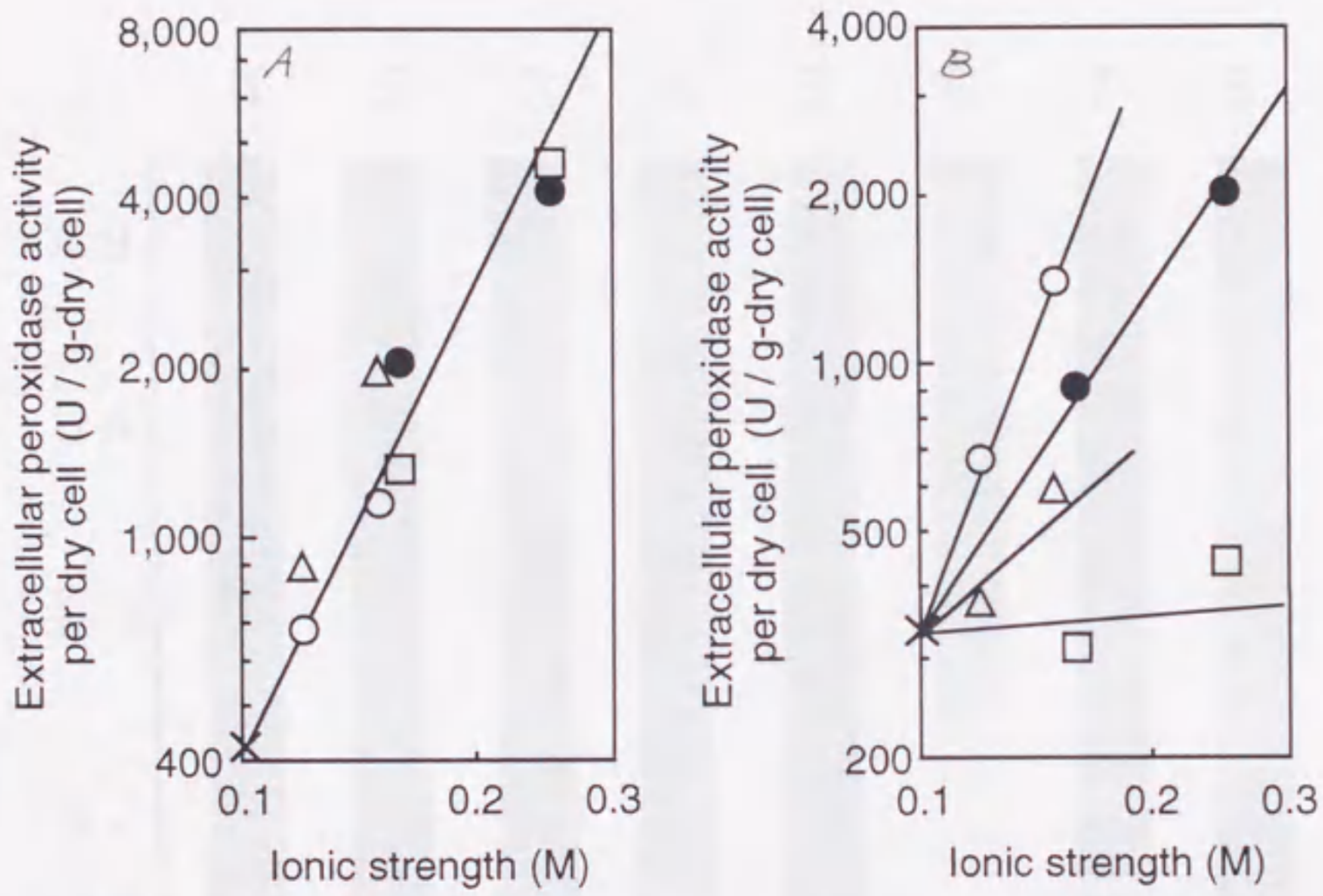

FIG. 2-3. Relationship between extracellular Peroxidase activity per dry cell and ionic strength. Each ionic strength was calculated from supplemented ionic strength and total ionic strength of components in MS medium. A, non-supplementation (X), Na^+ (○), K^+ (△), Mg^{2+} (□), Ca^{2+} (●) B, non-supplementation (X), Cl- (○), NO_3^- (△), SO_4^{2-} (□), CO_3^{2-} (●)

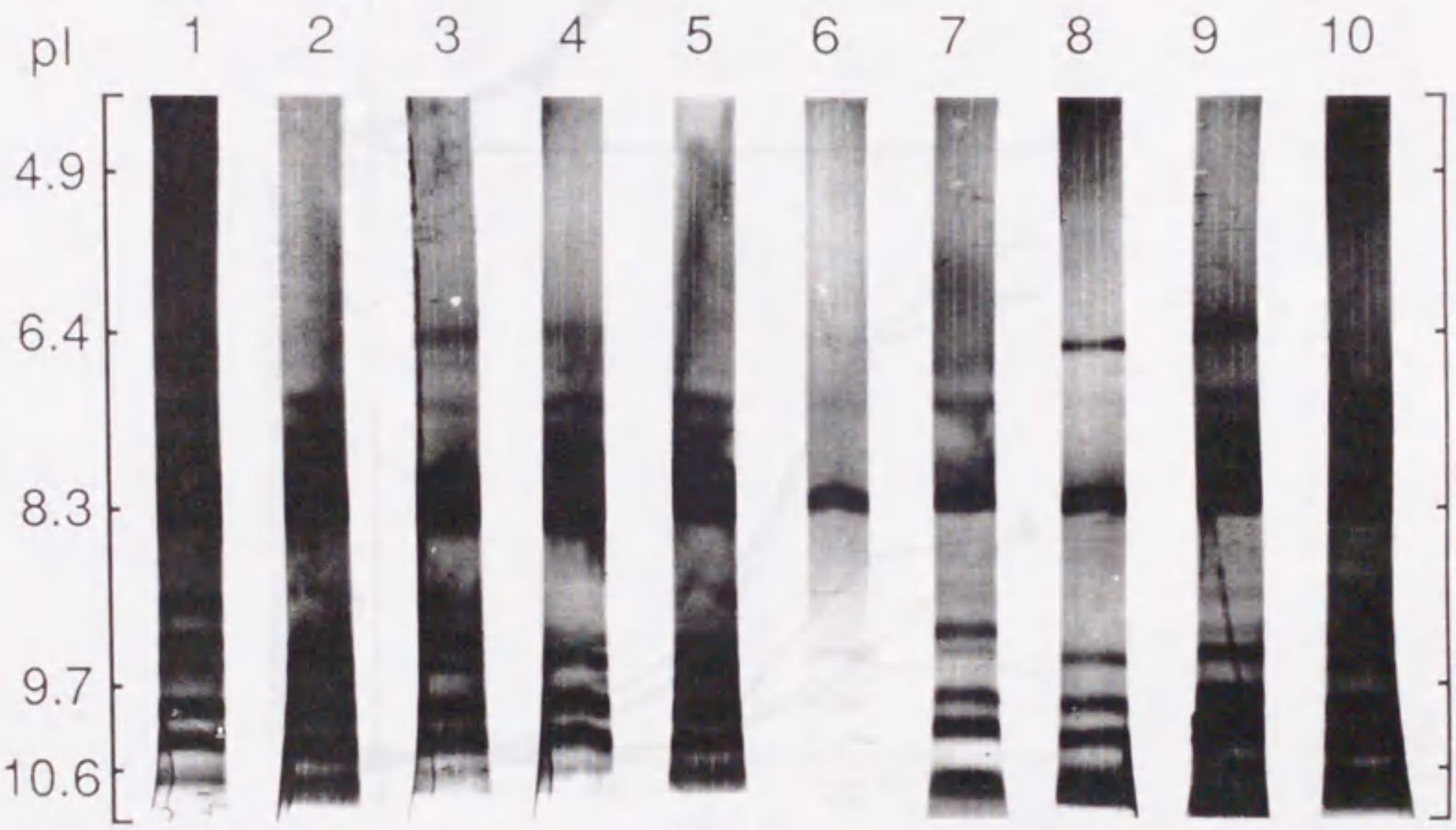

FIG. 2-4. Isoelectric focusing of the intracellular (lanes 1-5) and extracellular (supernatant) fractions (lanes 6-10) of culture with various cation supplementations (50 mM). lanes 1 and 6, None ; 2 and 7, K^+ ; 3 and 8, Na^+ ; 4 and 9, Mg^{2+} ; 5 and 10, Ca^{2+}

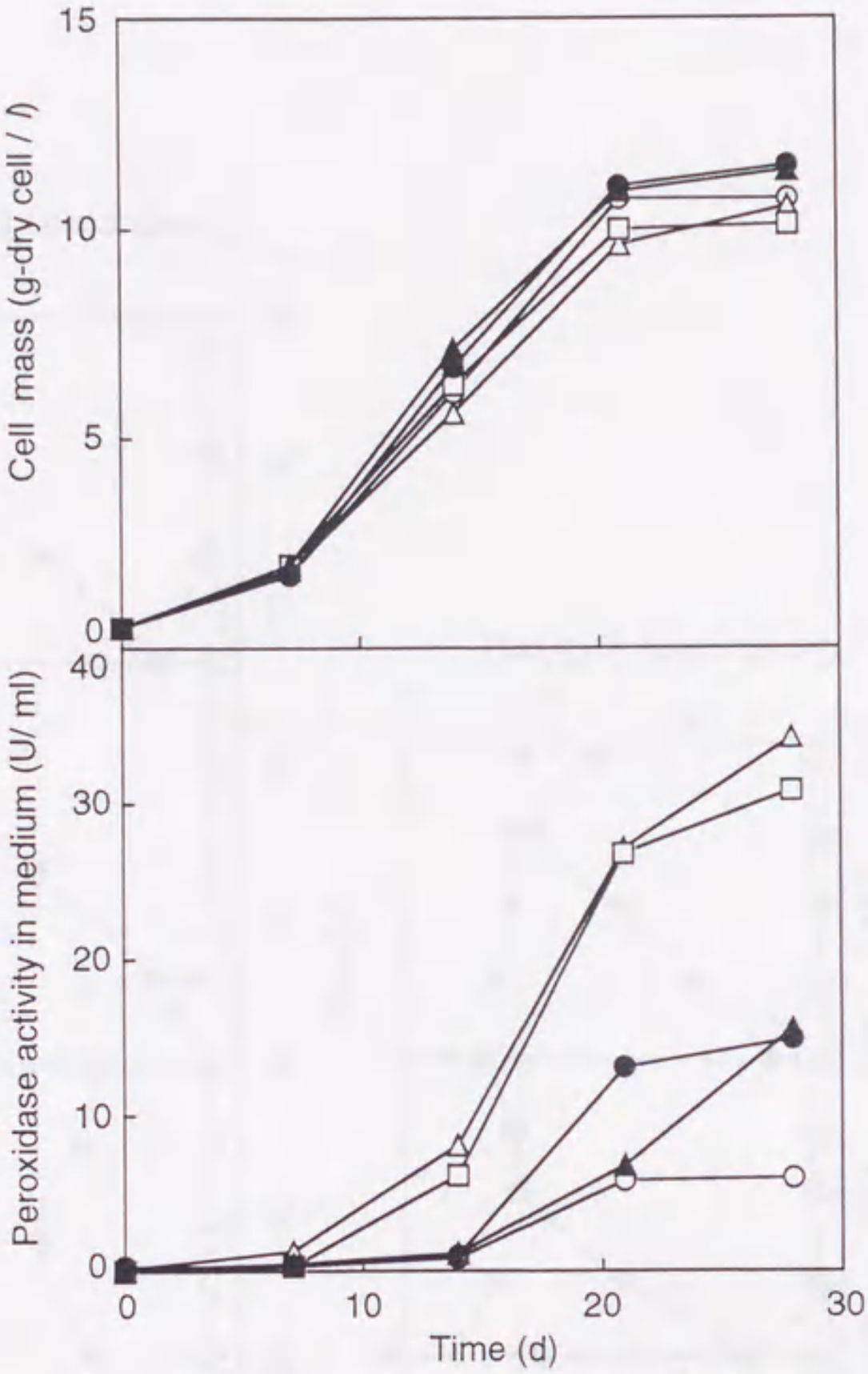

FIG. 2-5. The time course of horseradish hairy root growth (upper) and Peroxidase activity in medium with $CaCl_2$ addition at various stage of culture (lower). Cell mass was monitored by conductivity of medium. Final cell mass at 28 days was measured by dry cell weight (g /*l*). Non-supplementation (○), Supplementations with 50 mM $CaCl_2$ at the beginning of culture (△), 7 d (□), 14 d (●), 21 d (▲)

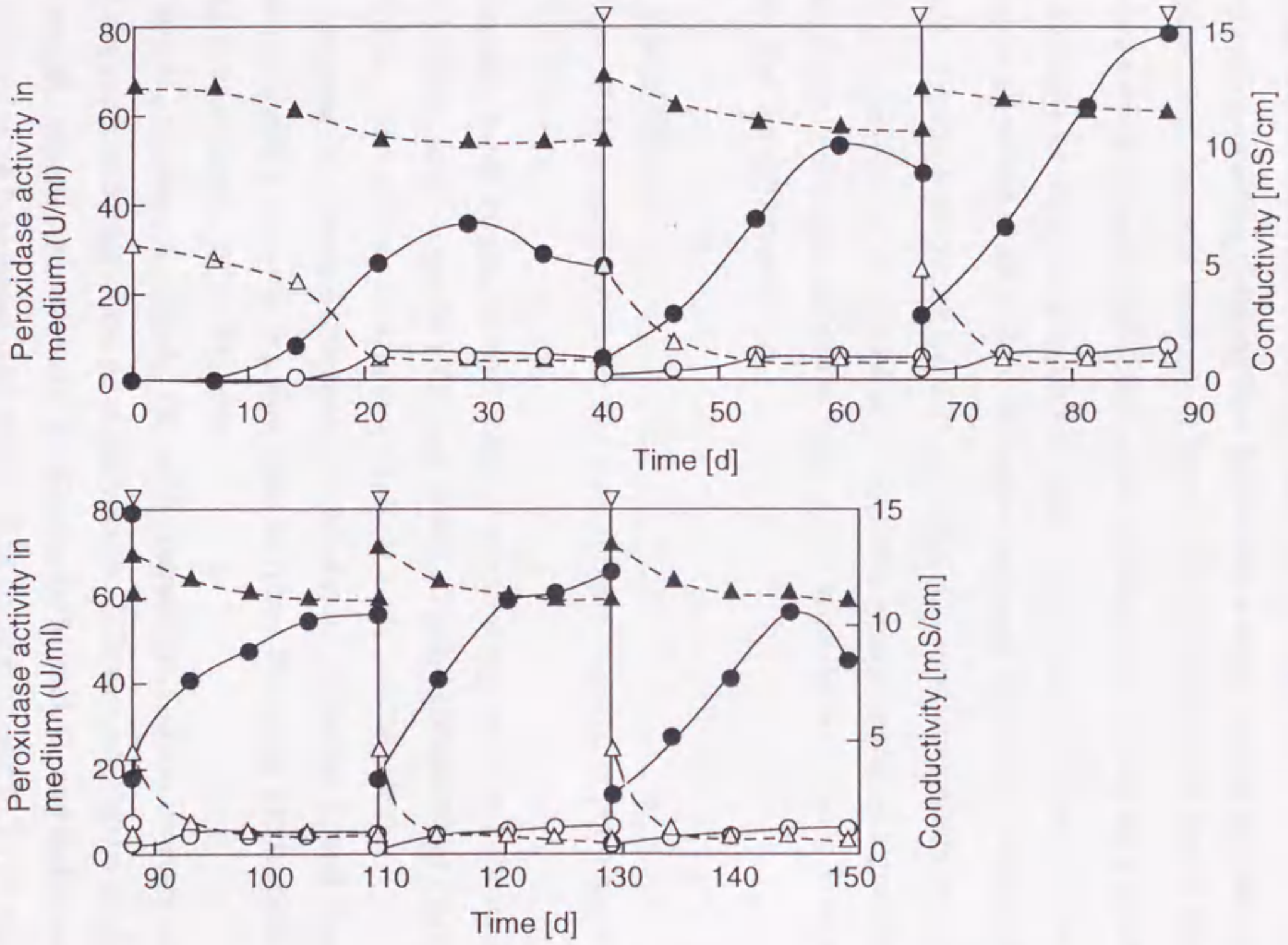

FIG. 2-6. Peroxidase activity in medium without $CaCl_2$ addition (○) and with 50 mM $CaCl_2$ supplementation (●) in repeated batch culture of horseradish hairy root in shake flask. Replacement of culture medium with 40 ml of fresh medium is indicated (▽) and was carried out when the conductivity of the medium in non-supplement (△) and 50 mM $CaCl_2$ supplement (▲) conditions did not decrease.

2. 5. SUMMARY

We tested that the effect of ion supplementation to medium on Peroxidase excretion, its application on fermenter scale culture and long term culture in chapter 2 and obtained the results as the following. Supplementation of mannitol instead of ion revealed that the excretion was stimulated, not by osmotic pressure in the medium, but by ionic properties. Extracellular Peroxidase activity per dry cell was proportionally correlated with the ionic strength of cation. $CaCl_2$ or $MgCl_2$ was found to be most effective excreting agent among any other combinations. $CaCl_2$ supplementation at the beginning of the culture exhibited higher Peroxidase production in the medium without a significant loss of final cell mass compared with $CaCl_2$ addition during the culture. Repeated batch culture with 50 mM $CaCl_2$ supplementation allowed a continuous retention of cell viability over 149 days and produced a great amount of extracellular Peroxidase, 12-fold as high as that achieved in a 40-day batch culture with 50 mM $CaCl_2$ supplementation.

2. 6. REFERENCES

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CHAPTER 3

Stimulation of Emergence of Root Apical Meristems in Horseradish Hairy Root by Auxin Supplementation and its Kinetic Model

3. 1. INTRODUCTION

Plant hairy roots, which are generated by integration of T-DNA on Ri plasmid of *Agrobacterium rhizogenes* into plant genomic DNA, are expected as the differentiated organs which have potential to produce valuable materials such as secondary metabolites (1-4). To obtain a large amount of hairy roots efficiently, we have developed a suitable bioreactor system for hairy root culture (5). Furthermore, we could obtain a high biomass yield of 30.1 kg-dry weight/m^3 at 38 d (carrot hairy root) and 27.2 kg-dry weight/m^3 at 39 d (*Ajuga* hairy root) with fed-batch culture using monosaccharide as a carbon source in a turbine blade reactor (6, 7). However, the dramatic improvement of the growth property would be difficult only via the technological approach because the growth rate is regulated by the number of root apical meristems of hairy roots.

we found that emergence of root apical meristems in horseradish hairy root was stimulated by auxin supplementation although hairy roots have been usually cultured without growth regulators (4). Since growth property of hairy root depends on both of the elongation rate and the number of root apical meristems, the increase in the number of root apical meristems of hairy root by auxin supplementation would increase growth rate significantly.

In this chapter, effects of auxin supplementation on growth behavior of horseradish hairy root were evaluated, and then the kinetic model for the growth was studied.

3. 2. MATERIALS AND METHODS

Plant materials and culture Horseradish hairy root (*Armoracia rusticana*) induced by the leaf disk method with *Agrobacterium rhizogenes* A4 as described previously (5) was used as a model hairy root in all experiments. The hairy root was maintained by regular subculture in the dark for 3 weeks at 25 ℃ on growth regulator-free Murashige and Skoog (MS) medium (8) supplemented with 2 % (w/v)

sucrose. For batch culture or repeated batch culture, about 1×10^{-4} kg hairy root (fresh weight) was inoculated into 4×10^{-5} m^3 MS medium containing 2 % sucrose and various concentrations of NAA in a 1×10^{-4} m^3 Erlenmeyer flask. The culture was maintained on a rotary shaker at 100 rpm in the dark at 25 ℃. Dry weight of the culture was estimated from the decrease in conductivity of the medium as described previously (5).

For measurement of root elongation rate under constant NAA concentration, one root fragment (5×10^{-3} m in length) with a root apical meristem excised by razor was inoculated on 5×10^{-5} m^3 solid MS medium containing 1 % (w/v) agar. Length of the fragment was manually measured photomicrographically every day until 2 d after the inoculation in order to evaluate the root elongation rate per day.

The number of root apical meristems in the liquid MS medium containing various NAA concentrations was measured at 7 d, and dry weight of the root was gravimetrically measured after drying the root at 60℃ for 24 h. The number of root apical meristems per meter was estimated from overall root length calculated from the dry weight using Eq. 16 described later.

Analytical methods Sucrose, glucose and fructose concentrations in the medium were assayed by a high performance liquid chromatography (HPLC) (Tri Rotor-V; JASCO, Tokyo) equipped with an Aminex HPX-87C column (BIO-RAD Laboratories, Richmond, CA) as described previously (5). NAA concentration was also determined by the HPLC with a Finepak Sil C-18 column (JASCO, Tokyo) and UV/VIS detector (870-UV; JASCO, Tokyo) at a wavelength of 280 nm. The solvent system used was 0.1% aqueous phosphoric acid: acetonitrile=40 : 60 (v/v %) with a flow rate of 1.0×10^{-6} m^3/min.

Scanning electron microscopy For scanning electron microscopy, the root was fixed with 2% glutaraldehyde in 10 mM phosphate buffer (pH 6.0) for 2 h. After drying by critical point dryer (HCP-2; Hitachi, Tokyo) and sputtering with ion sputter (E101; Hitachi, Tokyo), micrographs were taken using a Hitachi S-570 scanning electron microscope.

3. 3. RESULTS AND DISCUSSION

Selection of growth regulator for biomass increase At first, the effects of

growth regulator on the growth behavior were studied on MS medium supplemented with various growth regulators (1×10^{-3} kg/m^3). The growth regulators used in this study were three auxins which are indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA, three cytokinins which are kinetin, zeatin and 6-benzyladenine (BA), and abscisic acid (ABA). Supplementation of all auxins increased the number of root apical meristems, and NAA was the most effective. However, cytokinins caused corpulent of root apical meristems and inhibited the root growth. In the case of ABA, diameter of the root became small although the hairy root could grow (data not shown). In the subsequent experiments, the effects of NAA supplementation were studied in more detail.

Effect of NAA supplementation on the root growth behavior Figure 3-1 shows the effect of NAA on morphology of the hairy root. NAA supplementation stimulated the increase in the number of root apical meristems (Fig. 3-1B). To elucidate the effect of NAA on the root growth, the root was cultured in the medium supplemented with various NAA concentrations ranging from 0 to 5×10^{-3} kg/m^3 (Fig. 3-2A). NAA in the medium was rapidly absorbed by the root after inoculation, and was almost depleted within 4 d in all cases. After the depletion of NAA in the medium, the root began to grow. The cultures with 1×10^{-3} kg/m^3 of NAA reached the highest mass increase, 10.9 kg-dry weight/m^3 at 21 d with the maximum growth rate, 1.12 kg-dry weight/m^3/d. The cultures with 1×10^{-4} and 5×10^{-3} kg/m^3 of NAA also increased the growth rate compared with that of no NAA addition, although the maximum growth rates were lower than that of 1×10^{-3} kg/m^3 of NAA. Time courses for sugar concentrations in the culture medium without NAA and with 1×10^{-3} kg/m^3 of NAA are shown in Fig. 3-2B. In both cultures, sucrose was hydrolyzed to glucose and fructose during the culture, and glucose was consumed faster than fructose. The hydrolysis rate of sucrose in the culture with 1×10^{-3} kg/m^3 of NAA was higher than that of no NAA addition although biomass hardly increased during the initial culture phase (0-5 d). This indicates that NAA supplementation stimulated extracellular invertase production.

Figure 3-3 shows the root elongation rate under constant NAA concentration in the medium on the solid culture. Since only one root fragment with a root apical meristem (5×10^{-3} m long) was cultured on the solid medium in this experiment, more

than 98 % NAA was expected to remain in the medium at 2 d after inoculation based on Eqs. 4 and 16 (see next section). Thus, NAA concentration was considered to be kept almost constant for 2 d. Under the culture condition with 5×10^{-4} kg/m^3 of NAA, the root elongation was almost completely inhibited. Shen *et al.* also reported that *Catharanthus trichophyllus* hairy root was 100 to 1000 times more sensitive to NAA than untransformed root and more than 2×10^{-4} kg/m^3 of NAA inhibited its elongation completely (9). We also checked the root elongation rate in liquid culture without NAA in order to test influence of the environmental condition between solid and liquid cultures. Root elongation rate in the liquid culture was 30 % less than that on the solid culture as shown in Fig. 3-3. The root elongation rate in the liquid culture with NAA was estimated to be 30 % less value on the solid culture.

