Effects of Microgravity and Hypergravity on Plant Growth

植物の成長における微小重力と過重力の影響の解析

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Contents

List of abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
Acro	acropetal (hypergravity)
Ara	arabinose
AVG	2-aminoethoxyvinylglycine
Basi	basipetal (hypergravity)
Fuc	fucose
Gal	galactose
Glc	glucose
HC	hemicellulose
Man	mannose
PPFD	photosynthetic photon flux density
PS	pectic substances
Rha	rhamnose
Xyl	xylose

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Preface

The relationship between gravity and plant growth has been of significant interest to plant physiologists since Darwin's initial examination of the issue at the end of the last century (Darwin 1880). It has been known for very many years that plants sense the direction of gravity and control the direction of their growth. However, the mechanisms involved in this phenomenon remain to be fully elucidated.

One of the reasons for the slow progress in this field has been the difficulty associated with the construction of equipment for exposure of samples to various gravitational conditions. Such equipment is obviously essential for investigations of the effects of gravity on plants. In earlier studies, researchers devised a number of ways to expose plants to various gravitational conditions. Thus, for example, plants were set horizontally or upside down, plants were fixed on an axis that was rotated slowly (single-axis clinostat; Hoshizaki and Hamner 1962, Prasad and Cline 1987, Shen-Miller et al. 1968); or plants were exposed to hypergravity in a centrifuge for a relatively short period of time, for example, 15 minutes (Mineyuki and Furuya 1980).

There have been several problems related to abovementioned methods, as follows. For an examination of the