Figure 3-4 shows the relationship between the number of root apical meristems and the amount of NAA absorbed by the root at 7 d after the inoculation in the liquid medium with 0, 1×10^{-3}, 5×10^{-3} and 2×10^{-2} kg/m^3 of NAA. In all cases, depletion of NAA in the medium at 7 d was confirmed . The number of root apical meristems increased with increasing the uptake of NAA by the root and reached a plateau almost at 2×10^{-2} kg-NAA/kg-dry weight.

The effects of NAA on the growth of horseradish hairy root are summarized as follows: (1) Root elongation is strongly inhibited by NAA in the medium. (2) NAA is taken by the hairy root rapidly. (3) After NAA is absorbed by the root, the number of root apical meristems increases.

Kinetic model of hairy root growth treated with NAA Taya *et al.* have developed a kinetic model of hairy root growth (10). Schematic drawing of the growth model of hairy roots was shown in Fig. 3-5. On the basis of the experimental results, the kinetic model was extended to apply to hairy root growth treated with NAA. The following assumptions were made. 1) The hairy root elongates linearly at root apical meristem with length L_G. 2) Once root elongation at a root apical meristem becomes equal to the average length between basal portion of lateral root and next one (L_B), another root apical meristem emerges at somewhere on the root within negligible time. 3) Environmental factors such as shear stress cause decay of root apical meristems. 4) The root is regarded as a cylinder with diameter D and length L.

A linear growth law for the hairy root can be written as the following equation on

the basis of the assumption 1.

$$dL/dt=\mu L_G N \qquad (1)$$

where, N is the number of root apical meristems, L is overall length of the root, t is culture time and μ is the specific elongation rate of the root apical meristem.

The growth rate of hairy roots depends on not only the number of root apical meristems but also root elongation rate. Since the number of root apical meristems increased with increasing NAA absorbed by the root as shown in Fig. 3-4, higher concentration of NAA in the medium would promote higher growth rate from view-point of number of root apical meristems. However, since the activity of root apical meristem can be influenced by NAA absorbed, the following equation was applied for μ to represent the kinetic growth with inhibition by NAA supplementation.

$$\mu = \mu_{max} S\{(K_S+S)(1+A/K_1)(1+\int_0^t Adt/K_2)\} \qquad (2)$$

where S is sugar concentration, μ_{max} is the maximum specific rate of elongation of the root apical meristem, A is NAA concentration in the medium, K_S is the saturation constant, and K_1 and K_2 are the inhibition constants by auxin supplementation. For this equation, the non-competitive inhibition type equation was used. A factor of $(1+\int_0^t Adt/K_2)$ was used in order to represent the NAA inhibition which affects the root growth after the absorption of NAA by the root.

The uptake of NAA is expressed from the results shown in Fig. 3-2A by using the following equation:

$$(1/X)\,dA/dt = -K_3 A \qquad (3)$$

where X is dry weight of hairy root per m^3-medium and K_3 is the NAA uptake rate constant. From the experimental results in Fig. 3-2A, mass increase was negligible from 0 to 4 d (i.e., X=constant). Accordingly, Eq. 3 is integrated as the following equation.

$$A=A_0 \exp(-K_3 X_0 t) \qquad (4)$$

where A_0 and X_0 are the initial NAA concentration in the medium and the initial dry weight of hairy root per m^3-medium at $t=0$, respectively. Since this equation fits well with the results shown in Fig. 3-2A, the value of K_3 was calculated as 0.24 m^3/kg-dry

weight/h.

In the batch culture, the uptake of NAA per kg-dry weight (P) is expressed by the following equation.

$$P=(A_0-A)/X \qquad (5)$$

Since NAA was completely taken into the root from the liquid medium until 7 d after inoculation in the results shown in Fig. 3-4, P is equal to A_0/X after 7 d, and the number of root apical meristems per meter (B) and L_B are expressed as follows.

$$B=510P/(0.0018+P)+96 \qquad (6)$$

$$L_B=1/B \qquad (7)$$

Between the n-th and (n+1)-th lateral root formation ($t_{I,n} \leqq t < t_{I,n+1}$), the initial conditions are

$$t=0 \quad : \quad L=L_0, \quad N=N_0 \quad (n=1) \qquad (8)$$

$$t=t_{I,n} \quad : \quad L=L_{I,n} \quad N=N_{I,n} \quad (n \geqq 2) \qquad (9)$$

where the subscripts 0, I and n denote zero time, initial time for n-th lateral root formation and n-th lateral root formation, respectively. In this study, we assumed that when the length of elongation reached L_B value from the initial time of n-th lateral root formation, (n+1)-th lateral root formation occurred at somewhere on the root.

The time until the next formation of lateral root, Δt, is given by

$$\Delta t = \int_0^{L_B} dL_S / (\mu L_G) \qquad (10)$$

where L_S is length of elongation at root apical meristem from initial time of n-th lateral root formation.

The decay rate of root apical meristems is expressed as follows.

$$dN/dt = -k_d N \qquad (11)$$

where k_d is the decay rate constant of root apical meristems. When i of root apical meristems decay at $t=\theta_i$

$$\theta_i=(1/k_d)\ln\{N_{I,n}/(N_{I,n}-i)\}+t_{I,n} \qquad (12)$$

and hence

$$
\left.
\begin{array}{lcl}
t_{I,n} \leqq t < \theta_1 & : & N = N_{I,n} \\
\theta_1 \leqq t < \theta_2 & : & N = N_{I,n}-1 \\
\quad \vdots & & \quad \vdots \\
\theta_r \leqq t < t_{I,n+1} & : & N = N_{F,n} = N_{I,n}-r \\
 & & i = 0,1,2 \cdots, r
\end{array}
\right\} \quad (13)
$$

where the subscript F denotes final time for n-th lateral root formation. At the $(n+1)$-th lateral root formation, the initial number of root apical meristems, $N_{I,n+1}$, is calculated from Eq. 14.

$$N_{I,n+1} = 2N_{F,n} \quad (14)$$

Thus, overall root length L can be obtained by integrating Eq. (1).

The relationship between biomass increase and sugar consumption by hairy root is written in the following equation as described previously (7).

$$-dS/dt = mX + (1/Y_{X/S})dX/dt \quad (15)$$

where m and $Y_{X/S}$ are maintenance coefficient and biomass yield, respectively. Sugar consumption can be obtained by integrating Eq. 15 with respect to t.

Dry weight of hairy root can be calculated from Eq. 16.

$$X = \rho\pi(1-W_C)LD^2/(4V) \quad (16)$$

where ρ, D, W_C and V are density, diameter, water content of hairy root and culture volume, respectively.

Estimation of parameter based on the batch culture The values of the model parameters, μ_{max}, K_S, K_1, K_2 and k_d which were not determined from the above experimental results were estimated by simulation for the time courses of the batch cultures shown in Fig. 3-2A with Eqs. 1-3, 5-16 and the following initial values (at t=0) for X_0, N_0 and S_0 as 0.25 kg/m^3, 76 and 20 kg/m^3, respectively. Here, the culture volume was 4×10^{-5} m^3, and L_0 and N_0 are calculated from a given X_0 value based upon Eq. 16 and the result in Fig. 3-4 (N_0=96L_0). The kinetic parameters obtained from this simulation are μ_{max}=0.43 h^{-1}, K_S=4.1 kg-sugar/m^3, K_1=5.1×10^{-5} kg-NAA/m^3, K_2=7.6×10^{-2} kg-NAA · h/m^3 and k_d=4.6×10^{-3} h^{-1}. The parameters and constants used for the calculation are summarized in Table 3-1. Solid lines in Fig. 3-2A represent the growth simulation curves. The model describes accurately the root growth curve in the

culture with 0 to 5×10^{-3} kg/m^3 of NAA supplementation.

Root elongation rate in the culture with various NAA concentrations in the medium can be evaluated using Eqs. 1-3, 5, 15 and 16 and the initial dry weight (1.3×10^{-3} kg/m^3) calculated from length (5×10^{-3} m) of one root fragment, based upon Eq. 16. As shown in Fig. 3-3, calculated value fitted to the elongation rate in the liquid culture estimated from the experimental ones on the solid culture. Thus, the model could be considered to simulate the batch culture with the liquid medium.

Repeated batch culture Figure 3-6 shows repeated batch cultures with 1×10^{-3} kg/m^3 NAA supplementation and without NAA supplementation. The whole culture broth was replaced with the fresh medium every 5 d during the culture time (0-20 d) and every 4 d during 20-40 d. From 10 d through 32 d, NAA-treated root grew linearly at the rate of 2.27 kg-dry weight/m^3 /d, and then the growth rate decreased gradually. The decrease in the growth rate may be due to the decrease in dissolved oxygen concentration in high density culture. In the case of no NAA-treated root, the lag-phase was about 17 d and then the linear growth was observed at the rate of 1.44 kg-dry weight/m^3 /d. The culture with 1×10^{-3} kg/m^3 of NAA exhibited a 1.7-fold increase in dry weight, *i.e.* 57 kg/m^3 at 40 d compared with that of no NAA-treated root. Simulation for the time courses of the repeated batch cultures was carried out as shown by the solid lines in Fig. 3-6. Values of all the parameters used for the simulation in the batch culture were used for the repeated batch culture. At the replacement with the fresh medium, A and S were restored to initial values, A_0 (=0 or 1×10^{-3} kg/m^3) and S_0 (=20 kg/m^3), respectively. Between j-th and (j+1)-th medium replacement, Eqs. 5 and 6 were modified as follows.

$$P_0=(A_0-A)/X \qquad (j=0) \qquad (17)$$

$$P_j=\{P_{E,j-1}X_{E,j-1}+(A_0-A)\}/X \qquad (j\geqq1) \qquad (18)$$

$$B=510P_j/(0.0018+P_j)+96 \qquad (19)$$

where $P_{E,j}$ and $X_{E,j}$ are total amount of NAA uptake per kg-dry weight and dry weight of the root per m^3-medium at final time for j-th medium replacement, respectively. The growth simulation matched well to the experimental results in the repeated batch cultures as shown in Fig. 3-6.

In conclusion, we reported that NAA promoted horseradish hairy root growth due

to the increase in number of root apical meristems, and the high biomass of hairy root could be obtained by the repeated batch culture with NAA supplementation. The growth model simulated well the growth patterns of the root treated with NAA in batch and repeated batch cultures. This growth model would provide a conceptual framework for the effective hairy root culture by auxin supplementation.

Table 3-1. List of the model parameter and constant values

Parameter values evaluated from the simulation			
μ_{max}	$0.43\ h^{-1}$	K_2	7.6×10^{-2} kg-NAA · h/m^3
K_S	4.1 kg-sugar/m^3	K_3	0.24 m^3/kg-dry weight/h
K_1	5.1×10^{-5} kg-NAA/m^3	k_d	$4.6\times10^{-3}\ h^{-1}$
Constant values measured from the experimental results			
D	4.0×10^{-4} m	W_C	0.90
L_G	4.0×10^{-4} m	$Y_{X/S}$	0.67 kg-dry weight/kg-sugar
ρ	1.01×10^3 kg/m^3	m	2.9×10^{-3} kg-sugar/kg-dry weight/h

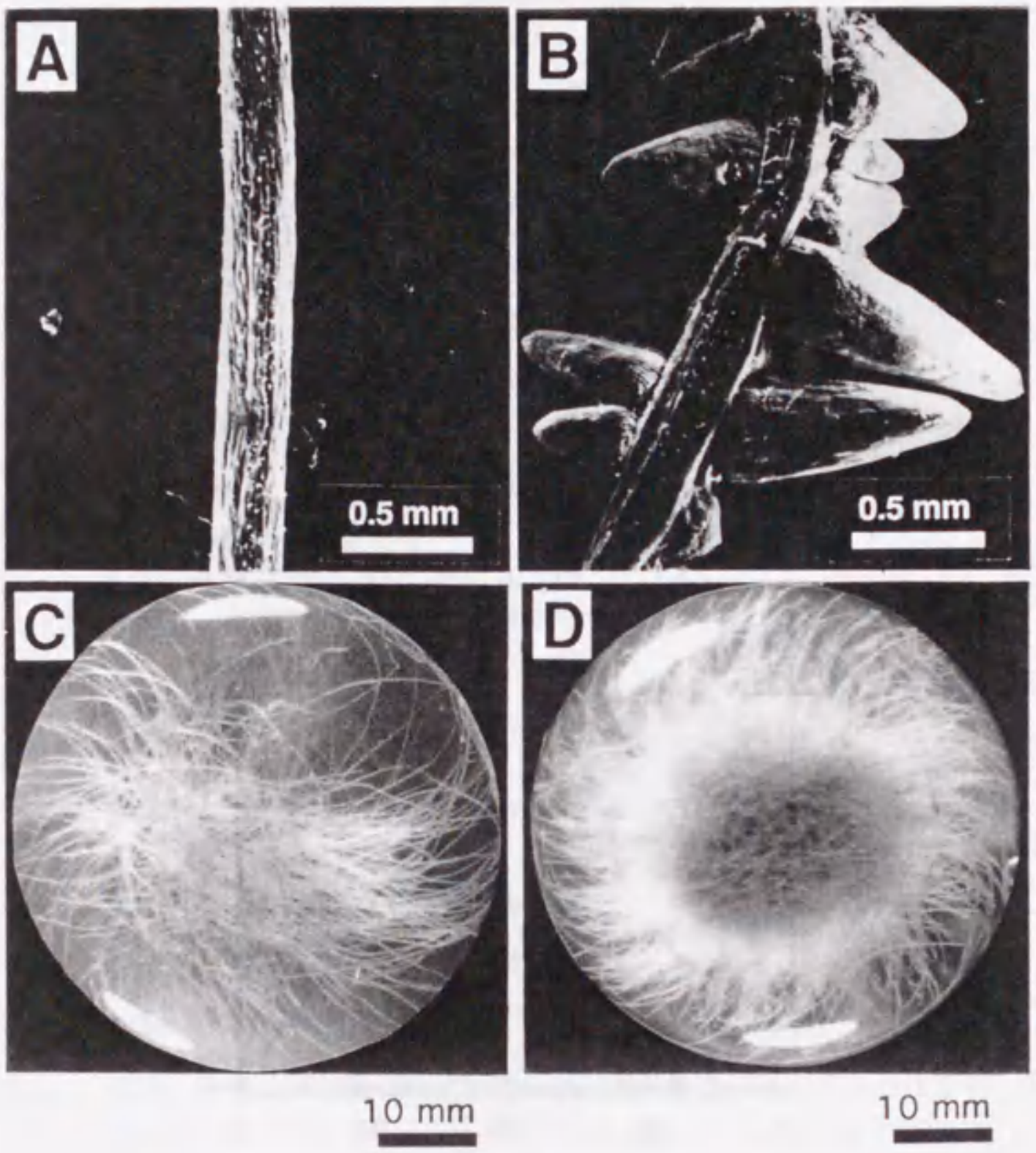


FIG. 3-1. Scanning electron micrographs of horseradish hairy root cultured in MS medium at 6 d (A and B) and photographs in flask culture at 15 d (C and D). NAA concentration: No addition of NAA (A and C); and 1×10^{-3} kg/m^3 of NAA (B and D).

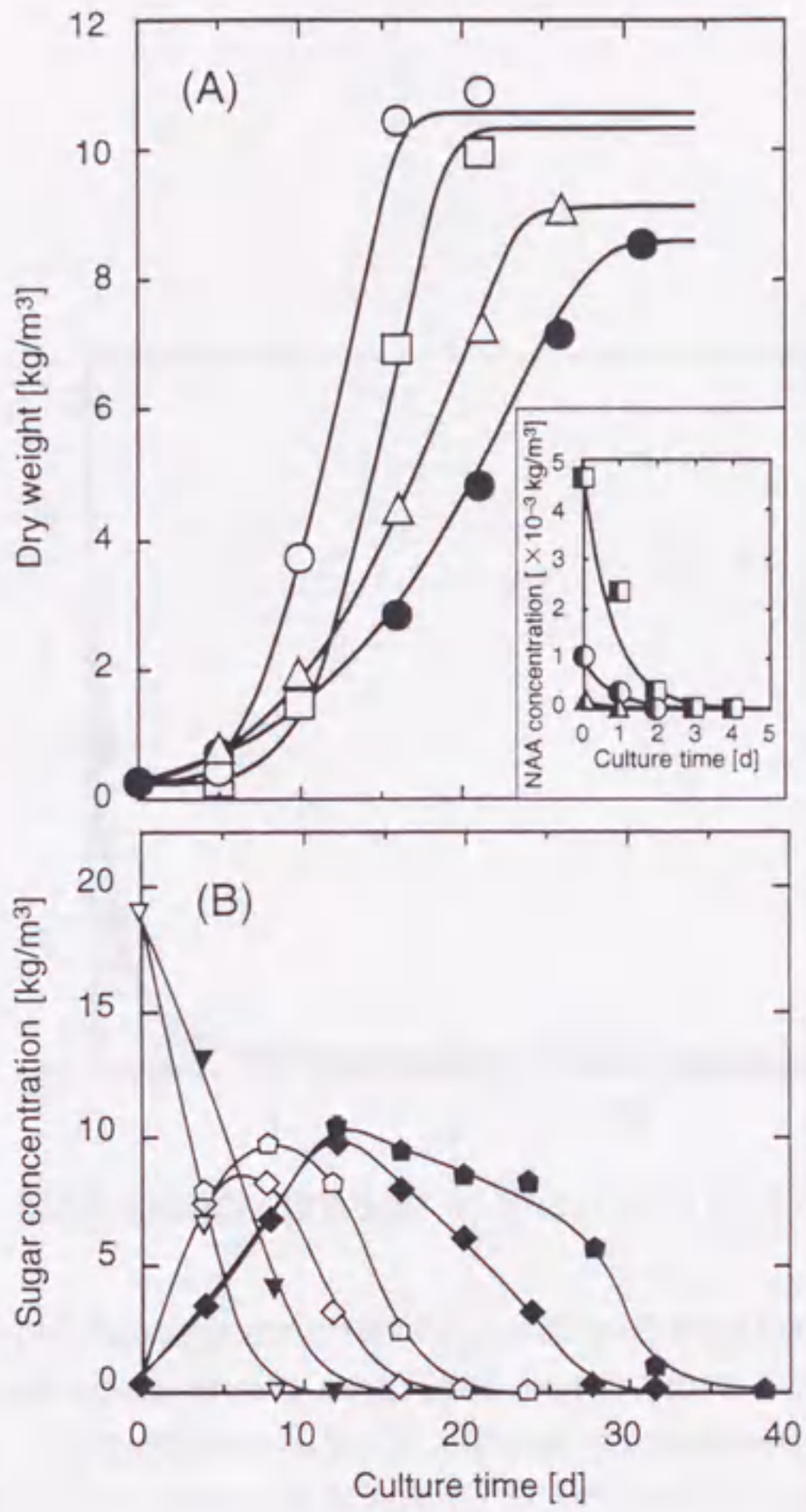


FIG. 3-2. Time courses of dry weight of horseradish hairy root and NAA concentration in medium (A), and sugar concentration in medium (B) of the batch culture with NAA supplementation. Lines for dry weight and NAA concentration depict the calculated results based on the kinetic model. Symbols in all figures represent experimental data. (A) No addition of NAA (● dry weight) ; NAA 1×10^{-4} kg/m^3 (△ dry weight, ◭ NAA); NAA 1×10^{-3} kg/m^3 (○ dry weight,◐ NAA); and NAA 5×10^{-3} kg/m^3 (□ dry weight,◧ NAA). (B) Sucrose (▼▽); glucose (◆◇); and fructose concentrations (⬟⬠); closed symbols, no addition of NAA; open symbols, NAA 1×10^{-3} kg/m^3.

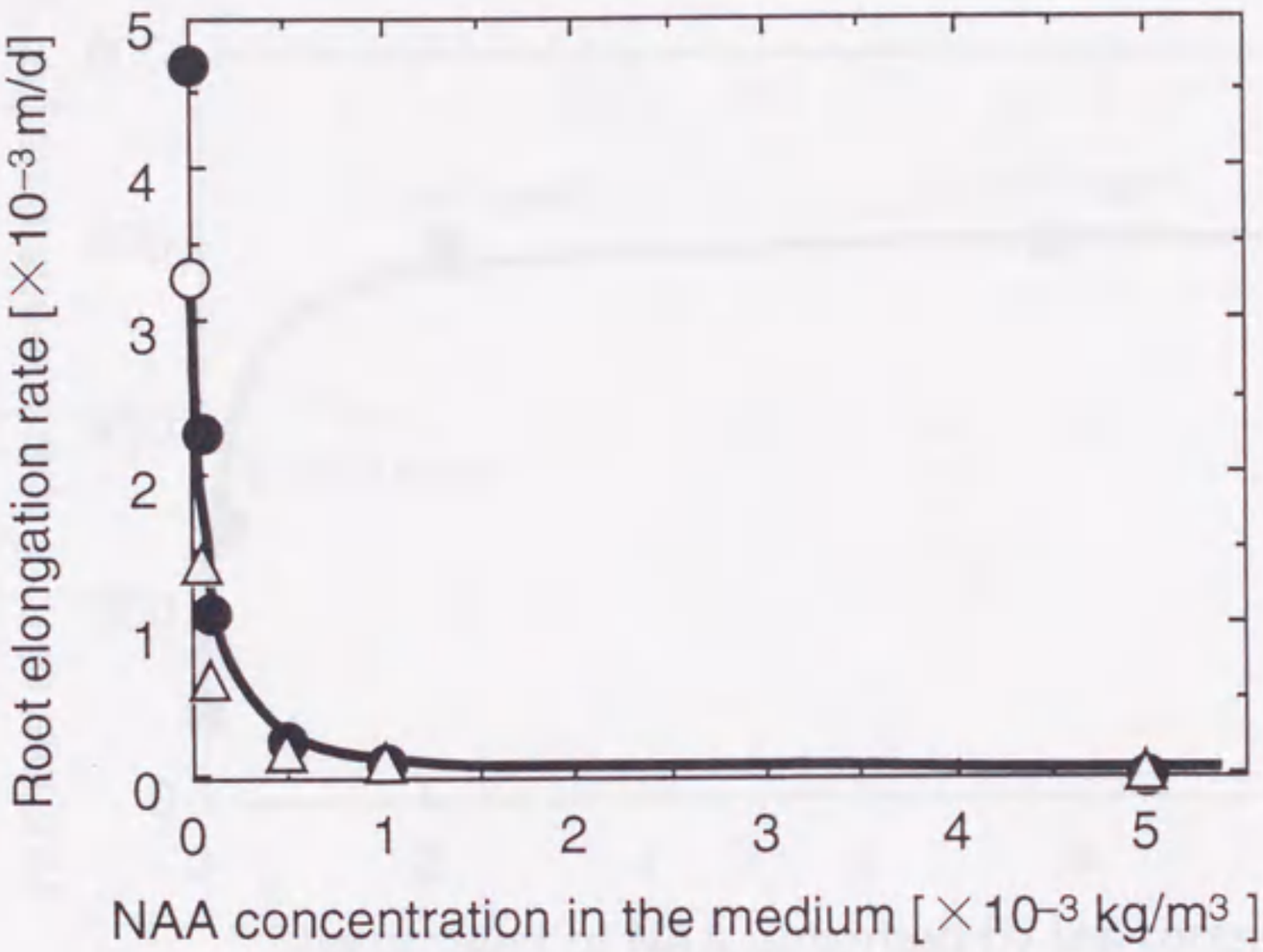


FIG. 3-3. Effect of NAA supplementation on the elongation rate of a root fragment with a root apical meristem. The root fragment was cultured on MS solid medium supplemented with various concentrations of NAA (●), or in MS liquid medium without NAA (○). Symbol (△) represents the elongation rate in MS liquid medium with NAA evaluated on the experimental data, and the line depicts the calculated results based on the kinetic model.

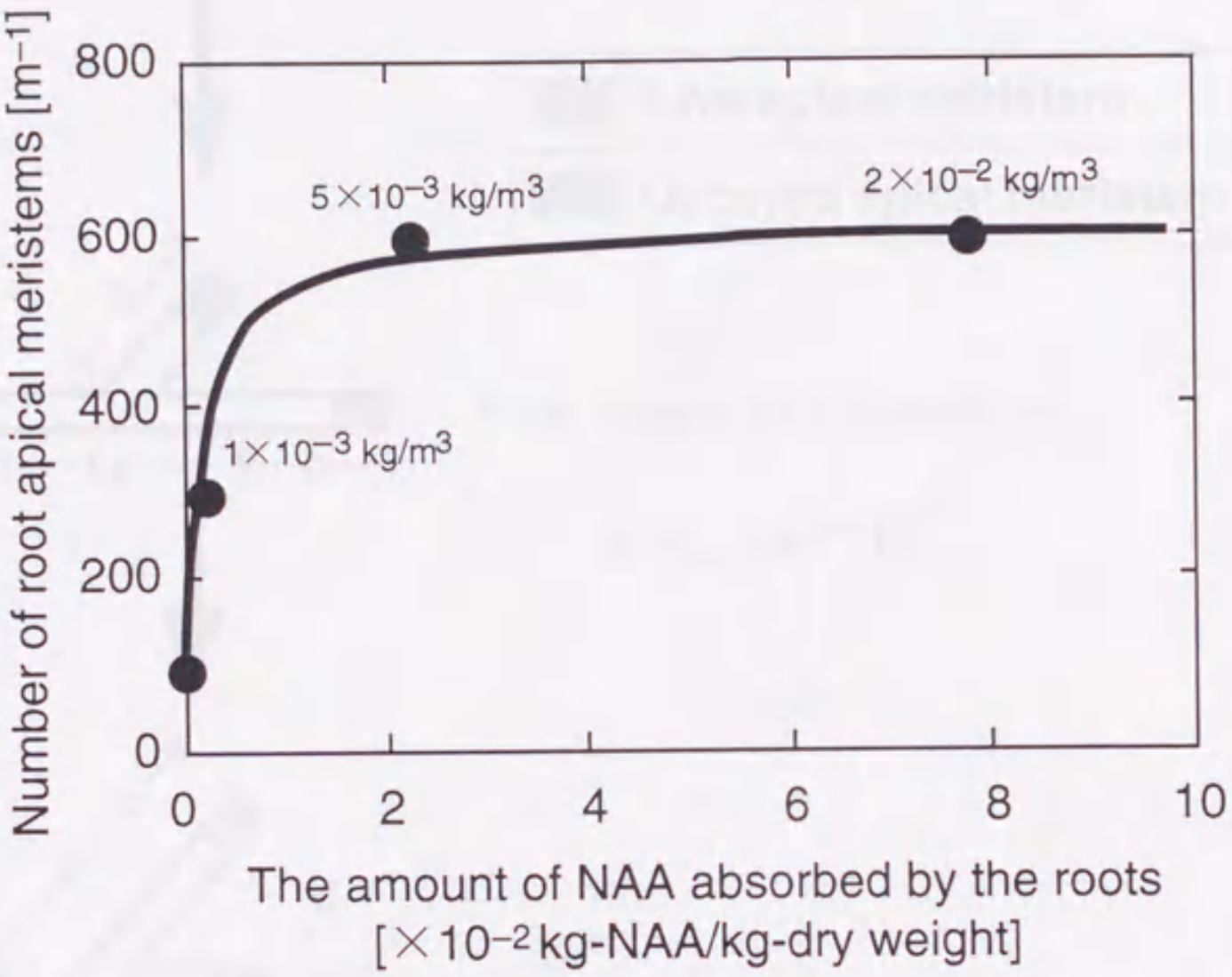
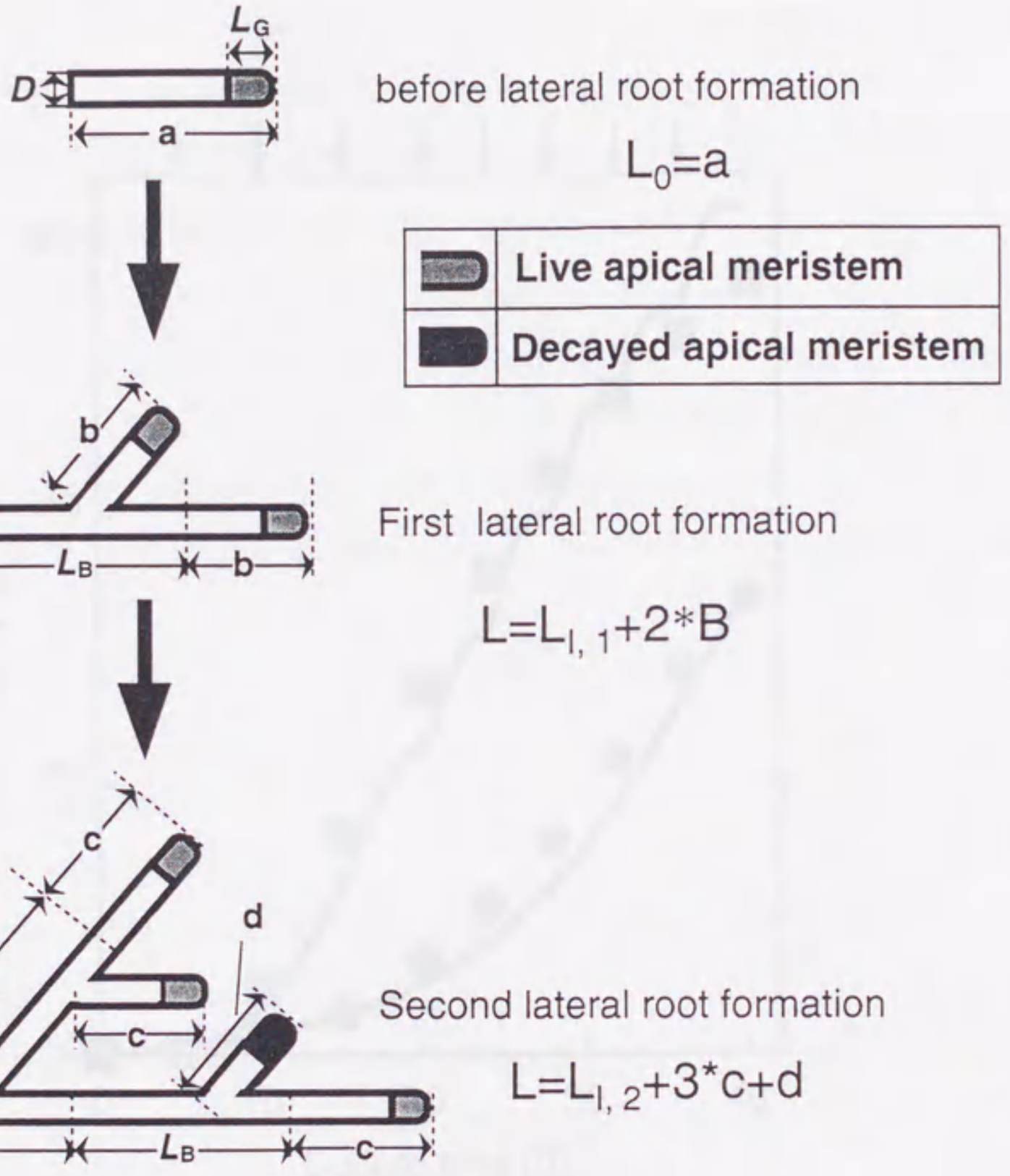


FIG. 3-4. The relation between the amount of NAA absorbed by the root and the number of root apical meristems. The number of root apical meristems and the amount of NAA absorbed by the root was evaluated at 7 d in the liquid medium. Symbols represent experimental data, and the solid line depicts the calculated result based on the kinetic model. The figures on the plots indicate the initial NAA concentration in the medium. .



before lateral root formation

$L_0=a$

	Live apical meristem
	Decayed apical meristem

First lateral root formation

$L=L_{l,\,1}+2*B$

Second lateral root formation

$L=L_{l,\,2}+3*c+d$

Fig. 3-5. Schematic drawing of the branching growth model of hairy roots.

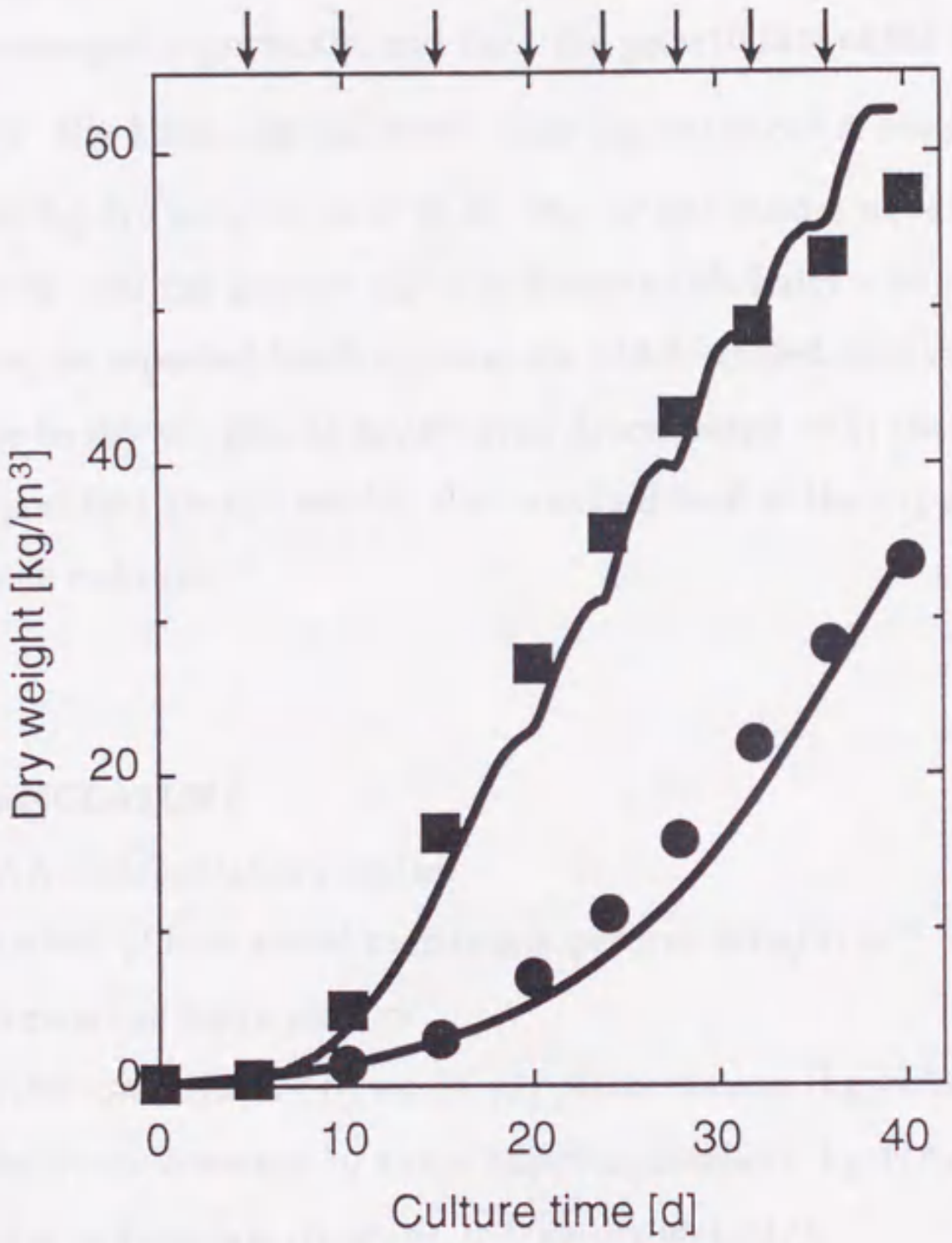


Fig. 3-6. Repeated batch cultures of horseradish hairy root with 1×10-3 kg/m3 NAA (■) and without NAA (●). Arrows indicate the timing of replacement with the fresh medium containing 1×10-3 kg/m3 of NAA or without NAA. Lines depict the calculated results based on the kinetic model.

3. 5. SUMMARY

Effects of auxin supplementation on growth behavior of horseradish hairy root were evaluated, and then the kinetic model for the growth was studied in chapter 3. 1-Naphthaleneacetic acid (NAA) in the medium strongly inhibited the horseradish hairy root elongation. In batch culture, NAA in the medium was absorbed by the root at the beginning of the culture (0-4 d). After the depletion of NAA, root apical meristems emerged vigorously, and then the growth rate of the root increased dramatically. The batch culture with 1×10^{-3} kg/m^3 of NAA reached the highest biomass, 10.9 kg-dry weight/m^3 at 21 d. The kinetic model developed previously was modified to fit well the growth curve of horseradish hairy root treated with NAA. Furthermore, in repeated batch culture, the NAA-treated root culture exhibited a 1.7-fold increase in dry weight, 57 kg/m^3 at 40 d, compared with that of no NAA-treated root. The modified kinetic model also matched well to the experimental results in repeated batch culture.

3. 6. NOMENCLATURE

A : NAA concentration, kg/m^3

B : number of root apical meristems per root length, m^{-1}

D : diameter of hairy root, m

K_1 : inhibition constant by auxin supplementation, kg-NAA/m^3

K_2 : inhibition constant by auxin supplementation, kg-NAA · h/m^3

K_3 : NAA uptake rate constant, m^3/kg-dry weight/h

k_d : decay rate constant of root apical meristems, h^{-1}

K_S : saturation constant, kg-sugar/m^3

L : overall length of hairy root, m

L_B : average length between basal portion of lateral root and next one, m

L_S : length of elongation at root apical meristem from initial time of n-th lateral root formation, m

L_G : length of root apical meristem, m

m : maintenance coefficient, kg-sugar/kg-dry weight/h

N : number of root apical meristems, -

P : amount of NAA absorbed per kg-dry weight of root , kg-NAA/kg-dry weight

r : number of root apical meristems decayed, -

S : sugar concentration in the medium, kg/m^3

t : culture time, h

W_C : water content of hairy root, -

X : dry weight of hairy root, kg/m^3

$Y_{X/S}$: biomass yield, kg-dry weight/kg-sugar

μ : the specific elongation rate of root apical meristems, h^{-1}

μ_{max} : maximum specific rate of elongation, h^{-1}

ρ : density of hairy root, kg/m^3

θ : time at which r root apical meristems decay, h

<subscripts>

0 : value at zero time

F : value at final time for n-th lateral root formation or j-th medium replacement

I : value at initial time for n-th lateral root formation or j-th medium replacement

j : j-th replacement with fresh medium

n : n-th lateral root formation of root apical meristems

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CHAPTER 4

Production of Artificial Seed from Horseradish Hairy Root.

4. 1. INTRODUCTION

Artificial seeds are expected to be a reliable delivery system for clonal propagation of elite plants. The delivery system has the potential for genetic uniformity, high yield and low cost of production. The candidate cells for artificial seeds have been somatic embryos, which were proposed by Murashige (1). The plant cells for artificial seeds require a good ability of regeneration and a high resistance against disease and mutation. Somatic embryogenic frequency is, however, usually low.

From some species of hairy roots, which are generated by integration of T-DNA on Ri plasmid in *Agrobacterium rhizogenes* (2), it has also been observed that the regeneration occurs under light conditions. Successful regeneration of the whole plant from Ri-transformed cells has been obtained with potato (3), apple tree (4), horseradish (5), *Allocasuarium verticillata* (6), and so on (7). Tanaka *et al.* reported that the productivity of an insect molting hormone synthesized in the whole plant regenerated from the hairy root was about 2.5 times higher than that in the original plant *Ajuga reptans* (8, Tanaka N. Japan Patent, No. Hei2-332775, 1990, Tanaka, N., and Matsumoto, T., Abstr. 111-th Pharmaceutical Soc., Japan, p.123, 1991). Generally, genetic improvement of plants through conventional breeding and selection methods takes a long period. Within an acceptable time period, new gene transfer technologies offer the opportunity to produce plants easily with desirable traits such as disease or herbicide resistance. The genetic modification using *A. rhizogenes* plasmids as vector seems to be feasible for improvement of the plant properties and for production of transgenic plants. Above successful reports on elite transgenic plant cells and their advantageous properties stimulated interest in developing regeneration and delivery system of hairy roots.

The use of the cultured hairy roots has consistently been focus on the large scale production of useful products or secondary metabolites, such as pigments and alkaloids (9, 10, 11, 12, 13). However, the artificial seed system sheds light on another potential of hairy roots for agronomic application. The proper system of plant regeneration is required to gain the transgenic plant from the hairy root efficiently. In

particular, the production process should be constructed and improved by developing a novel production system to realize these plants to the artificial seed in industrial scale.

Taking into consideration these hairy root potentials, hairy roots can be applied to produce artificial seeds. In this study, the regeneration frequency from horseradish hairy roots is reported by using excision and encapsulation combined with supplementation of growth regulators. The aim of this work is to develop the manipulation of hairy roots for an artificial seed and to estimate the efficiency of hairy roots as a seed system.

4. 2. MATERIALS AND METHODS

Plant cell culture and medium. The plant used in all experiments was horseradish (*Armoracia rusticana*) hairy root induced by the leaf-disc method (11). The hairy root was maintained by regular subculture in dark, during 3 weeks at 25°C, on hormone-free Murashige and Skoog (MS) medium (14) supplemented with 2 % (w/v) sucrose, and then was transferred aseptically into a 100-ml Erlenmeyer flask containing a 40 ml of MS medium with 2 % sucrose (the final inoculum size of hairy roots was about 2g/*l* in fresh weight). The solid medium contained 1.5 % agar. Under light conditions, the photoperiod (ca. 3500 lux) with fluorescent white light and fish light was 14 h a day.

Encapsulation and regeneration conditions. The root was sergically excised with blade. A suspension of the excised root fragments in 2-fold concentrated MS medium containing 4 % of sucrose was mixed at room temperature with the same volume of 4 % (w/v) sterilized sodium alginate (Wako Chemical Ltd.) solution. The mixture was added dropwise to a sterile solution of 100 mM $CaCl_2$, using pipetes, thus forming calcium alginate beads of the size as large as needed to cover an entire root fragment. The resultant beads were rinsed with a small amount of water and then directly placed on agar plates containing MS medium and 2 % sucrose under the light conditions. When the beads were placed indirectly, a plastic sheet (5x5 mm) was set between the bead and the agar. In this report, plantlet development was defined by two leaves emergence.

Dependency of shoot formation frequency on portions of hairy roots

From the preliminary experiments, the shoot formation occurred throughout the entire root except for the apical meristem. This agreed with the data reported by Noda *et al.* (5). We found that a larger number of shoot occurred at the portion far from apical meristem rather than nearby the meristem.

When the root was excised to various fragments in length from 1.0 to 10 mm and encapsulated with alginate, the shoot formation frequency was examined (Fig. 4-1). In this case, the excised root fragment including apical meristem exhibited a high shoot formation frequency. The shoot emerged on the root surface apart from apical meristem, after the root fragment elongated vigorously out of the bead. In the case of the root fragment containing branch, shoot formation was observed at the center of the beads (the photograph will be shown later), and the shoot formation frequency was comparable with that of the apical meristem fragment. The shoot formation frequency of root fragments without apical meristem and branch (intermediate portion) was significantly low.

The appropriate root length for regeneration should be determined for the encapsulation process. We determined the minimum root length enabling shoot formation, choosing the root fragment with apical meristem as the encapsulated root fragment (Fig. 4-2). The shoot formation frequency increased with the increasing root length up to 5 mm. The fragment whose length was more than 5 mm was almost constant in the frequency. The results indicated that the fragment of 5 mm length was suitable for encapsulation. In the subsequent experiments, the root fragment of approximately 5 mm was used.

Effects of auxin on root morphology and shoot formation frequency

For somatic embryogenesis, the callus is maintained in the medium supplemented with auxin, and then is transferred to the auxin-free medium. In preliminary experiments, we found that auxin stimulated the hairy root to meristem and branch emergence. To harvest a large amount of meristem and branch portions from the whole root culture, we examined the effects of auxin on morphological change of the root. After the root was treated with various concentrations of auxin, the randomly picked root fragments were encapsulated and transferred to phytohormone-free MS

medium under the light conditions. The root treated with 1 naphthaleneacetic acid (NAA)- or indole-3-butyric acid (IBA) showed a markedly high shoot formation frequency, compared with the non-treated root (Fig. 4-3). In particular, the highest shoot formation frequency was obtained when the root was placed on the medium containing 0.1 mg/*l* of NAA. On the other hand, 2,4-dichlorophenoxyacetic acid (2,4-D) and indole-3-acetic acid (IAA) resulted in decrease of shoot formation frequency. There were significant effects of auxin on the number of branch emergence. Branch numbers of NAA (0.1 and 1 mg/*l*) and IBA (1mg/*l*)-treated roots were larger than that of the non-treated root, while 2,4-D (1mg/*l*)-, IAA (1mg/*l*) and NAA (10mg/*l*)-treated roots showed no drastic morphological change. This result suggested that the number of branch induced by auxin seemed to be an accurate indication of shoot formation frequency when the whole root was used as the seed.

To explain the effect of auxin-treatment on shoot formation, the frequency of NAA-treated root with or without branch was also examined (Table 4-1). With the hairy root containing branch at day 24, shoot formation was significantly higher as compared with the root without branch. There was apparent correlation between morphological change and shoot formation frequency. The higher frequency of shoot formation on the 0.1- 1 mg/*l* of NAA and 1 mg/*l* of IBA-treated roots was due to a large number of branch induced by NAA or IBA treatment.

Effects of preculture conditions on the regeneration frequencies The above results were obtained in a solid state culture. Next, the effect of a liquid culture was examined on the regeneration frequency. There was no significant difference in the shoot formation frequency between the root derived from the liquid culture and that from the solid state culture (data not shown). The liquid culture provides significant increase in cell biomass compared with the root on agar. From these results, we concluded that the liquid culture was superior to the solid state culture.

To achieve an efficient liquid culture, the root was at first grown in MS medium devoid of auxin to produce the normal linear hairy root for 14 d, and then transferred for 10 d into MS medium containing NAA. As expected, the number of branch obtained with this two step culture was larger than that with none-treated culture. When NAA was incorporated into the basal medium at 0.1-5 mg/*l*, the shoot formation frequency increased slightly compared with that without NAA, as shown in

Fig. 4-4. In subsequent morphological development, two leaves emerged (plantlet development) from the shoot. The plantlet development frequencies decreased with a higher NAA concentration as shown in Fig. 4-4 and 0.1 mg/*l* of NAA was optimum in the two step culture.

As described previously, the shoot was formed from the branch portion of the root fragment in the bead, and then two leaves came out in the liquid preculture without NAA (Fig. 4-5). Healthy plantlets were also grown in the preculture with 0.1 mg/*l* and 1 mg/*l* of NAA as shown in Fig. 4-6, which appeared larger than non-treated plantlet. However, abnormal morphologies of the plantlets were observed in the liquid preculture with 5 mg/*l* of NAA (Fig. 4-6). From the plantlet development frequency and morphological observation, the optimum NAA concentration in the preculture was thereby determined to be at 0.1 mg/*l*.

Requirement of NAA elimination after the excise and encapsulation

Figure 4-7 shows the effect of NAA in beads on the shoot formation frequency. Although 1 mg/*l* of NAA supplementation under the light conditions (B in Fig. 4-7) resulted in decrease of shoot formation frequency, supplementation of NAA (0.1 mg/*l*) under the light conditions (A in Fig. 4-7) showed shoot formation frequency similar to that obtained without NAA (C and D in Fig. 4-7). However, all of the shoots failed to develop into the plantlet at the prolonged culture, while most of the shoots in which NAA was not supplemented in the beads under the light conditions (C and D in Fig. 4-7) developed to the plantlet (data not shown). This implied that NAA inhibited plantlet development from shoot extensively. Hence, plantlet development required removal of NAA from the medium under the light condition after the preculture with NAA under dark conditions for the shoot formation.

Effects of carbon source concentration on shoot formation For application of the artificial seeds, carbohydrate should be contained only in the beads. Various concentrations of sucrose in the beads placed on the plastic sheet were tested without sucrose supplementation in agar (Fig. 4-8). High shoot formation frequency of the root was observed when sucrose concentration in the beads was above 3%. Once leaf emerges from the root, the energy of differentiation and proliferation could be supplied by the photosynthesis. Thus supplementation with 3 % of sucrose would be considered sufficient for plantlet development.

4. 4. DISCUSSION

The present study examined the feasibility of artificial seeds using horseradish hairy root. The plant cell candidate for artificial seeds has been exclusively considered as somatic embryos derived from plant tissue or organ. We could propose the application of the transgenic plant to artificial seeds for the plant propagation, and also evaluated the shoot formation and plantlet development frequencies from the root in combination with excise and encapsulation since handling of hairy roots in encapsulated artificial seed system becomes easier compared with that of hairy roots themselves or root fragments. The regenerated plants from hairy roots often exhibit aberrant features such as wrinkled leaves, shortened internode distances, reduced fertility and lateral root initiation (15, 16, 17). Application of their properties is suggested by the recent reports showing agronomic potential of *A. rhizogenes* to induce root formation. For example, the plant regenerated from hairy roots possessed a large amount of root mass, with a resulting improvement in the drought resistance of the plant (18). The plants derived from hairy roots will be generated to meet individual requirements.

Transgenic plants such as hairy roots have been restricted to the test pots inside laboratory. However, some of the transgenic plants have recently been tested outside in USA. Transgenic plants will be acceptable to grow outside in the near future, and in that situation the artificial seed system using hairy roots would be more useful.

In the horseradish root fragment containing apical meristem, the shoot formation occurred progressively at the end portion opposite to the meristem. Since the shoot formation seemed to be correlated with root age, it is impossible to have at the same time both a high shoot formation frequency and a good proliferation. It is interesting to note that the branch portion exhibited a high frequency of shoot formation. The bud primodium may exist nearby the branch area. The above observations imply that the selection of the fragments is required to obtain an efficient regeneration from the non-treated hairy root.

When the length of the root fragment without apical meristem and branch was shorter than 5 mm, some root fragments did not elongate and became to death, and some elongated very slowly and the shoot formation occurred after the root length

became longer than a critical one (about 20 mm). The present process, which consists of excision and culturing 5 mm long root fragment, might provide initial favorable conditions for encapsulation in alginate beads.

A novel feature of plant development from somatic embryogenesis is controlled by exogenous concentration of auxin (19) or of cytokinin. The synergistic effect of transfer to the light conditions and control of growth regulators, such as phytohormone, seemed to trigger shoot formation. The reasons why the removal of auxin had an effect on phenotypic characters of the hairy root are unknown. We have also tested the effects of auxin on the morphological changes of the root. NAA and IBA treatments caused increase in the number of branch emergence. There was a relationship between the number of branch induced by these auxin treatment and shoot formation frequency. Therefore these results suggested that improvement of the number of shoot formation by the auxin-treatment was due to increase of the bud primodium around the branch induced by auxin. Although shoot formation from the hairy root was observed under no growth regulator control, the preculture in combination with NAA (0.1mg/*l*) improved the shoot formation frequency from randomly picked root fragments to use the whole root culture efficiently. Furthermore, the two step liquid culture divided into growth phase and auxin-treatment phase enhancing the number of branch portion contributed to increase the number of shoot formation. Shen *et al.* (20) reported that hairy root was about 100 times more sensitive to extracellularly supplied auxins than the normal cells. The gene derived from Ri plasmid might influence the auxin-mediated signal transduction pathway, and the root response to auxin may be different from that of the somatic cells. Further elucidation of auxin role in regeneration may require genetic and molecular biological approach in combination with biosynthesis inhibitors or determination of endogenous auxin concentrations.

Determination of sucrose concentration is one of the critical parameters to support the regeneration from the root. Redenbaugh *et al.* (21, 22) reported that alginate beads containing sufficient nutrient for differentiation to plantlet were very suitable system for the propagation of plant cells. The regeneration process in the beads must avoid the contamination invading from outer surface of the capsule and harmful effect of highly condensed nutrients. To prevent the capsule contents from bacteria or fungi

attack, concentration of carbon source, which is also an nutrient for microorganisms, should be as low as possible. Brown *et al*. (23) reported that the appropriate concentration of carbohydrate supported the shoot emergence. Assuming that dependency of proliferation on carbon source declines after the leaf emerged on the root, 3 % sucrose would be sufficient to be stored in the beads for shoot formation. Development to the healthy plantlet was retarded by a lower sucrose concentration (less than 3 % sucrose). If the secreted amylase is expressed in the hairy root by means of *Agrobacterium*-mediated transformation, starch will be supplemented in the beads in stead of sucrose.

The results described here shows the efficiency for regenerating transgenic horseradish in combination with excise, encapsulation and auxin supplementation. This type of protocol will be applied to product artificial seed using other hairy roots, thus contributing to the transgenic elite plant propagation in agronomic and industrial fields. Attempts to integrate this regeneration protocol with other factors and appropriate conditions are now in progress.

TABLE 4-1. Effects of auxin treatment on branch emergency and shoot formation at 24 d

	Non-treated	NAA	NAA	NAA	IAA	IBA	2,4-D
		(mg/*l*)					
		0.1	1	10	1	1	1
Shoot formation frequency (%)	21	72	60	19	3	60	0
Branch (%) [a]	17	70	58	16	3	60	0
Others (%) [b]	4	2	2	3	0	0	0
Percentage of branch portion (%) [c]	60	85	88	53	58	88	50

[a] Shoot formation frequency from root fragments containing branch

[b] Shoot formation frequency from root fragments without branch

[c] The number of beads included root fragments with branch / the total number of beads

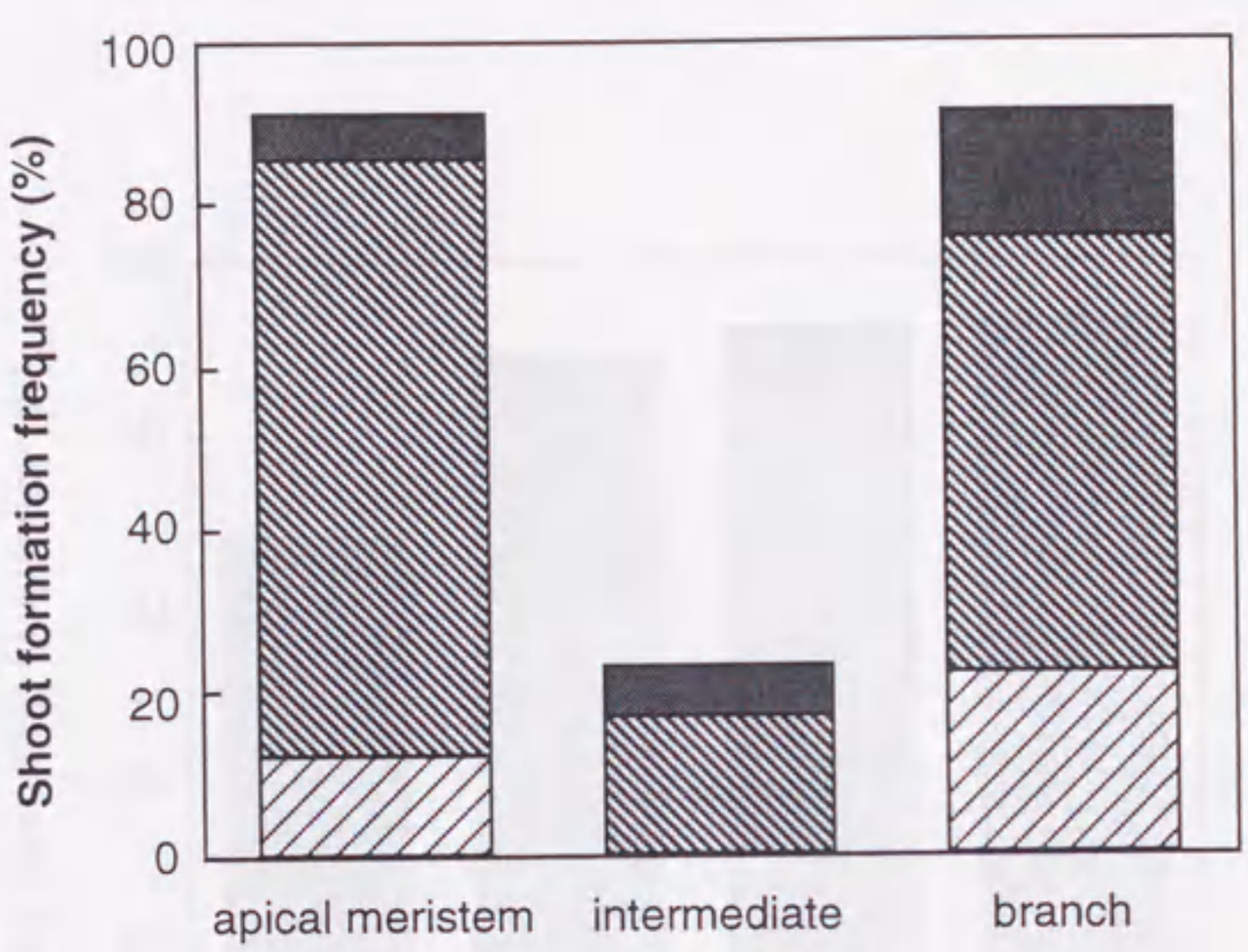

FIG. 4-1. Shoot formation frequency on the fragments containing various portion of horseradish hairy root at 15 d (▨), 30 d(▧) and 45 d (■). The root was excised to fragments in length from 5 to 7mm and then encapsulated with the beads as described in MATERIALS AND METHODS.

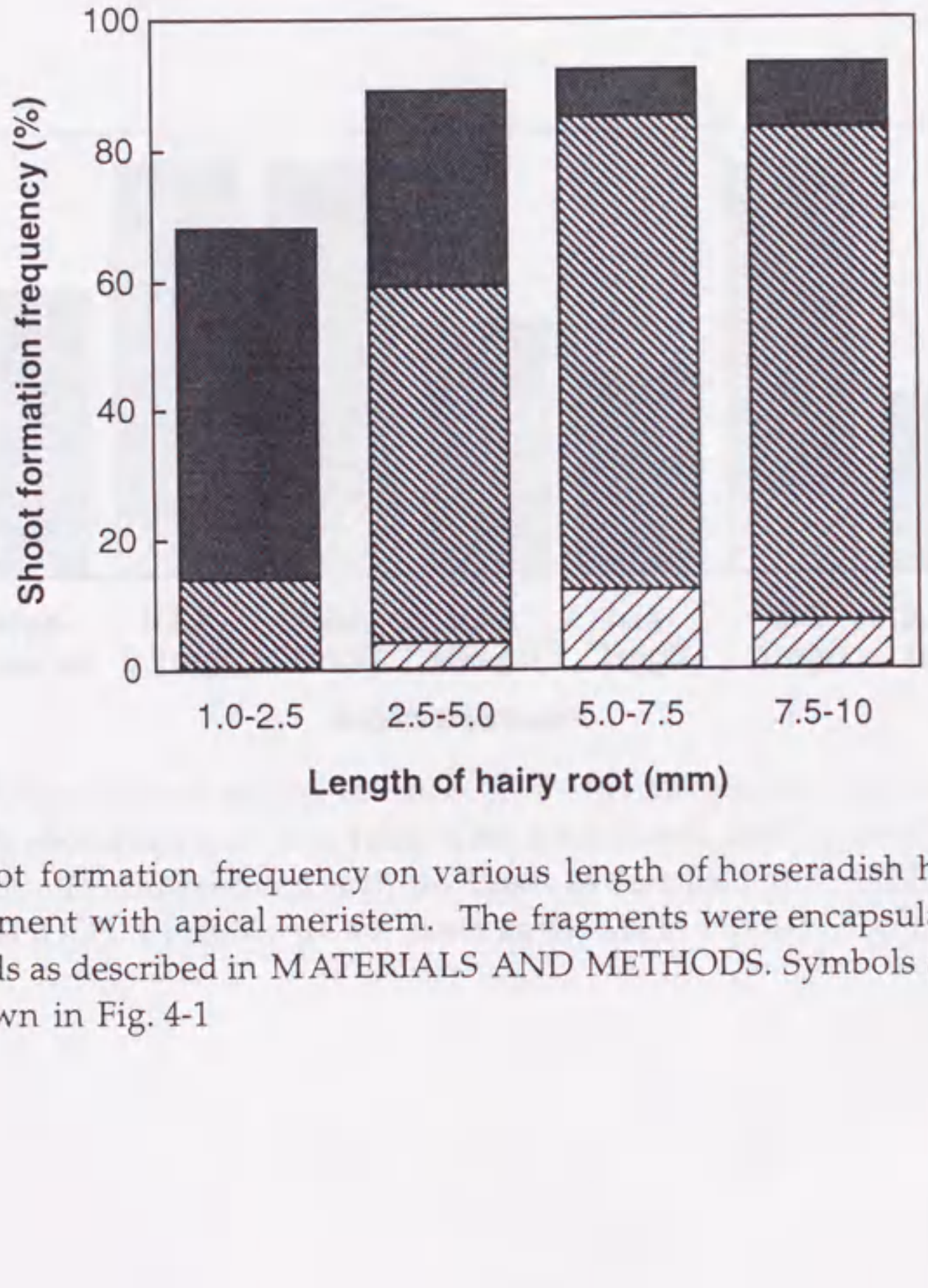

FIG. 4-2. Shoot formation frequency on various length of horseradish hairy root fragment with apical meristem. The fragments were encapsulated with the beads as described in MATERIALS AND METHODS. Symbols are the same as shown in Fig. 4-1

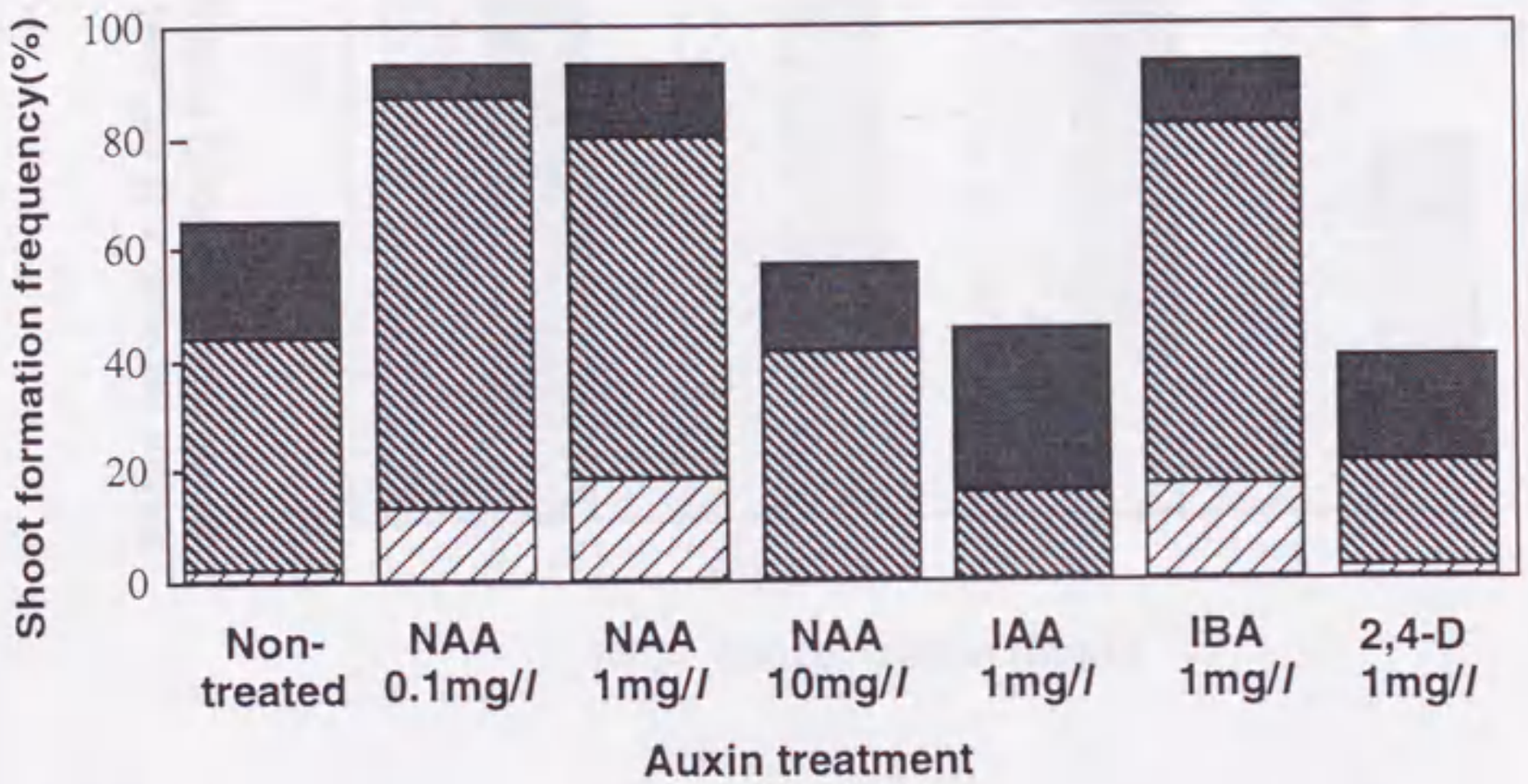

FIG. 4-3. Effects of various auxins on shoot formation frequency from horseradish hairy root fragment. The roots were precultured for 10 days with various auxin and encapsulated with the beads as described in MATERIALS AND METHODS. Symbols are the same as shown in Fig. 4-1

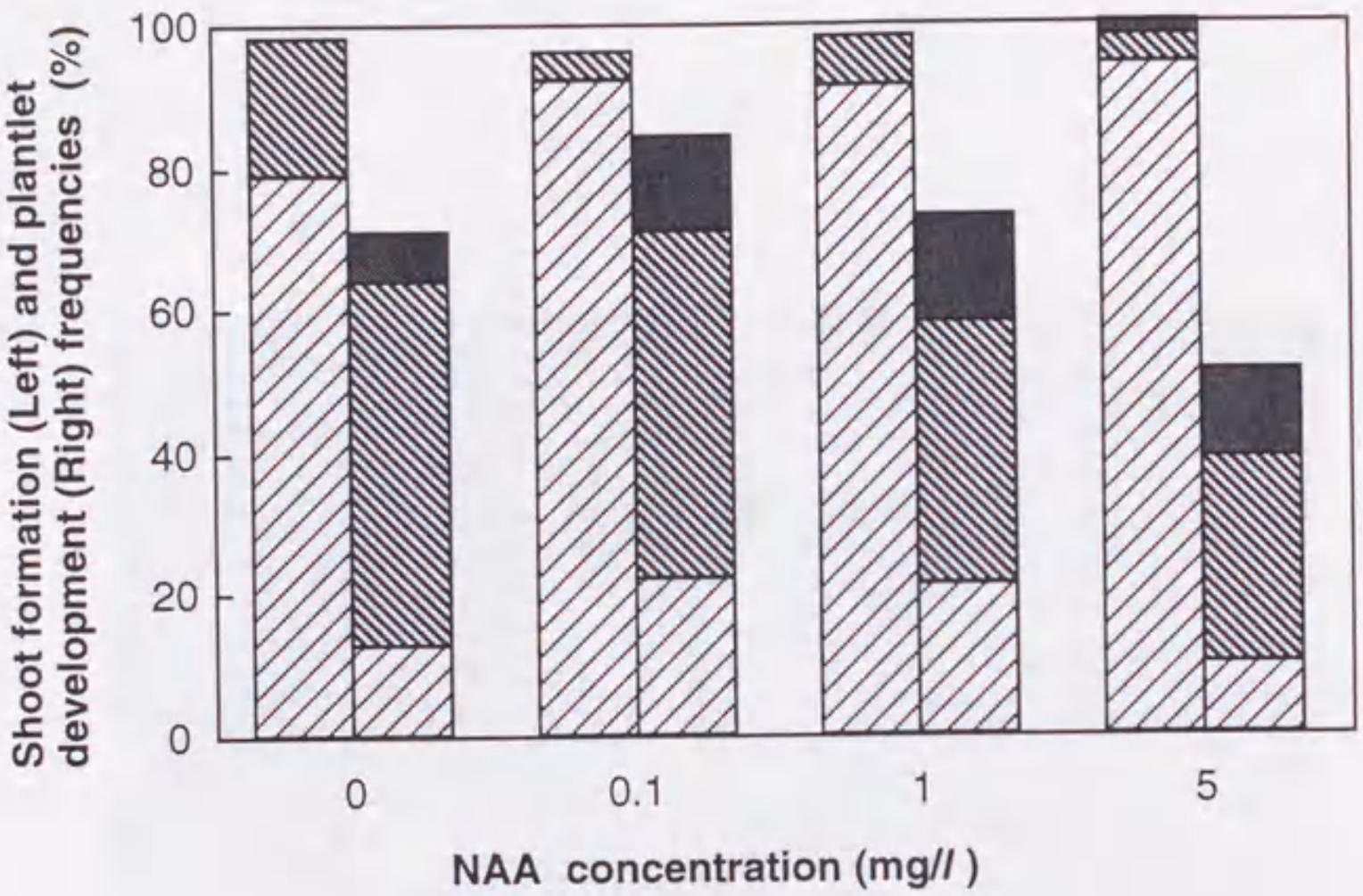

FIG. 4-4. Effects of liquid preculture in combination with NAA on shoot formation and plantlet development frequencies. Two step culture (non-NAA treatment for 14 d and later NAA-treatment for 10 d) was performed in dark, followed by encapsulation and regeneration of hairy root fragment without NAA under the light condition. Symbols are the same as shown in Fig. 4-1

FIG. 4-5. Two leaves emergence from hairy root fragment with branch. The root was cultured for 24 d in MS medium without NAA and the fragment was encapsulated with alginate containing MS medium and 2 % sucrose.

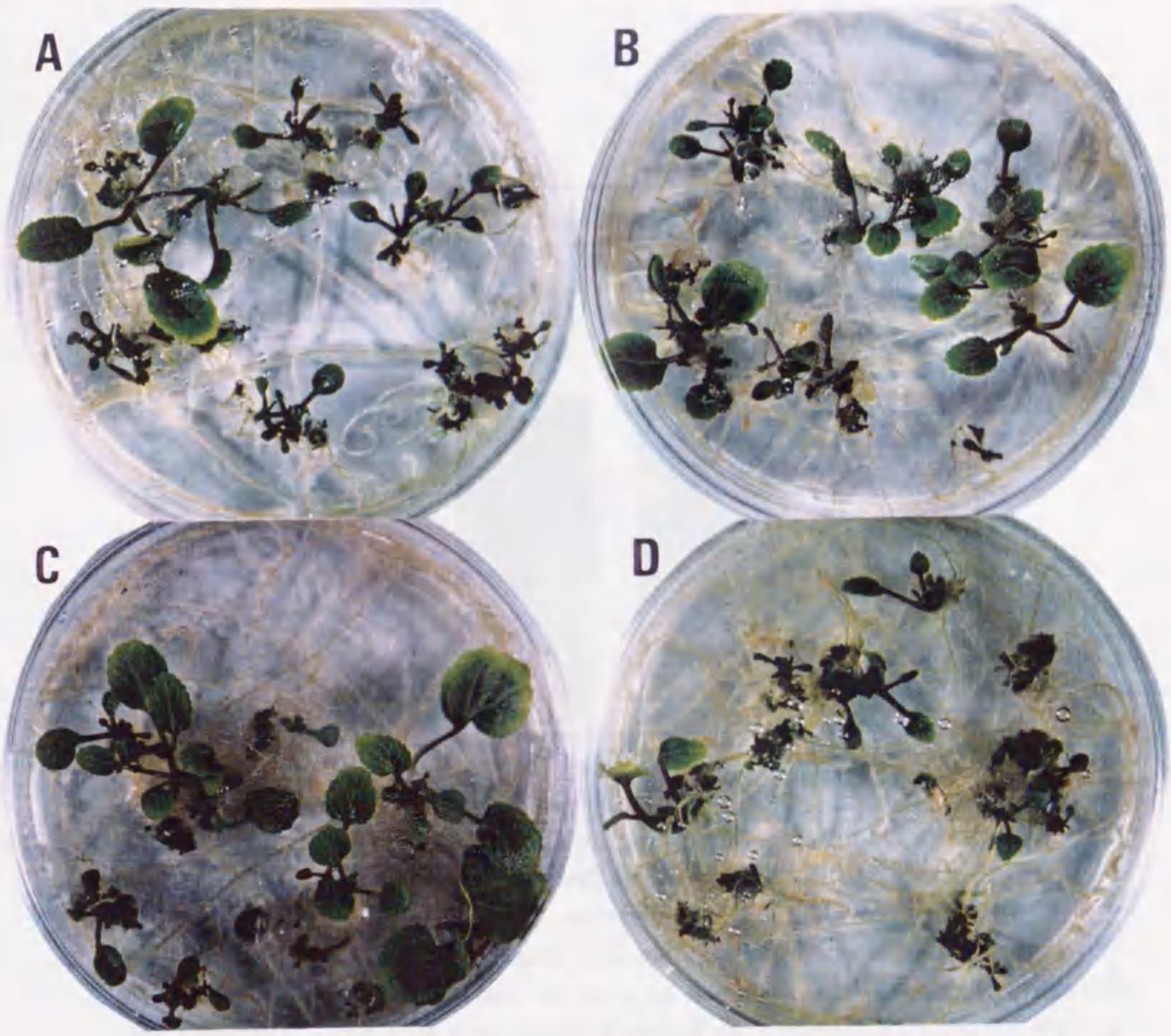

FIG. 4-6. Plantlet development from horseradish hairy roots treated according to the legend of Fig. 4-4. NAA treatments : without NAA (A), 0.1 mg/*l* of NAA (B), 1.0 mg/*l* (C), 5.0 mg/*l* (D).

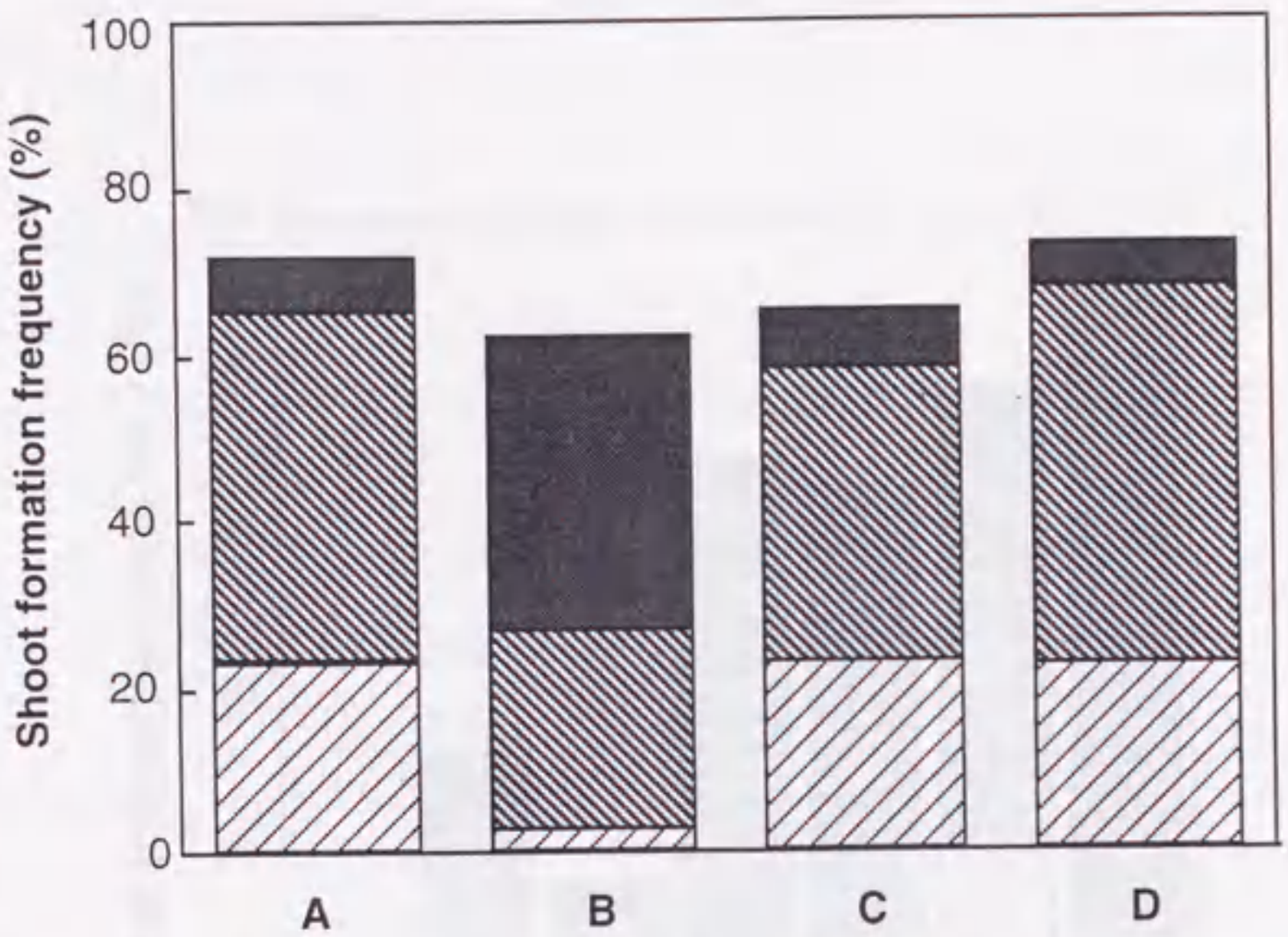

FIG. 4-7. Effects of NAA treatment under light condition on shoot formation frequency. A : The root was cultured under dark condition with 0.1 mg/*l* of NAA. The excised root was then encapsulated with alginate and the resultant fragments were cultured under light condition with 0.1 mg/*l* of NAA. B : All conditions were the same as A except that NAA concentration was 1 mg/*l*. C : The root was cultured under dark condition with 0.1 mg/*l* of NAA. The excised root was then encapsulated with alginate and the resultant fragments were cultured under the light condition without NAA. D : All conditions were the same as C except that NAA concentration was 1 mg/*l*. Symbols are the same as shown in Fig. 4-1

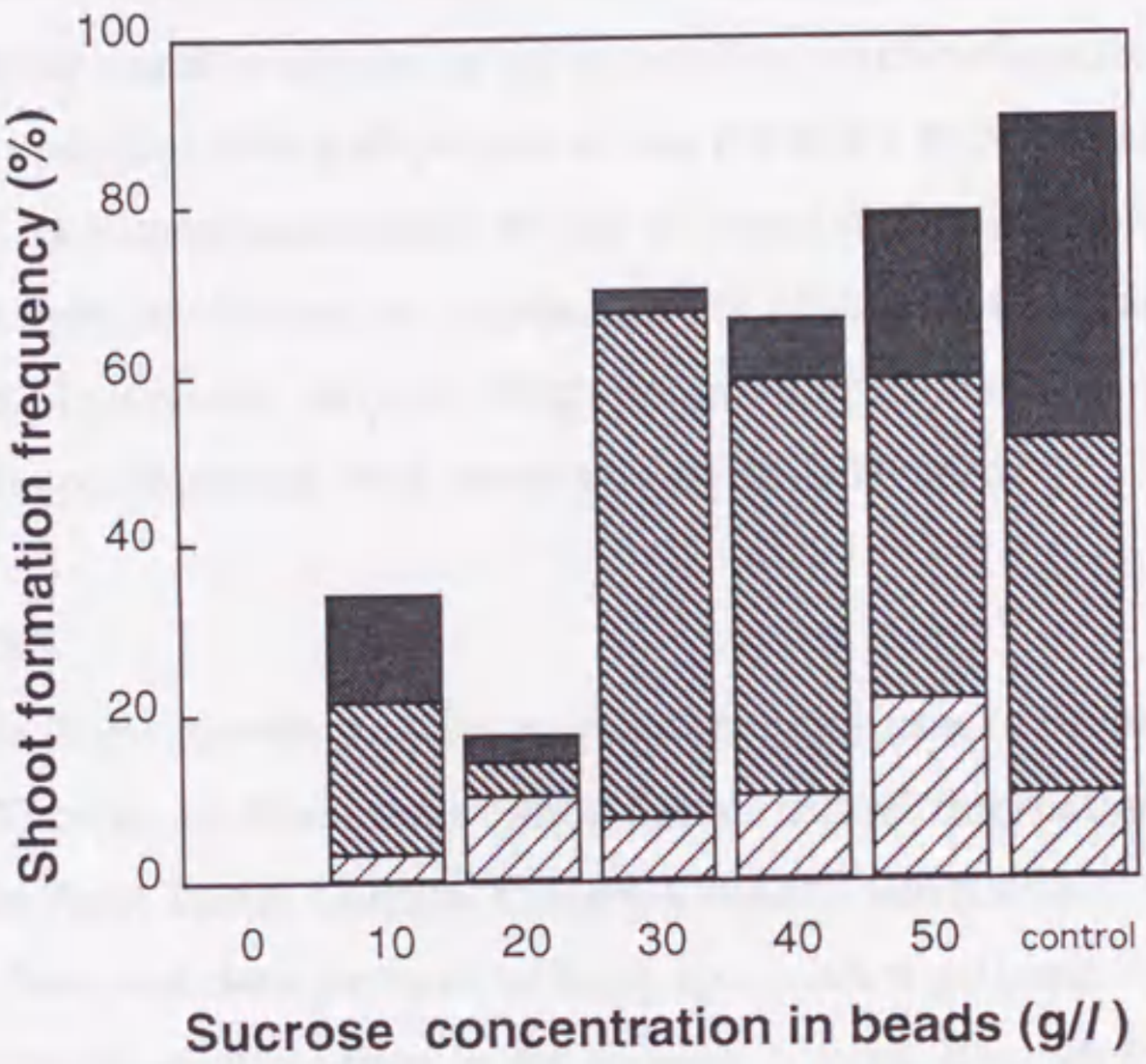

FIG. 4-8. Dependency of sucrose concentration in the beads on shoot formation frequency of horseradish hairy root fragments. Control : 2 % sucrose was contained in both the beads and the agar. Symbols are the same as shown in Fig. 4-1

4. 6. SUMMARY

Horseradish hairy roots were excised with a blade and encapsulated in alginate beads. The root fragments with an apical meristem or a branch efficiently regenerated to whole plants and root fragments of more than 5 mm possessed a high ability of shoot formation. We found that naphthaleneacetic acid (NAA) or indole-3-butyric acid treatment showed the stimulation of branch emergence from the root and many shoot formations on the root. These results indicated that the root fragment with branch induced by auxin was the most reactive portion. After the two step liquid culture (14 d without NAA and 10 d with NAA), the root fragment with high shoot formation frequency could be chosen as the material for artificial seeds. The development to a plantlet after encapsulation was inhibited by NAA, and the auxin was not required for the encapsulation. When encapsulated root fragments were placed under the light conditions, an addition of 3 % of sucrose in beads was sufficient for regenerations. The results showed a high potential of the hairy root for propagation by the combination with formation of artificial seeds.

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CHAPTER 5

Production of Plantlets for Use as Artificial Seeds from Horseradish Hairy Roots Fragmented in a Blender

5. 1. INTRODUCTION

The infection of dicotyledonous plants by *Agrobacterium rhizogenes* causes the formation of hairy roots. This response results from the integration of a part of the Ri plasmid (T-DNA) in *A. rhizogenes* into the plant genomic DNA (1). Hairy roots have the potential, as a differentiated organ, to produce useful materials such as enzymes (2-3) and secondary metabolites (4). Another use of hairy roots is micropropagation of elite plants. Plant regeneration from hairy roots has been reported in several plant species including the apple tree (5), horseradish (6), *Catharanthus roseus* (7), morning glory (8), potato (9) and *Allocasuarina verticillata* (10). Plants regenerated from hairy roots have altered phenotypes such as wrinkled leaves, plagiotropic roots, reduced apical dominance and short internodes (11), some of which have a potential of agronomic application (12). In addition, the use of *A. rhizogenes* offers the opportunity of introducing foreign genes into plant genomes at the same time (13). The development of a micropropagation process using elite hairy roots would have an impact on agriculture and biochemical production of useful materials.

Somatic embryo obtained from callus has been investigated for use in the production of artificial seeds (14-16). We have extended this concept to hairy roots. To date, fragmented hairy root segments (17), and adventitious shoot primordia of hairy roots (18) of horseradish have been studied for their application in the production of artificial seeds (Fig. 5-1). To use hairy roots in artificial seed preparation, fragmentation of the roots before encapsulation is required because they elongate at the root apical meristem. In order to develop a hairy-root-based artificial seed system for commercial applications, mechanical fragmentation of the roots is advantageous.

In this study, we report that mechanical fragmentation of hairy roots was suitable for efficient production of plantlets. In addition, plant growth regulator supplementation and dehydration led to an increase in productivity of plantlets for artificial seeds.

5. 2. MATERIALS AND METHODS

Plant material and culture Horseradish (*Armoracia rusticana*) hairy roots induced by the leaf disk method with *Agrobacterium rhizogenes* A4 as described previously (19) were used in all experiments. The hairy roots were maintained by regular subculture in the dark for 3 weeks at 25 ℃ on growth-regulator-free Murashige and Skoog (MS) medium (20) supplemented with 2% (w/v) sucrose and 1% agar. Hairy roots (0.1 g fresh weight) were inoculated into 40 ml of MS medium containing 2% sucrose and various concentrations of naphthaleneacetic acid (NAA) in a 100-ml Erlenmeyer flask. These were then cultured on a gyratory shaker at 100 rpm in the dark at 25 ℃. Dry weight of the roots was measured gravimetrically after they were dried at 60 ℃ for 24 h.

For plantlet formation, 0.1 g fresh weight of the root fragments was inoculated into 60 ml of MS medium containing 2% sucrose and various concentrations of kinetin, in a 300-ml Erlenmeyer flask. The culture was maintained on a gyratory shaker (100 rpm) at 25 ℃ for 10 d under illumination (14 h/d) with a light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from fluorescent white light tubes.

The media used for all experiments were adjusted to pH 6.0 before autoclaving at 121 ℃ for 20 min.

Hairy root fragmentation with a blender Root fragments were obtained using a blender with blades (MX-30, Matsushita Electric Industrial Co., Osaka), shown diagrammatically in Fig. 5-2. Eight g fresh weight of the roots was fragmented in the blender (11,000 rpm) containing 200 ml of MS medium with 2% sucrose. Root fragments less than 100 μm in length were removed by sieving through a stainless steel mesh and discarded. The root fragments were then transferred aseptically to sterile filter paper to remove excess moisture and their fresh weight was measured. The root fragments were suspended in the medium for plantlet formation stage. The longest length of each root fragment was measured from photographs to determine size distribution of the fragments after the root fragments were resuspended in sterilized water.

Measurement of plantlet formation The plantlets formed during the culture period were separated into size categories of 0.4, 1.0, 2.0 and 4.0 mm by sieving.

The number of plantlets was counted and the dry weights of the plantlets and roots were measured gravimetrically, after drying at 60 ℃ for 24 h.

Encapsulation of plantlets The plantlets (2-4 mm in length) were suspended in MS medium containing 2% sucrose and 2% sodium alginate prepared as described previously (17). Individual plantlets in the suspension were dropped into a 100 mM calcium chloride solution using a pipette (with 5 mm tip diameter). After 10 min, encapsulated pellets were separated from the solution.

Dehydration procedure Plantlets (2-4 mm in length) were subjected to one of the following two dehydration treatments: (1) exposure for 0.2 to 3 h in an open Petri dish to an air flow (air flow rate = 0.41 m/s) in a laminar flow cabinet (CCV-1301EC, Hitachi Co., Tokyo), or (2) exposure for 0.5 to 11 d in a Petri dish sealed with Parafilm under the fluorescent light conditions at 25 ℃.**Determination of plantlet development frequency** Plantlets encapsulated in calcium alginate beads and non-encapsulated plantlets were placed on MS agar plates containing 2% sucrose under the fluorescent light conditions with illumination of 14 h/d and incubated at 25° C. The plantlet development frequency was defined as the percentage of plants with two or more leaves on the basis of the total number of plantlets encapsulated.

Scanning electron microscopy For scanning electron microscopy, the plantlets were fixed with 2% glutaraldehyde in 10 mM phosphate buffer (pH 6.0) for 2 h. After drying in a critical point dryer (HCP-2; Hitachi Co.) and sputtering with an ion sputter (E101; Hitachi Co.), micrographs were taken using a scanning electron microscope (S-570; Hitachi Co.).

5. 3. RESULTS

The procedure used for plantlet formation from horseradish hairy roots fragmented in the blender and for production of artificial seeds is shown in Fig. 5-3. First, hairy roots were cultured until the maximum weight of the hairy roots was obtained (hairy root growth stage), and then fragmented in the blender for various treatment times. The root fragments were then transferred into the liquid plantlet formation medium, and cultured for 10 d under illumination (plantlet formation stage). Finally, plantlets encapsulated in calcium alginate beads were cultured on solid medium (plantlet development stage).

Fragmentation of hairy roots using the blender Since the hairy roots elongate at the root apical meristem, root fragmentation before plantlet formation is required to obtain plantlets suitable for use as artificial seeds. Mechanical fragmentation is preferable for large-scale production of artificial seeds. In this study, hairy root fragmentation was performed using a blender. Hairy roots cultured for 35 d without addition of plant growth regulator were fragmented in the blender for 10, 20, 30 or 60 s. As the duration of fragmentation increased, the mean fragment size decreased (Fig. 5-4). The root fragments obtained after fragmentation for 10 or 20 s were 1625 μm or less in length, and those obtained after fragmentation for 30 or 60 s were 1000 μm or less in length. Approximately 54% of the root fragments (≧100 μm) on a fresh weight basis was recovered in the case of fragmentation for 30 s. After fragmentation, the root fragments were transferred into plantlet formation medium without plant growth regulator. Plantlets were formed at day 4, after which secondary roots began to elongate from the plantlets.

Table 5-1 shows the effects of root fragmentation time on plantlet formation at day 10. Under all culture conditions, the plantlets were less than 2 mm in length. Fragmentation for 30 s resulted in a slightly higher number of plantlets, compared with that for 10 s or 20 s. Fragmentation for 60 s reduced the number of plantlets formed. The number of plantlets formed from the root fragments cut manually with a razor was 1.5-fold as high as that formed from the roots which were fragmented mechanically in the blender for 30 s. However, it took approximately 10 min to cut the roots manually to achieve a degree of fragmentation comparable to that achieved by fragmentation in the blender for 30 s. Although use of the blender for fragmentation resulted in a decrease of plantlet formation frequency as compared with that in the case of manual fragmentation, use of a blender is advantageous for rapid and large-scale root fragmentation. Therefore, fragmentation for 30 s in the blender was used in subsequent experiments.

Effects of NAA supplementation in hairy root growth stage on plantlet formation

We reported previously that auxin supplementation stimulated the emergence of root apical meristems in horseradish hairy roots, and led to an increase in growth rate (21). In addition, NAA supplementation led to an increase in plantlet formation

frequency of horseradish hairy roots (17). Therefore, effects of NAA supplementation in the medium on plantlet formation were investigated (Table 5-2). After the hairy roots were cultured until the stationary phase in MS medium supplemented with various concentrations of NAA, the hairy roots fragmented by the blender for 30 s were cultured in the medium without plant growth regulator for 10 d. NAA supplementation (0.1 mg/*l* or 1.0 mg/*l*) led to an increase not only in the root growth rate but also in plantlet formation frequency compared with those of non-treated roots. The frequency of plantlet formation from NAA (1.0 mg/*l*)-treated roots was increased about 1.2-fold as compared with that of non-treated roots. However, supplementation with 5 mg/*l* of NAA led to a decrease in plantlet formation frequency. The plantlet productivity of NAA (1.0 mg/*l*)-treated roots was 79 $l^{-1}d^{-1}$ which was 1.8-fold that of non-treated roots. Hairy roots were cultured in the medium supplemented with 1.0 mg/*l* of NAA for 20 d at the hairy root growth stage, and they were used in the subsequent experiments.

Effect of kinetin supplementation on plantlet formation The effects of kinetin supplementation at the plantlet formation stage on plantlet formation were tested because kinetin stimulates plantlet formation from the hairy roots. Root fragments were cultured in the medium supplemented with kinetin for 10 d (Table 5-3). Kinetin supplementation from 0.01 to 1.0 mg/*l* led to an increase in the number of plantlets as compared to that of root fragments cultured without kinetin supplementation. The highest plantlet formation frequency was achieved in the medium supplemented with 0.1 mg/*l* of kinetin. On the other hand, supplementation of 5 mg/*l* of kinetin resulted in inhibition of plantlet formation and supplementation of 10 mg/*l* of kinetin caused callus formation (data not shown).

Kinetin supplementation also resulted in an increase in plantlet size. Size distributions of plantlets grown in the medium without kinetin and with 0.01 mg/*l* of kinetin were similar (0.4-2 mm). Supplementation with higher concentrations of kinetin (0.1-5 mg/*l*) resulted in a broader size distribution (0.4-10 mm). The optimal plantlet size for encapsulation is considered to be 2-4 mm, and control of plantlet size by adjusting the kinetin concentration is necessary for efficient production of artificial seeds. Moreover, since plantlets with multiple shoots do not develop into healthy plants, plantlets with single shoots are suitable for production of artificial seeds.

Supplementation of 0.1 mg/*l* of kinetin resulted in the largest number of plantlets 2-4 mm in size (Table 5-3), and 91 % of plantlets (2-4 mm) had single shoots (Table 5-1).

Figure 5-4 shows morphological changes during plantlet development stage from the root fragment to plantlet in the case of supplementation with 0.1 mg/*l* of kinetin. The presence of bud primordium was observed in root fragment in the blender (Fig. 5-5A). After 4 d of culture, development of two leaves from bud primordium was observed and chlorophyll was detected (Fig. 5-5B). At 6 d of culture, more extensive leaf development from bud primordium was observed (Fig. 5-5C). The photograph of healthy plantlets at 10 d of culture was shown in Fig. 5-6A.

Root elongation was suppressed during plantlet formation stage. Inhibition of root elongation by kinetin supplementation is advantageous for handling and encapsulation of the plantlets. Percentage of leaves and shoots of plantlets which were cultured without kinetin supplementation was 11.7 %, whereas that cultured with kinetin supplementation of more than 0.1 mg/*l* was above 85.5 % (Table 5-3).

On the basis of the above results, the hairy roots should be cultured with 1.0 mg/*l* of NAA before fragmentation, and the fragmented roots should be grown in the medium supplemented with 0.1 mg/*l* of kinetin to achieve optimal plantlet formation frequency. In Table 5-1, we presented data indicating the optimal fragmentation time to obtain plantlets under the conditions of 1.0 mg/*l* of NAA and 0.1 mg/*l* of kinetin supplementation. Fragmentation for 30 s resulted in formation of the highest number of plantlets 2-4 mm in size which had a single shoot, compared with that for 10, 20 or 60 s.

Effect of plantlet dehydration on frequency of plantlet development

Frequency of plantlet development from encapsulated plantlets in size of 2-4 mm (Fig. 5-6B) is shown in Fig. 5-7. At the plantlet development stage, roots emerged, and two new leaves appeared. Frequency of plantlet development from encapsulated plantlets was only 10% at 15 d culture and 58% at 45 d. On the other hand, non-encapsulated plantlets developed into healthy plants at high frequency (92% at 30 d). These results indicated that encapsulation resulted in a decrease in plantlet development frequency. When the plants were observed under a microscope, leaves were found to be water-soaked, translucent and glassy. These abnormal morphologies are characteristic of hyperhydricity (22). We considered that the inhibition of plantlet development from

encapsulated plantlets was due to prevention of dehydration after encapsulation.

Plantlets dehydrated by exposure to an air flow in a laminar flow cabinet were encapsulated and their plantlet development frequency was measured (Table 5-4). After 3 h of dehydration in the air flow, the plantlet weight had decreased by 81% compared with the initial weight. Dehydration treatment improved plantlet development frequency in the case of a weight decrease to 35% or less of the initial weight. Dehydration treatment of longer than 2 h caused serious damage to the plantlets. Following dehydration treatment for 5 h, most plantlets had turned brown and did not develop into healthy plants (data not shown). Optimal duration of dehydration treatment seems to be 1 h, since 74% of encapsulated plantlets had developed into healthy plants at 30 d of culture.

The dehydration treatment was also performed for plantlets cultured in sealed Petri dishes under light conditions (Table 5-5). When plantlets were subjected to the dehydration treatment of 7 d (23 % decrease in weight), plantlet development frequency at 15 d culture reached the highest level (98%) and was higher than that of the plantlets subjected to the dehydration treatment in the laminar flow cabinet. Slow dehydration was suitable for achieving high plantlet development frequency. In summary, the optimal conditions determined in this study are shown in parentheses in Fig. 5-3.

5. 4. DISCUSSION

In this study, production of plantlets derived from horseradish hairy roots fragmented in the blender was examined for application to production of artificial seeds. Since the hairy roots elongate at the root apical meristem, fragmentation of the roots has to be done. Use of the blender for fragmentation decreased the plantlet development frequency as compared with manual fragmentation with a razor (Table 5-1). Use of a razor allows proper cutting of roots into slices, whereas fragmentation in the blender results in random cutting, which inevitably leads to a decrease in plantlet development frequency. However, from the point of view of fragmentation time and large-scale treatment, use of the blender for fragmentation has significant advantages.

Since plantlets with multiple shoots do not develop into healthy plants, the optimal fragmentation time for obtaining root fragments with the potential to develop

into single shoot plantlets must be determined. In the present study, fragmentation for 30 s was found to be optimal for production of the highest number of single shoot plantlets.

Plantlet development through somatic embryogenesis and adventitious bud formation can be controlled by supplementation of auxin and/or cytokinin (23, 24). In the present study, NAA supplementation of 1.0 mg/*l* in the root growth medium promoted hairy root growth and plantlet formation (Table 5-2). This improvement is considered to be due to an increase of branching number because the branching number is correlated with shoot number as previously reported (17). Saitou *et al.* reported that the number of plantlets increased on MS medium with NAA supplementation under light conditions (25). Although the plantlet formation medium in the present study was not supplemented with NAA, residual NAA in the roots might have caused a promotive effect on plantlet formation. Kinetin addition in the plantlet formation stage promoted plantlet formation and led to an increase in dry weight of plantlets and inhibition of root elongation (Table 5-3). The size of the plantlets could be controlled by kinetin concentration in the medium. Such control is very important in the production of artificial seeds because the optimal plantlet size for encapsulation is considered to be 2-4 mm in length.

The non-encapsulated plantlets exhibited a higher plantlet development frequency as compared with encapsulated plantlets. Water content of the non-encapsulated plantlets seemed to decrease more rapidly during the culture on the agar plate than that of the encapsulated plantlets. It has been reported that dehydration of somatic embryos could recover germination ability (26). From these results, we concluded that dehydration treatment can strongly improve plantlet development frequency (Tables 5-4 and 5-5). In particular, dehydration treatment of 7 d before encapsulation promoted the plantlet development as shown in Table 5-5, which indicates that this dehydration treatment can be used to shorten the plantlet development duration. Plantlets subjected to rapid dehydration treatment in a laminar flow cabinet exhibited lower plantlet development frequency than those with slow dehydration treatment. The rapid dehydration procedure may be too severe for plantlets.

In the previous study, we proposed use of root fragments encapsulated in beads as hairy-root-based artificial seeds (17). For artificial seeds planted in a field, the duration

of plantlet development should be as short as possible to decrease the risk of contamination by microorganisms and efflux of nutrients from the capsule. Use of encapsulated plantlets as artificial seeds can shorten the time required for development into healthy plants, and the number of plantlets which develop into healthy plants can be increased by dehydration treatment, as compared with direct development from the root fragments.

TABLE 5–1. Effects of fragmentation using the blender or a razor on plantlet formation

NAA concentration [a] [mg/l]	Kinetin concentration [b] [mg/l]		Number of plantlets at 10 d [f] [l^{-1}]				
			Size of plantlet [mm]				Total
			0.4-1.0	1.0-2.0	2.0-4.0	4.0-10	
		with a razor [c] root length [mm]					
		≦1	600	2400	0	0	3000
		in a blender [d] fragmentation time [s]					
0	0	10	580	1370	0	0	1950
		20	830	1150	0	0	1980
		30	770	1230	0	0	2000
		60	230	210	0	0	440
		in a blender [d] fragmentation time [s]					
1.0	0.1	10	1040	1270	1270 (1100) [e]	710 (310)	4290
		20	1930	1800	1540 (1390)	460 (280)	5730
		30	2170	2150	1750 (1600)	520 (340)	6590
		60	2190	1900	1000 (980)	460 (430)	5550

[a] NAA concentration added into the medium at hairy root growth stage.

[b] Kinetin concentration added to the medium at plantlet formation stage.

[c] The hairy roots were placed on a scaled glass slide with a scale, and then manually cut with a razor.

[d] The hairy roots were fragmented in the blender as described in Materials and Methods.

[e] Figures in parentheses are the number of plantlets which have a single shoot in the total number of plantlets.

[f] Numbers of plantlets represent the means of duplicate experiments.

TABLE 5-2. Effects of NAA supplementation at the root growth stage on plantlet formation

NAA concentration [mg/l]	Culture time [a] [d]	Dry weight [b] [g/l]	Number of plantlets [c] [l^{-1}]			Productivity [d] [$l^{-1} \cdot d^{-1}$]
			Size of plantlet [mm]		Total	
			0.4-1.0	1.0-2.0		
0	35	8.5	770	1230	2000	44
0.1	25	9.2	630	1630	2260	65
1.0	20	10.9	1310	1060	2370	79
5.0	25	10.0	420	450	870	25

[a] Culture time when the maximum weight of the hairy roots was attained at the root growth stage.

[b] Maximum weight of hairy roots at the root growth stage.

[c] Number of plantlets represent the means of duplicate experiments.

[d] Productivity=number of plantlets formed/culture time (root growth stage +plantlet formation stage).

TABLE 5-3. Effects of kinetin supplementation at plantlet formation stage on plantlet formation

Kinetin concentration [mg//]	Number of plantlets [a] [$/^{-1}$]						Dry weight [a, b] [g//]		Percentage of leaves and shoots [c] [%]
	Size of plantlet [mm]					Total	Leaves and shoots	Roots	
	0.4-1.0	1.0-2.0	2.0-4.0	4.0-10	>10				
0	1310	1060	0	0	0	2370	0.14	1.06	11.7
0.01	1980	1630	0	0	0	3610	0.36	0.98	26.9
0.1	1630	1630	1780	600	0	5640	3.54	0.60	85.5
1	270	710	740	710	320	2750	4.30	0.15	96.6
5	130	680	630	240	0	1680	1.65	0.05	97.1

[a] Number of plantlets and dry weights represent the means of duplicate experiments.

[b] Dry weight of plantlets or hairy roots obtained after culture of the root fragments for 10 d in the plantlet formation medium with various concentrations of kinetin.

[c] Percentage of leaves and shoots=dry weight of leaves and shoots obtained by culture for 10 d/total dry weight measured after culture for 10 d $\times$ 100

TABLE 5-4. Weight decrease of plantlets and plant development frequency, as function of the dehydration duration (DD) with air flow in a laminar flow cabinet and culture time

DD [h]	Weight decrease [a] [%]	Plantlet development frequency [c] [%]	
		Culture time [b] [d]	
		15	30
0	0	10	52
0.2	14	12	56
0.5	23	16	58
1	35	28	74
2	62	28	50
3	81	4	6

[a] Weight decrease=(1– weight of plantlets after dehydration / initial weight of plantlets)×100

[b] Culture time of encapsulated plantlets on the solid medium under light conditions.

[c] Fifty encapsulated plantlets were used for each experiment.

TABLE 5-5. Weight decrease of plantlets and plant development frequency, as functions of the dehydration duration (DD) in a sealed Petri-dish and culture time

DD [d]	Weight decrease [a] [%]	Plantlet development frequency [c] [%]	
		Culture time [b] [d]	
		15	30
0	0	10	52
0.5	8	28	66
1	14	62	92
2	19	88	98
7	23	98	98
11	25	94	96

[a] Weight decrease=(1– weight of plantlets after dehydration / initial weight of plantlets)×100

[b] Culture time of encapsulated plantlets on the solid medium under light conditions.

[c] Fifty encapsulated plantlets were used for each experiment.

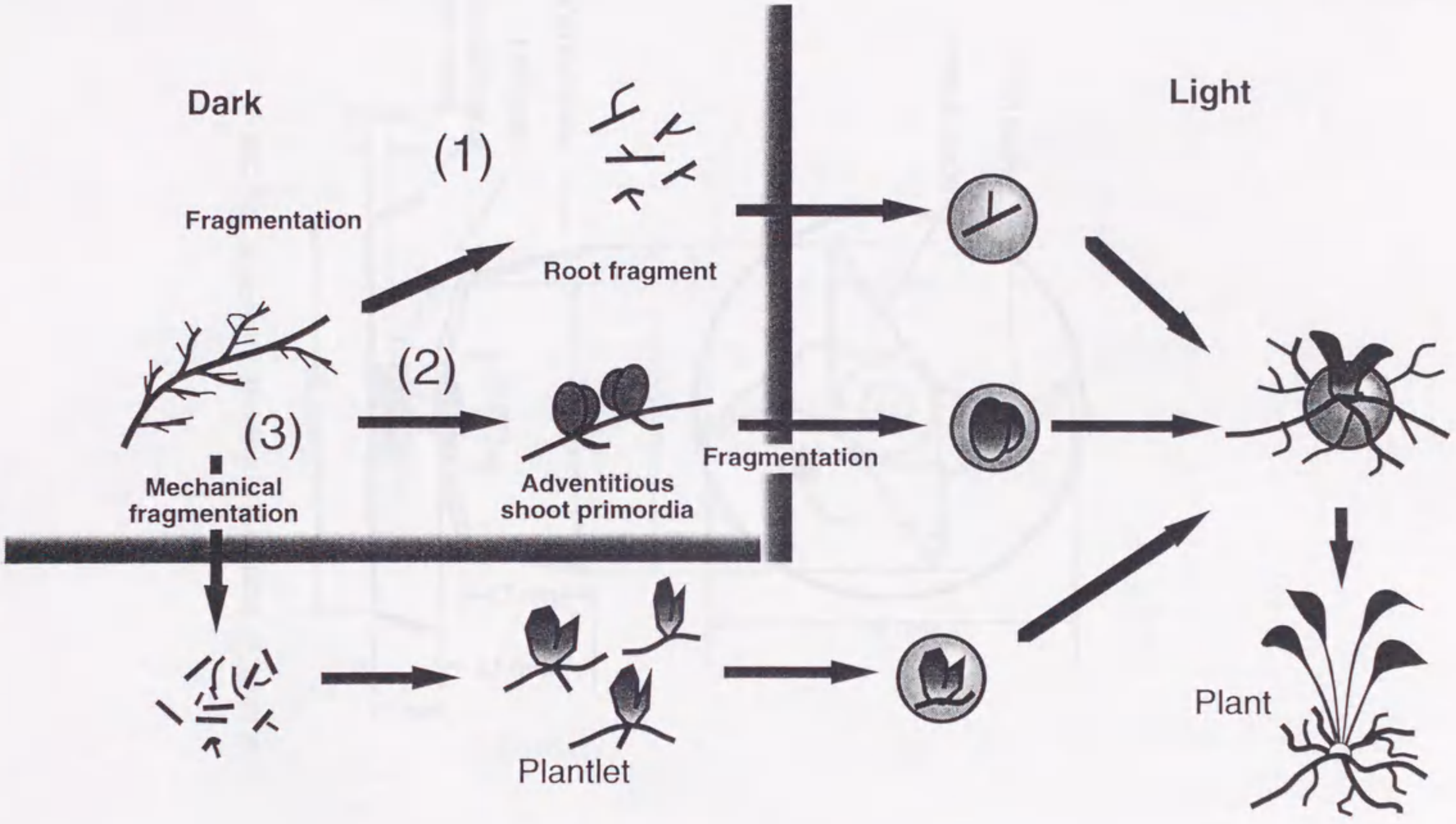

FIG. 5-1. The culture methods for artificial seed production using hairy roots developed in our laboratory.

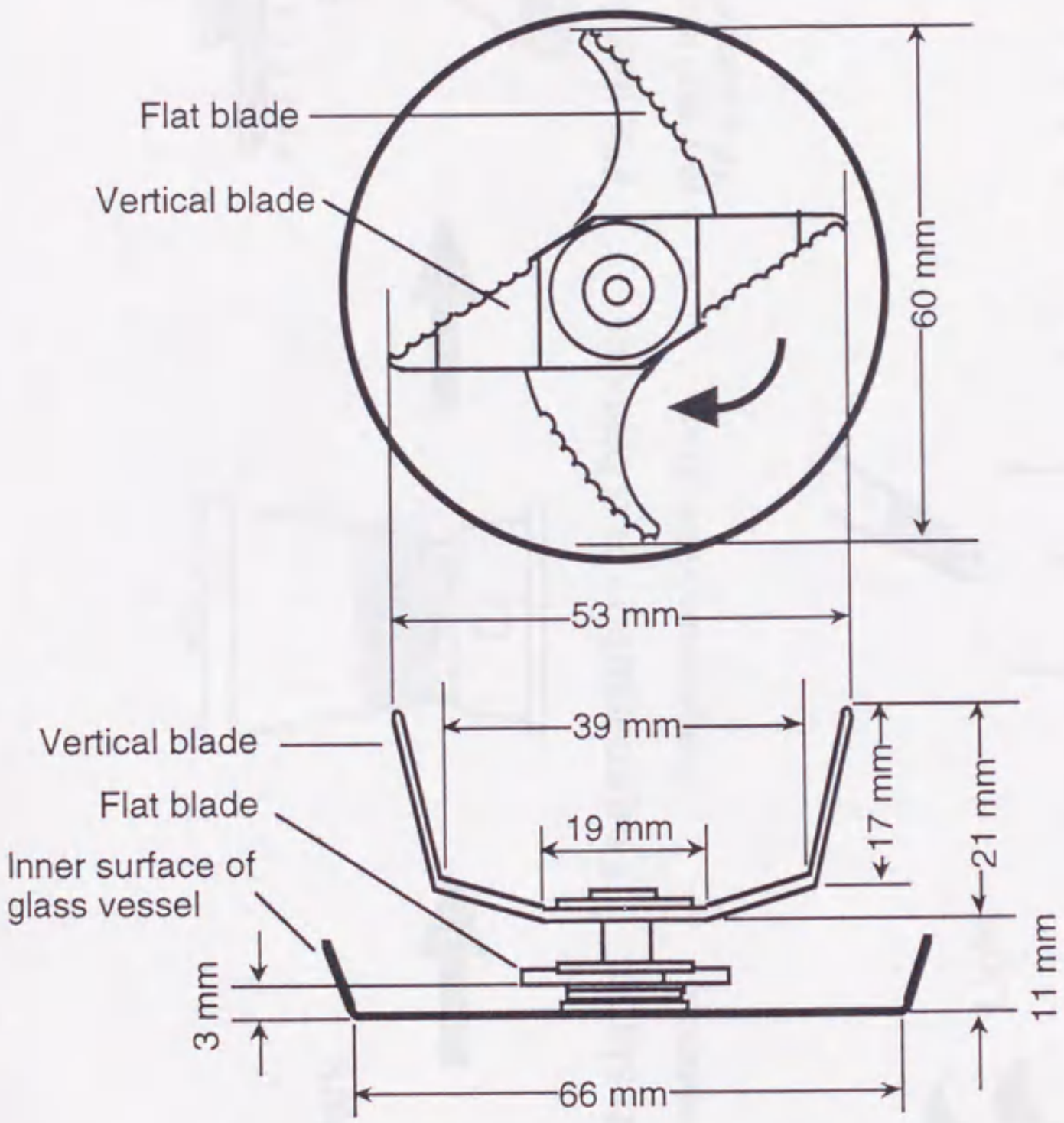

FIG. 5-2. Schematic drawing of the blades of the blender.

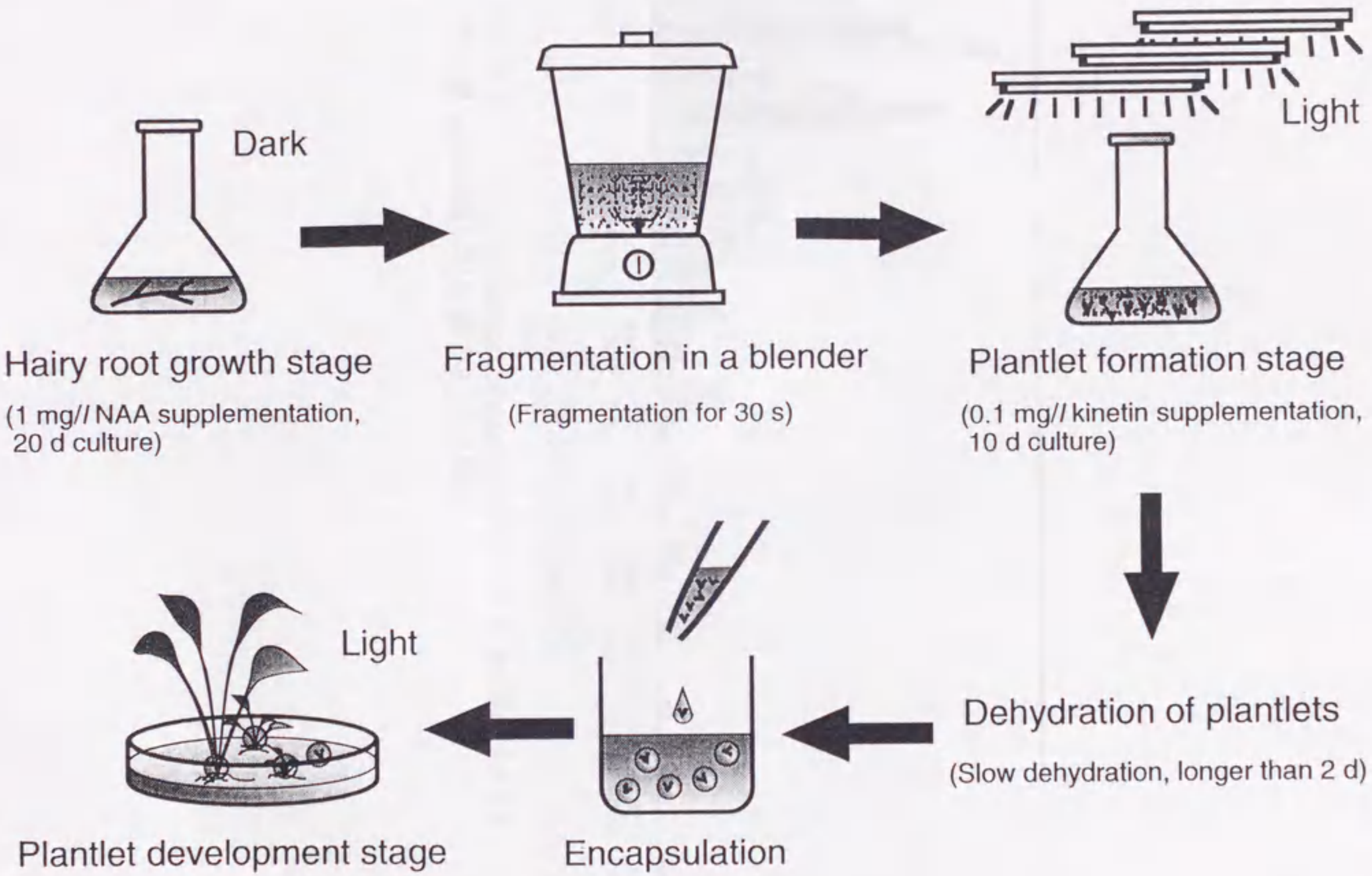

FIG. 5-3. Artificial seed production system using plantlets derived from horseradish hairy roots fragmented in a blender. Optimal conditions determined in this study are enclosed in parenthesis.

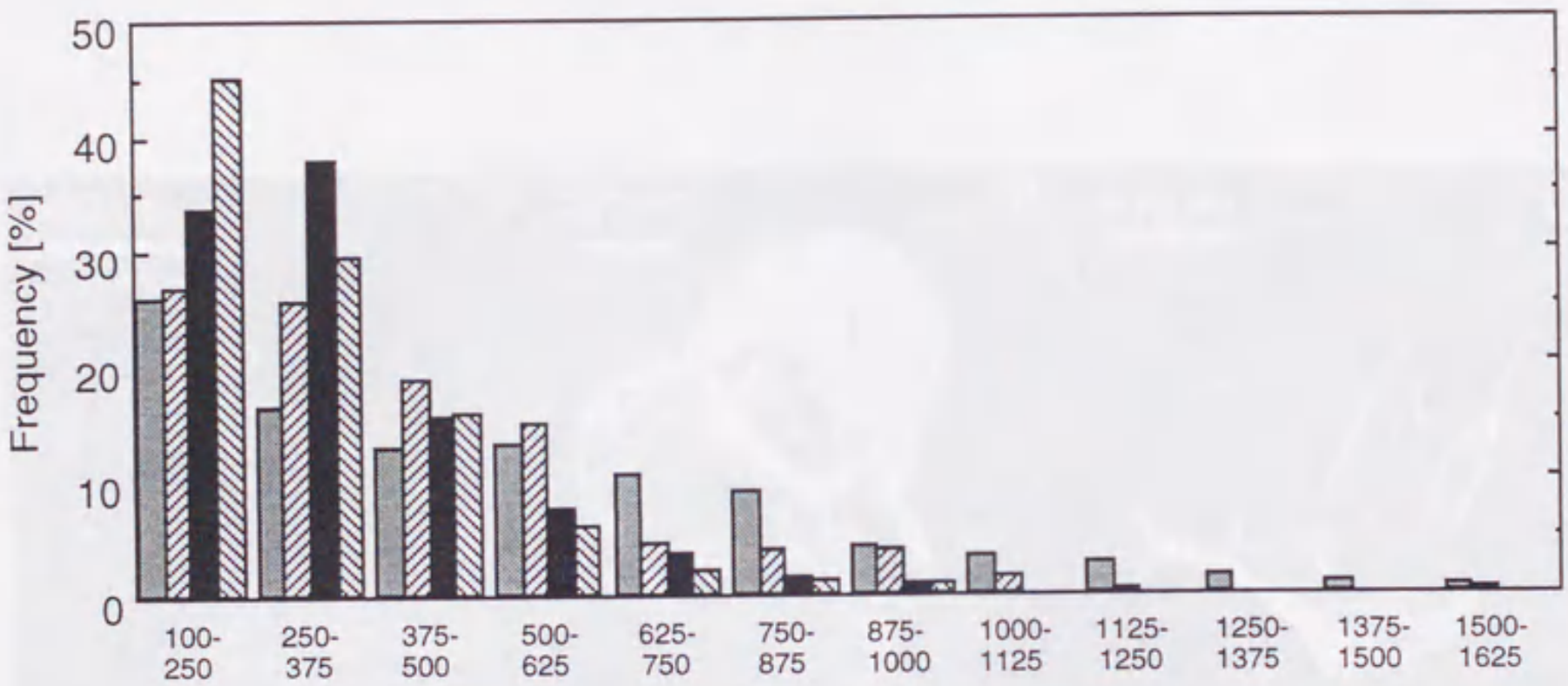

FIG. 5–4. Size distribution of roots fragmented in the blender for 10 s (▒), 20 s (▨), 30 s (■) or 60 s (▧)

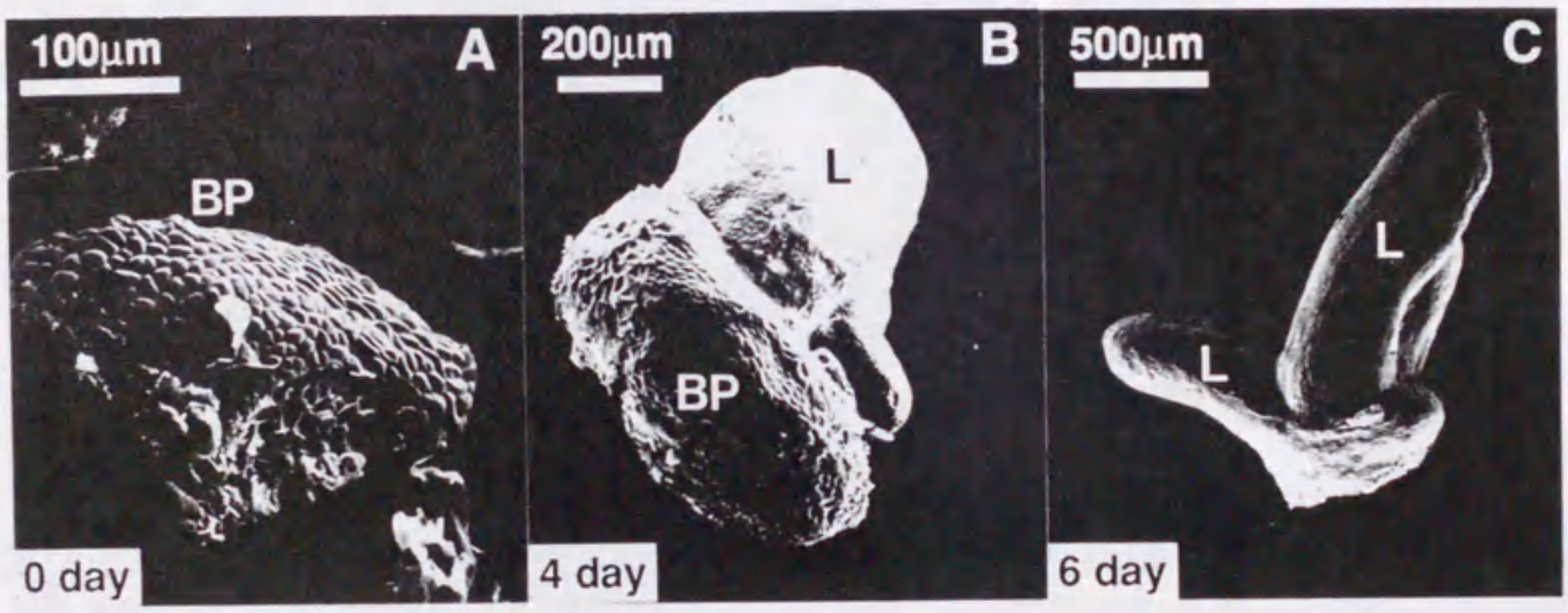

FIG. 5-5. Scanning electron micrographs of plantlet development from root fragment cultured in MS medium supplemented with 0.1 mg/*l* of kinetin at 0 d (A), 4 d (B) and 6 d (C). BP and L in micrographs represent bud primordium and leaf formed from bud primordium, respectively.

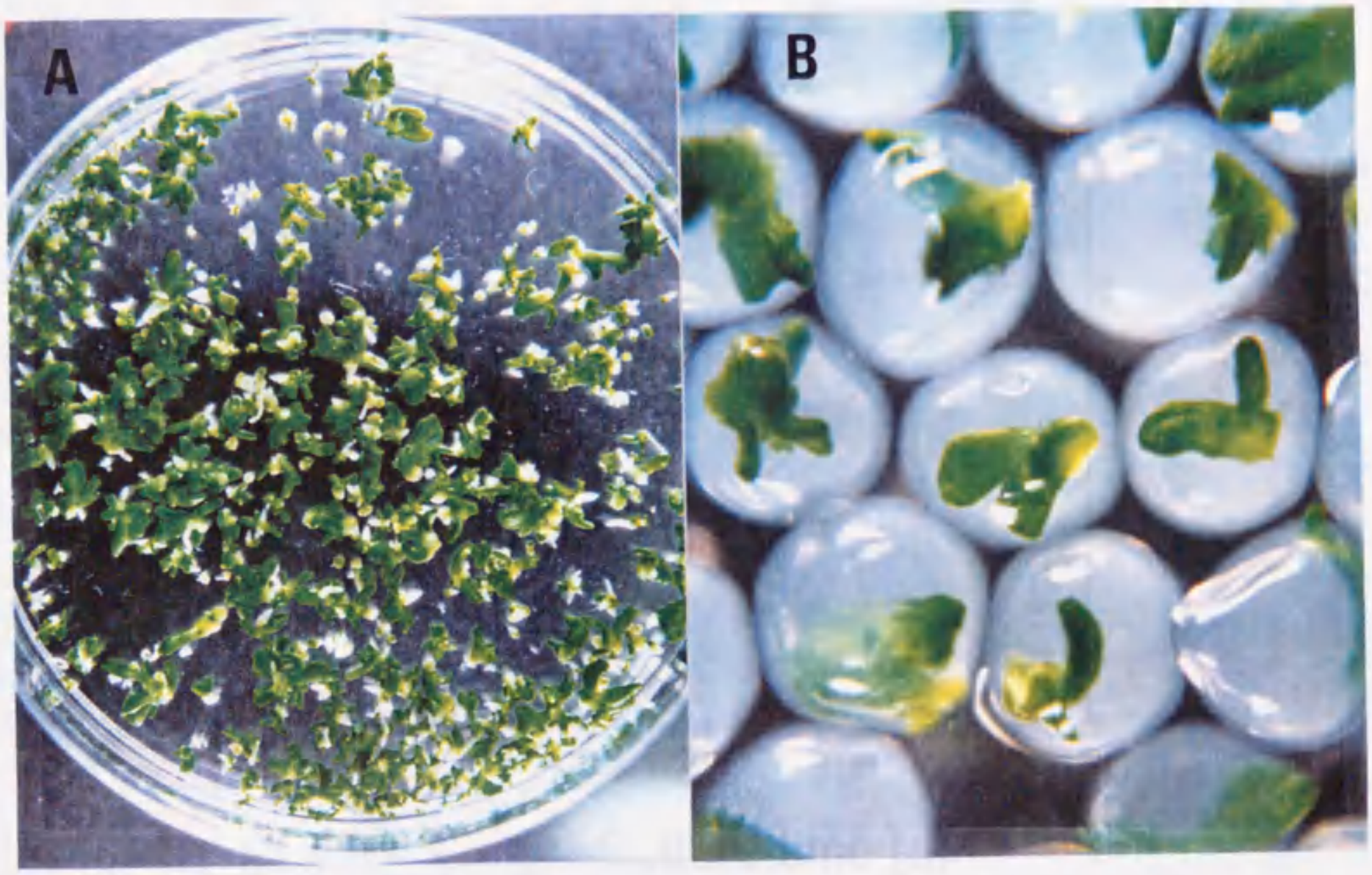

FIG. 5-6. The photographs of plantlets (A) from root fragment cultured in MS medium supplemented with 0.1 mg/*l* of kinetin at 10 d of culture, and plantlets encapsulated with 2 % of calcium alginate gel (B).

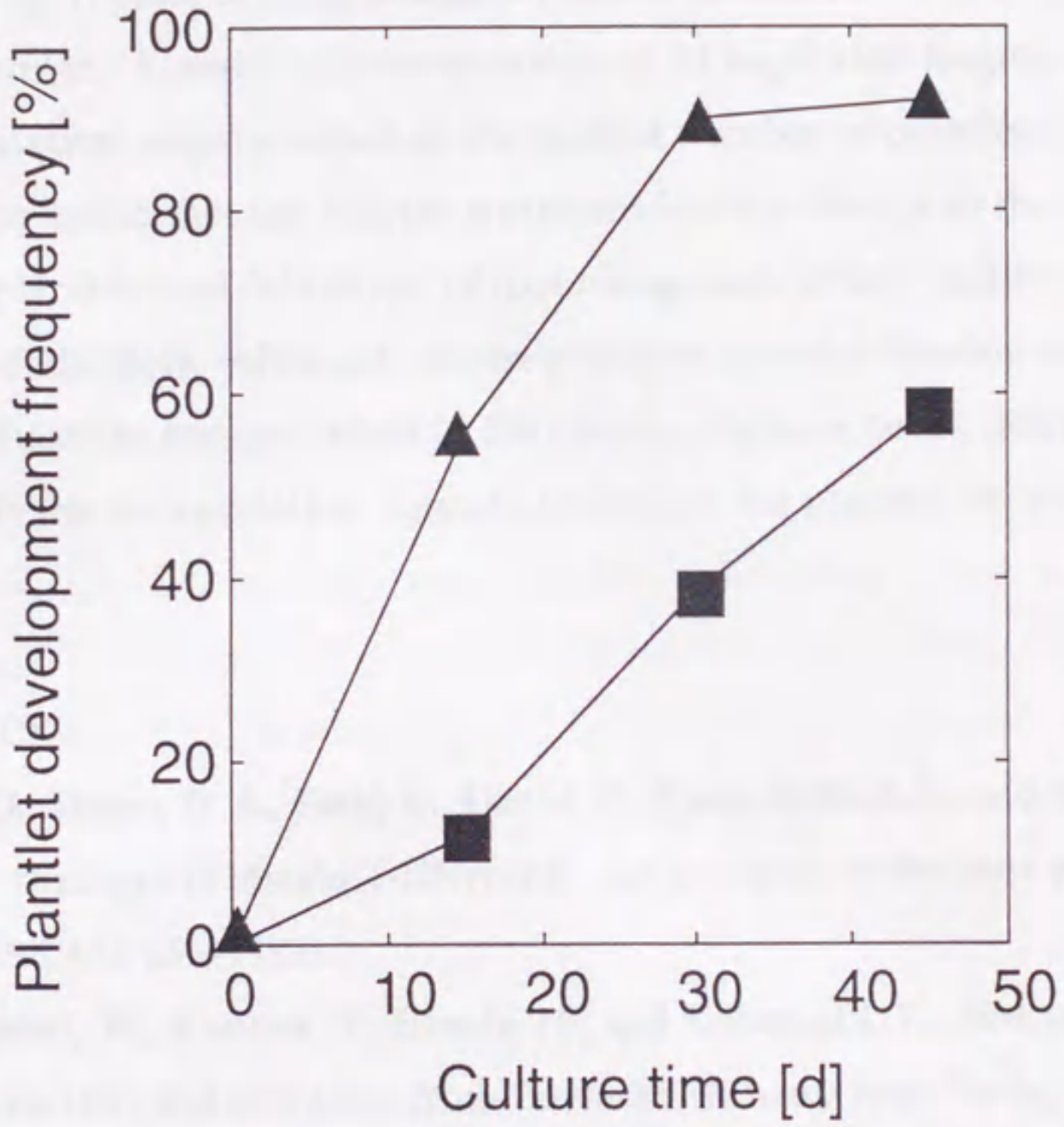

FIG. 5-7. Effect of encapsulation on plantlet development. Fifty encapsulated or non-encapsulated plantlets were used for each experiment. Symbols: (▲) non-encapsulated plantlets, (■) plantlets encapsulated in 2% calcium alginate beads.

5. 6. SUMMARY

We tested mechanical fragmentation of hairy roots suitable for efficient production of plantlets, plant growth regulator supplementation to enhance plantlet formation and dehydration to increase the plantlet development frequency from encapsulated plantlets. Hairy root fragments were obtained by fragmentation of hairy roots in a blender after the root growth. A fragmentation time of 30 s gave the best result on the plantlet formation. Treatment of the hairy roots with naphthaleneacetic acid (NAA, 1.0 mg/*l*) prior to fragmentation resulted in a decrease in the time required for hairy root growth. Kinetin supplementation of 0.1 mg/*l* after fragmentation (in the plantlet formation stage) resulted in the highest number of plantlets. Furthermore, we found that the kinetin treatment led to a change in the size distribution of plantlets and inhibition of root elongation, which facilitated encapsulation of plantlets. Although the frequency of plantlet development was significantly reduced by encapsulation in 2% calcium alginate beads, dehydration treatment before the encapsulation strongly improved the plantlet development frequency.

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CHAPTER 6

Culture Methods for Mass Production of Plantlets for Use as Artificial Seeds from Mechanically Fragmented Horseradish Hairy Roots

6.1. INTRODUCTION

We previously reported that mechanical fragmentation of hairy roots using a blender was suitable for production of plantlets derived from horseradish hairy roots (1). High density culture of hairy roots and plantlets is needed to obtain large amount of artificial seed. We have already developed a suitable bioreactor system for high density culture of hairy roots (2). However, a efficient culture method for mass production of plantlets for use as artificial seed was not developed. Therefore, efficient culture methods using plant growth regulator and nutrient's feeding were investigated in the case of high density culture of plantlets by the increase in initial density of mechanically fragmented roots in this chapter.

6.2. MATERIALS AND METHODS

Horseradish *(Armoracia rusticana)* hairy roots induced by the leaf disk method with *Agrobacterium rhizogenes* A4 as described previously (2) were used in all experiments. The hairy roots were maintained by regular subculture in the dark for 3 weeks at 25 ℃ on growth-regulator-free Murashige and Skoog (MS) medium (3) supplemented with 2% (w/v) sucrose and 1% agar. Hairy roots (0.1 g fresh weight) were inoculated into 40 ml of MS medium containing 2% sucrose and 1 mg/*l* of concentrations of naphthaleneacetic acid (NAA) in a 100-ml Erlenmeyer flask. These were then cultured on a gyratory shaker at 100 rpm in the dark at 25 ℃ for 20 d. Harvested roots were fragmented for 30 s using a blender with blades as described previously (1).

For plantlet formation, 0.1 and 0.8 g fresh weight of the root fragments were inoculated into 60 ml of MS medium containing 2% sucrose and various concentrations of kinetin in a 300-ml Erlenmeyer flask. The culture was maintained on a gyratory shaker (100 rpm) at 25 ℃ under illumination (14 h/d) with a light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from fluorescent white light tubes. Dry weight of the

plantlets and roots were measured gravimetrically after they were dried at 60 ℃ for 24 h. On fed-batch culture of nutrients, dry weight of the culture was estimated and feeding of the nutrients was controlled using the decrease in conductivity of the medium as described previously (4). The number of plantlets was counted and the dry weights of the plantlets and roots were measured by the method shown in previous paper (1). The media used for all experiments were adjusted to pH 6.0 before autoclaving at 121 ℃ for 20 min.

Sucrose, glucose and fructose concentrations in the medium were assayed by a high performance liquid chromatography (HPLC) as described previously (2). Kinetin concentration was also determined by the HPLC (Tri Rotor-V; JASCO, Tokyo) with a F-411A column (Shodex, Tokyo) and UV/VIS detector (870-UV; JASCO, Tokyo) at a wavelength of 274 nm. The solvent system used was 25 % methanol solution with a flow rate of 1.0ml/min.

6. 3. RESULTS AND DISCUSSION

In previous paper, we considered above 0.4 mm of adventitious shoots as plantlets. However, since development frequency of 0.4-1 mm shoots was very low, number of above 1 mm of adventitious shoots was measured as plantlets in this research.

Figure 6-1 shows time courses of plantlet formation under the optimum condition (initial density ; 1.7 g-f.w./*l*, kinetin concentration ; 0.1 mg/*l*) decided previously (1). Sucrose was hydrolyzed to glucose and fructose during the culture, and glucose was consumed faster than fructose. All sugars were consumed at 20 d of culture. Kinetin in the medium was consumed at 8 d of culture. After plantlets (1-2 mm) were formed at 4 d, the plantlets grew to above 2 mm of plantlets. Dry weight of plantlets simply increased after 6 d of culture. Although plantlets with roots began to increased after 10 d of culture, most of plantlets were without root elongation. Root elongation from plantlet is disadvantageous for handling and encapsulation of the plantlets. The highest number (4400 l^{-1}) of plantlets without roots available to use as artificial seed was obtained at 9 d of culture.

Next, initial density of the fragmented roots was increased to 13.3 g-f.w./*l*, expecting the increase in the number of plantlets. Figure 6-2 shows time courses of the

culture when initial density of fragmented roots and initial kinetin concentration were 13.3 g/*l* and 0.1 mg/*l*, respectively. Sugar was consumed at 15 d of culture. Kinetin in the medium was consumed at 4 . Dry weight of plantlets began to increase after 4 d, and dry weight of roots rapidly increased after 8 d of the culture. The total number of plantlets simply increased and 27300 l^{-1} of plantlets was formed at 15 d. However, the number of plantlets with roots dramatically increased after 8 d, and 35 % (9400 l^{-1}) of total plantlet at 15 d was without root elongation.

We reported that the increase in kinetin concentration in medium led to the increase in the number of plantlets, expansion of the plantlets and reduce the root elongation from plantlets in previous paper (1). Therefore, kinetin concentration was also increased to 0.8 mg/*l* in proportion to the increase to 13.3 g/*l* of the initial density of the fragmented roots. Time courses of the culture when initial density of fragmented roots and initial kinetin concentration were 13.3 g/*l* and 0.8 mg/*l* , respectively were shown in Fig. 6-3. All sugars were consumed at 15 d. Kinetin in the medium consumed at 6 d of culture. Dry weight of plantlets increased dominantly. Roots hardly grew during the culture periods. Formation of plantlet (1-2 mm) reached to plateau at 6 d, and the total number of plantlets (2-4 mm) began to increase at 5 d of culture. At 15d, 16500 l^{-1} of total plantlets was harvested. High concentration of kinetin might give toxic effect to the plant cells. Since root elongation from plantlets was reduced, 91 % (15000 l^{-1}) of the plantlets at 15 d was without roots .

To test effects of initial density of the roots on plantlet formation, when kinetin concentration was increased in proportion to the increase in initial density of the fragmented roots, plantlet formation was investigated (Table 6-1). The number of plantlets increased as the initial density of the roots increased. Since the increase in the number of plantlets between the inoculation of 13.3 and 17.6 g/*l* of the roots was small, the critical initial density of the roots would be 13.3 g/*l*. As the initial density of the roots increased, the mean plantlet size decreased.

Increase in initial density of the fragmented roots might cause the consumption of the nutrients during early phase of culture by which plantlet formation might inhibited. Therefore, fed-batch culture was done to test effect of nutrients on plantlet formation in the case of the initial culture condition of 13.3 mg/*l* of the roots initial density and 0.8 mg/*l* of kinetin. The contents of ammonium, nitrate and phosphate

ion in MS medium to adjust cell yield of sugar was modified (Table 6-2), and all nutrients contained in the modified MS medium and sugars were fed to keep the initial value using the decrease in electric conductivity of the medium as described previously (4). The results are shown in Fig. 6-4. The contents of ammonium, nitrate and phosphate ion in the modified MS medium and sugar kept the initial value. The fed-batch culture was finished at 12 d. The number of the plantlets and size distribution at 12 d are shown in Table 6-3 with other results under culture conditions tested in this chapter. The fed-batch culture didn't give the positive effect on the increase in the number of the plantlets, but contributed to the increase in the number of plantlets in size of 2-4 mm.

High concentration of kinetin might give toxic effect to the plant cells. Therefore, when 13.3 g-f.w./*l* of fragmented roots were inoculated, 0.1 mg/*l* of kinetin was supplemented into the medium at first day of culture, and then kinetin was added into the medium every day from 4 d to 10 d until final concentration of kinetin in the medium reached to 0.1 mg/*l*. Sum of the added kinetin was 0.8 mg/*l*. Figure 6-5 shows time courses of plantlet formation under fed batch culture of kinetin. All sugars were consumed at 12 d of culture. The total number of plantlets (18730 l^{-1}) at 12 d increased compared with that when 0.8 mg/*l* of kinetin was supplemented at initial day of the culture. Roots began to elongate from plantlets at 8 d of culture, which is faster than that in the culture condition of 13.3 g/*l*-initial density of roots and 0.8 mg/*l* of kinetin. The highest number of plantlets without root elongation was obtained at 12 d of culture (15300l^{-1}). The number of plantlets in size of 2-4 mm at 12 d was 7750 l^{-1}, which was the highest number of plantlets (2-4 mm) in the culture conditions tested in this study, and 6270 $l^{-1}$of the roots was without roots available to use as artificial seed. Plantlets in size of 2-4 mm obtained in the culture for 12 d were dehydrated in sealed Petri dishes under light conditions for 7 d and encapsulated with calcium alginate gel as described previously. Plantlet development frequency from encapsulated plantlets at 15 d culture was 93 % and the plantlets would be available to artificial seed.

TABLE 6-1. Effect of Initial density on plantlet formation

Initial density of the roots [g-f.w. /l]	Kinetin concentration [mg/l]	Number of plantlets [l^{-1}]				
		Size of adventitious shoot [mm]				Total
		0.4-1.0	1.0-2.0	2.0-4.0	4.0-10	
1.7	0.1	1,630	1,630	1,780	600	5,640
5.0	0.3	2,200	4,400	2,560	490	9,650
8.3	0.5	3,980	4,850	2,800	430	12,060
13.3	0.8	7,080	10,080	3,180	10	20,360
16.7	1.0	8,080	9,100	3,320	0	20,500

TABLE 6-2. Content of modified MS medium

	Cell yield [mg/g-dry weight]	Content in MS medium [mg/*l*]	X_{max} [g-dry weight/*l*]	Content in modified MS medium [mg/*l*]
NO_3^+	176	2440	13.9	1,920
NH_4^-	35.9	370	10.3	390
PO_4^{3-}	32.2	120	3.7	350
Sucrose	1.83	20,000	10.9	20,000

Table 6-3. Comparisons of plantlet formation at 12 d under various culture conditions

Culture condition	Number of plantlets [l^{-1}]			
	Size of plantlets [mm]			Total
	1-2	2-4	>4	
Batch (ID[a] 1.7, Kin[b] 0.1)	2230	1570	850	4650
Batch (ID 13.3, Kin 0.1)	17900	3390	60	21350
Batch (ID 13.3, Kin 0.8)	9360	4640	1030	15030
Fed-batch of nutrients (ID 13.3, Kin 0.8)	7620	6780	380	14780
Fed-batch controlled at the concentration of Kin 0.1mg/l [c] (ID 13.3, Kin 0.1)	7030	7750	3950	18730

[a] Initial density of the fragmented roots, g-f.w./*l*.
b Initial concentration of kinetin in the medium, mg/*l*.
c Sam of kinetin fed into the medium was 0.8 mg/*l*.

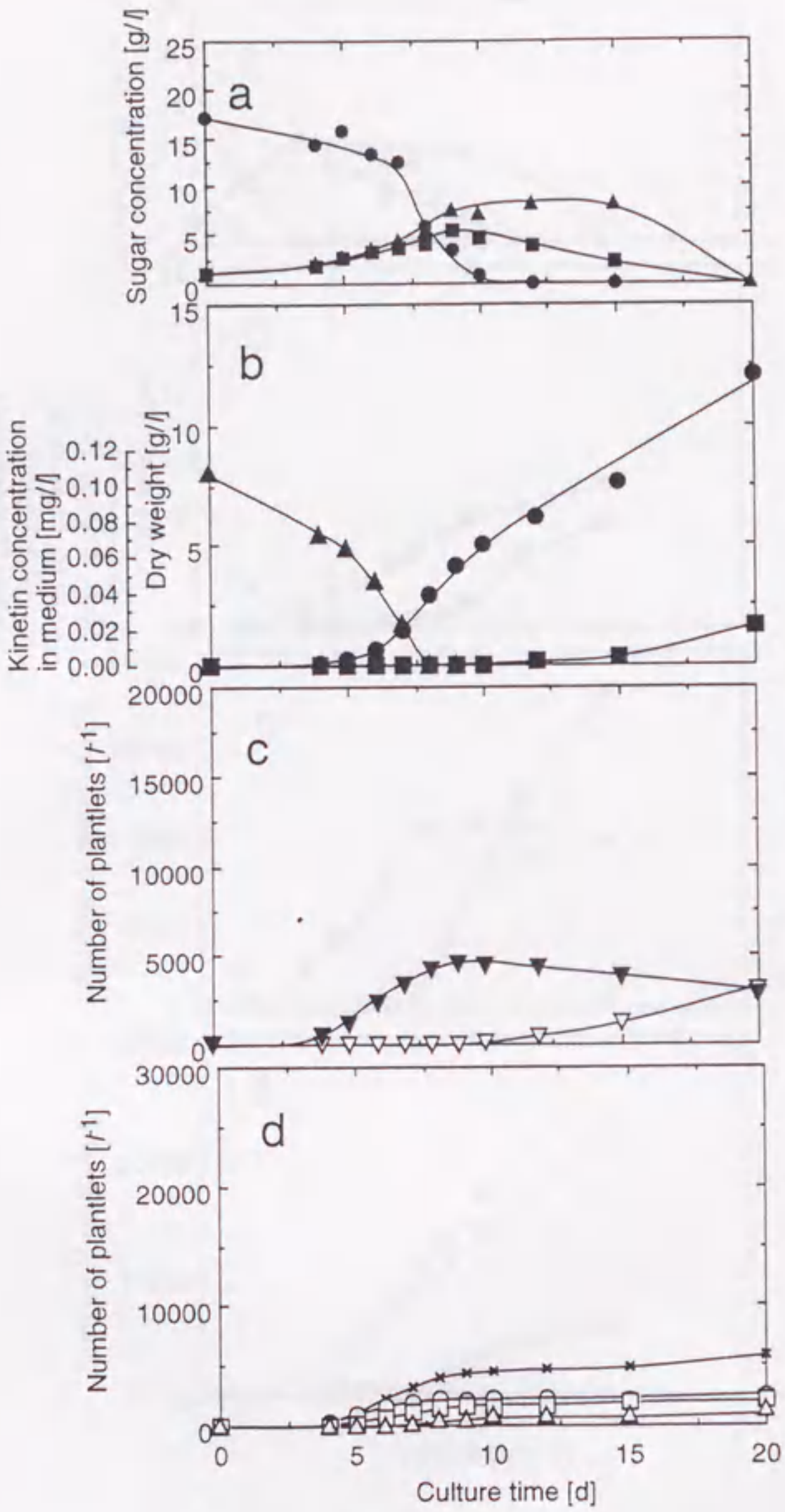

FIG. 6-1. Time courses of plantlet formation, sugar consumption and dry weight of biomass under the culture condition of combinations between 1.7 g-f.w./*l* of initial density of the roots and 0.1 mg/*l* of kinetin concentration in media. Symbols; a) Sucrose (●), glucose (■) and fructose (▲), b) Dry weight of plantlets (●) roots (■) and kinetin concentration (▲), c) The number of plantlets with roots (▽) and without roots (▼), d) Number of plantlets in size of 1-2 (○), 2-4 (□), above 4 mm (△), and total number of plantlets (×).

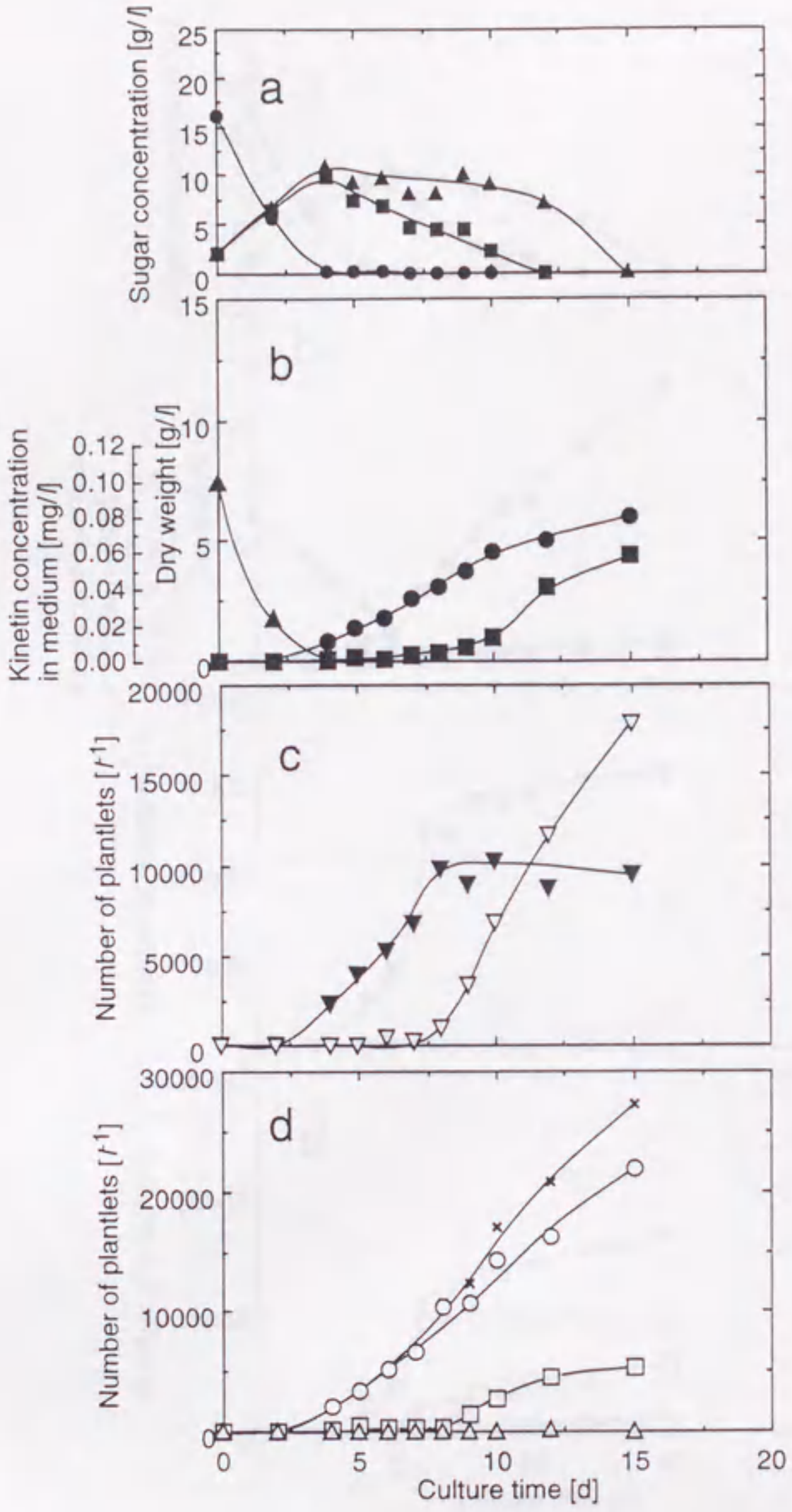

FIG. 6-2. Time courses of plantlet formation, sugar consumption and dry weight of biomass under the culture condition of combinations between 13.3 g-f.w./*l* of initial density of the roots and 0.1 mg/*l* of kinetin concentration in media. Symbols are the same as shown in Fig. 6-1.

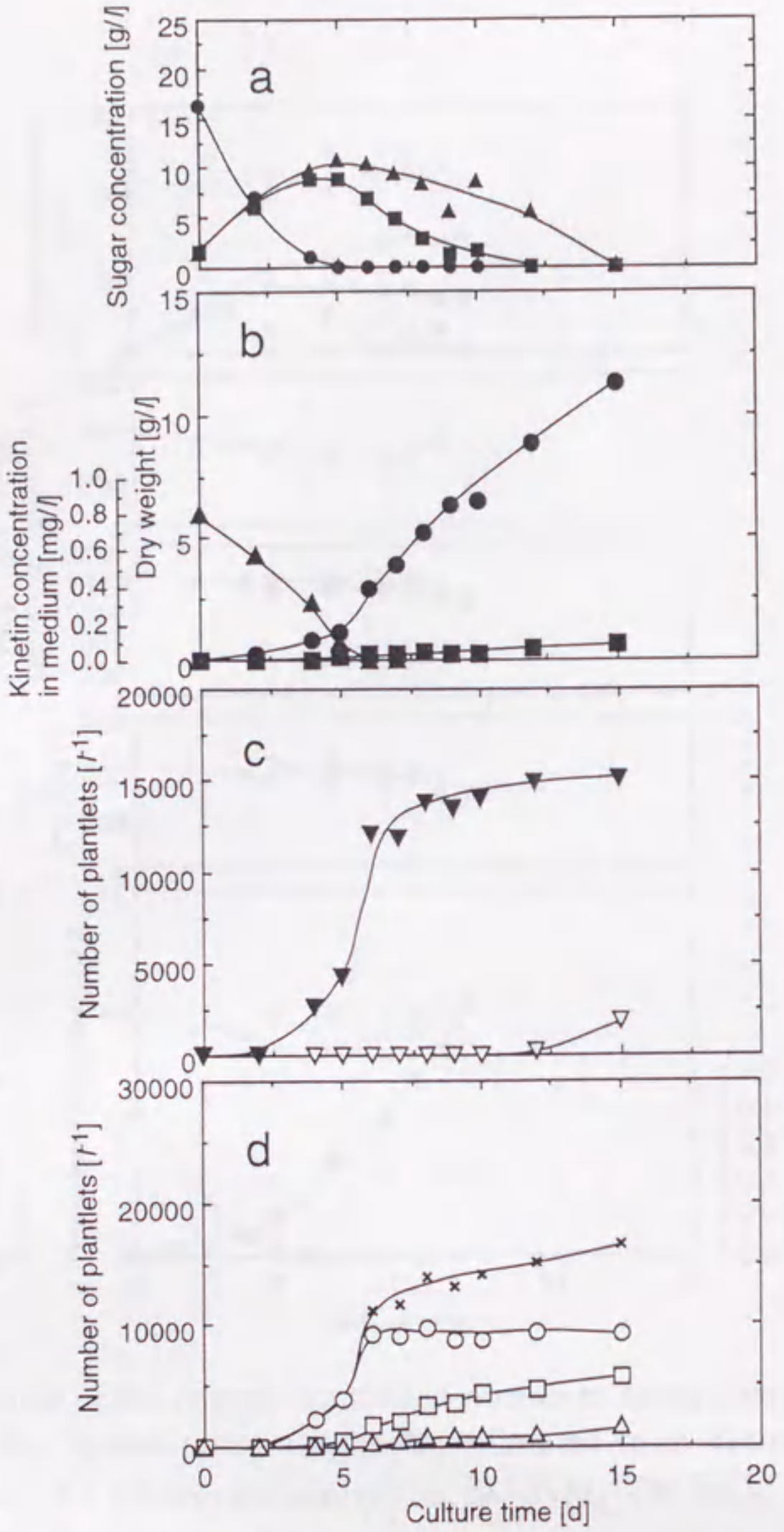

FIG. 6-3. Time courses of plantlet formation, sugar consumption and dry weight of biomass under the culture condition of combinations between 13.3 g-f.w./*l* of initial density of the roots and 0.8 mg/*l* of kinetin concentration in media. Symbols are the same as shown in Fig. 6-1.

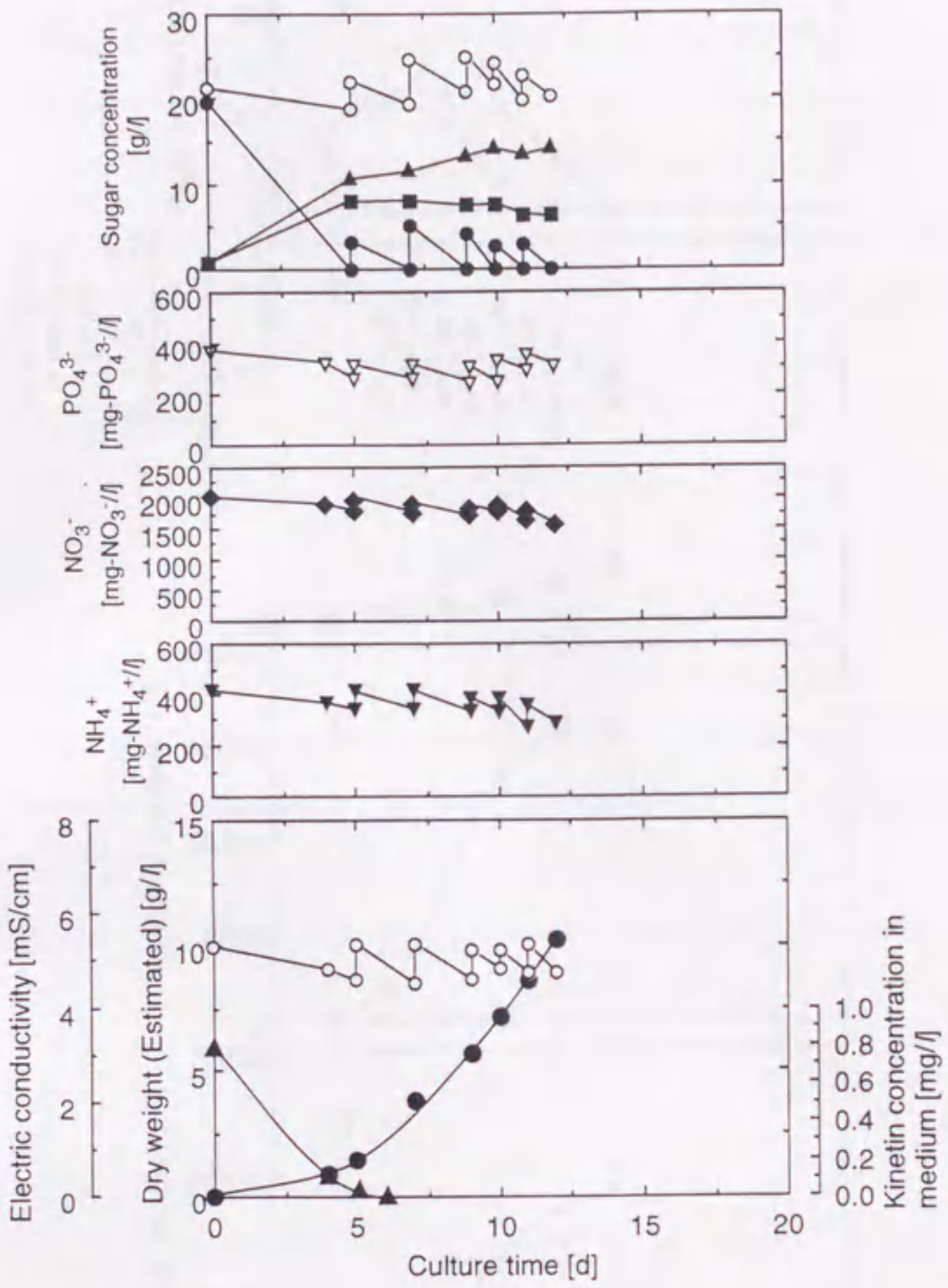

FIG. 6-4. Time course of the culture controlled nutrients using changes of electric conductivity. Symbols; dry weight (●) estimated from decrease in electric conductivity (○), kinetin concentration (▲), NH_4^+ (▼), NO_3^- (◆) PO_4^{3-} (▽), sucrose (●), glucose (■) and fructose (▲).

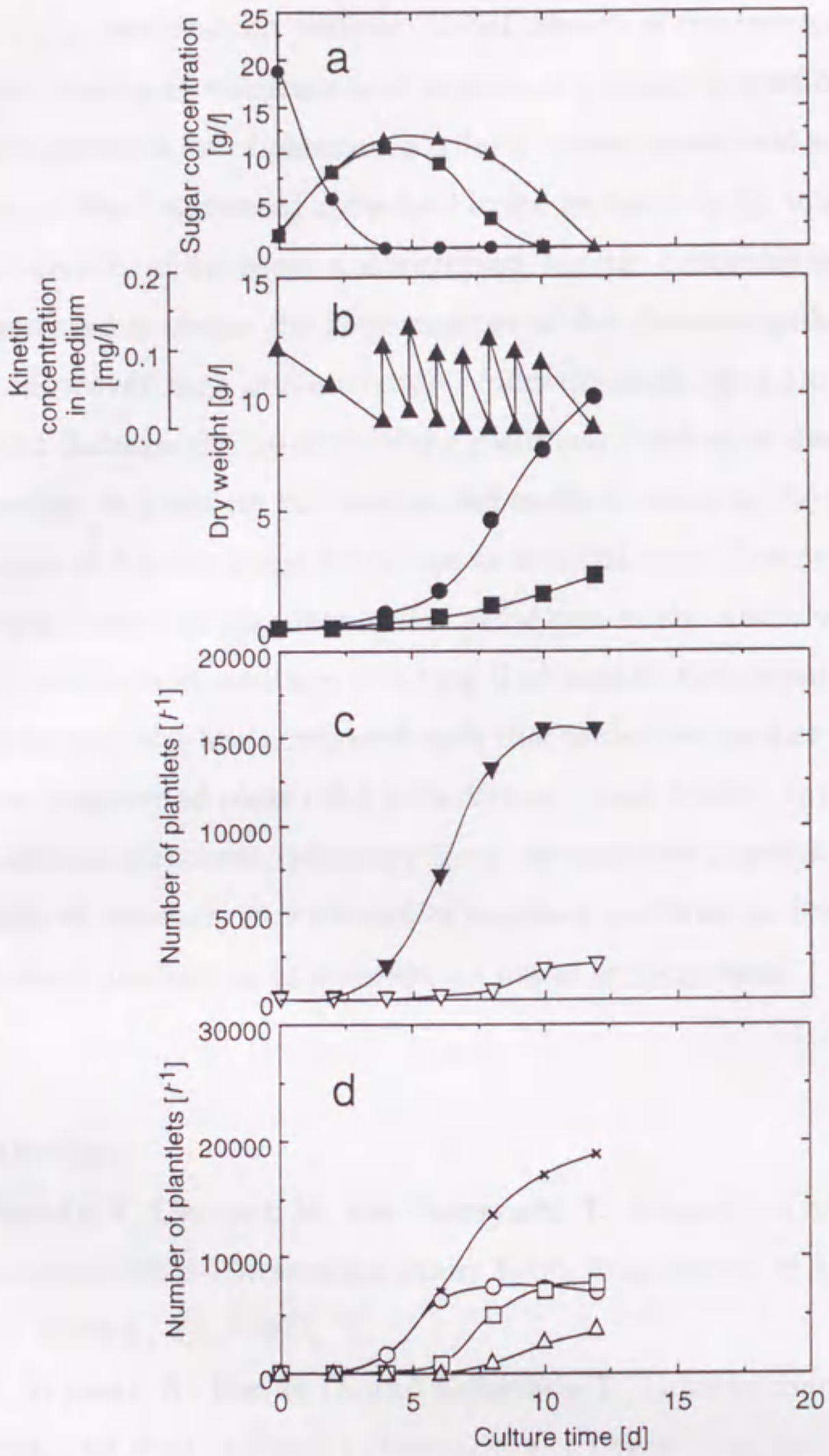

FIG. 6-5. Time course of the culture controlled at 0.1 mg/*l* of kinetin in the medium. Symbols are the same as shown in Fig. 6-1.

6. 5. SUMMARY

Effects of the combination between initial density of fragmented roots and kinetin concentration, feeding of nutrients and kinetin on plantlet formation were investigated to obtain a lot of plantlets available to use as artificial seed. Increase in initial density of the fragmented roots lead to the increase in the number of plantlets. When initial density of the roots was increased, kinetin concentration in the medium should be increased to obtain the large number of the plantlets without root elongation. However, high concentration of kinetin might give toxic effect to the plant cells, and decrease the number of the plantlets. Feeding of the nutrients did not affect the number of plantlets but contributed to the increase in the number of plantlets in size of 2-4 mm available to use as artificial seed. The most efficient method for production of plantlets in size of 2-4 mm in this study was fed-batch culture with intermittent addition of 0.1 mg/*l* of kinetin concentration although total number of plantlets was low compared with that under the culture condition of initial density of the fragmented roots (13.3 g-f.w./*l*) and initial kinetin concentration (0.1 mg/*l*). Plantlet development frequency from encapsulated plantlets at 15 d culture was 93 %. It might be necessary that control of nutrients and kinetin concentration at low level for efficient production of plantlets for use as artificial seed.

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CHAPTER 7

CONCLUDING REMARKS

Hairy roots have become of interest because of their indefinite and fairly active proliferation, and their capacity to synthesize the products similar to that of the original plants. Furthermore, plants regenerated from hairy roots have altered phenotypes which have a potential of agronomic application and the use of hairy roots offers the opportunity of introducing foreign genes into plant genomes at the same time with the root induction. The development of a micropropagation process using elite hairy roots would have an impact on production of useful materials and agricultural industry compared with that with somatic embryos and plant callus.

Based on the above views, efficient methods for production of useful metabolites and artificial seed using plant hairy roots were studied in this thesis. The conclusions obtained in each chapter are summarized as follows.

1) Supplementation of mannitol instead of ion revealed that the excretion was stimulated, not by osmotic pressure in the medium, but by ionic properties. Extracellular Peroxidase activity per dry cell was proportionally correlated with the ionic strength of cation. $CaCl_2$ or $MgCl_2$ was found to be most effective excreting agent among any other combinations. $CaCl_2$ supplementation at the beginning of the culture exhibited higher Peroxidase production in the medium without a significant loss of final cell mass compared with $CaCl_2$ addition during the culture. Repeated batch culture with 50 mM $CaCl_2$ supplementation produced a great amount of extracellular Peroxidase.

2) 1-Naphthaleneacetic acid (NAA) in the medium strongly inhibited the horseradish hairy root elongation. In batch culture, NAA in the medium was absorbed by the root at the beginning of the culture. After the depletion of NAA, root apical meristems emerged vigorously, and then the growth rate of the root increased dramatically. The kinetic model developed previously was modified to fit well the growth curve of horseradish hairy root treated with NAA. Furthermore, in repeated batch culture, the NAA-treated root culture exhibited a 1.7-fold increase in dry weight compared with that of no NAA-treated root. The modified kinetic model also matched well to the experimental results in repeated batch culture.

3) Horseradish hairy roots were excised with a blade and encapsulated in alginate beads. The root fragments with an apical meristem or a branch efficiently regenerated to whole plants and root fragments of more than 5 mm possessed a high ability of shoot formation. We found that NAA or indole-3-butyric acid treatment showed the stimulation of branch emergence from the root and many shoot formations on the root. These results indicated that the root fragment with branch induced by auxin was the most reactive portion. After the two step liquid culture, the root fragment with high shoot formation frequency could be chosen as the material for artificial seeds. When encapsulated root fragments were placed under the light conditions, an addition of 3 % of sucrose in beads was sufficient for regenerations. The results showed a high potential of the hairy root for propagation by the combination with formation of artificial seeds.

4) Horseradish hairy roots were fragmented in a blender after the root growth. A fragmentation time of 30 s gave the best result on the plantlet formation. Treatment of the hairy roots with NAA prior to fragmentation resulted in a decrease in the time required for hairy root growth and a increase in the plantlet formation. Kinetin supplementation after fragmentation resulted in the highest number of plantlets. Furthermore, the kinetin treatment led to a change in the size distribution of plantlets and inhibition of root elongation, which facilitated encapsulation of plantlets. Although the frequency of plantlet development was significantly reduced by encapsulation in 2% calcium alginate beads, dehydration treatment before the encapsulation strongly improved the plantlet development frequency.

5) At last, effects of the combination between initial density of fragmented roots and kinetin concentration, feeding of nutrients and kinetin on plantlet formation were studied. Increase in initial density of the fragmented roots lead to the increase in the number of plantlets. , Although kinetin concentration in the medium should be increased to obtain a lot of plantlets without root elongation when initial density of the roots was increased, high concentration of kinetin inhibited plantlet formation. Feeding of the nutrients did not affect the number of plantlets but contributed to the increase in the number of plantlets in size of 2-4 mm. The most effective method for production of plantlets available to use as artificial seed was fed-batch culture with intermittent addition of 0.1 mg/*l* of kinetin concentration. Plantlet development

frequency from encapsulated plantlets at 15 d culture was 93 % and the plantlets would be available to artificial seed.

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LIST OF PUBLICATIONS FOR DISSERTATION

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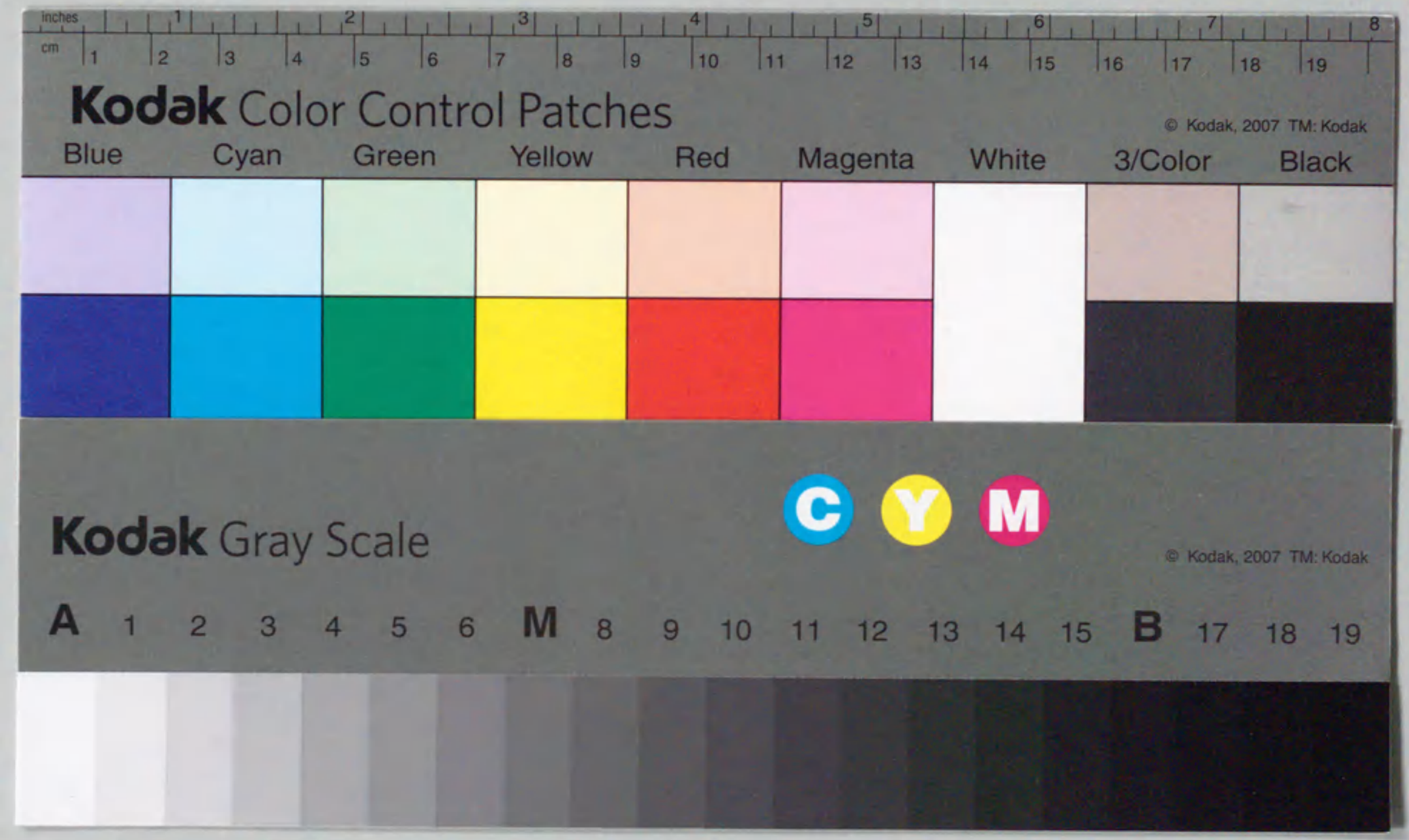
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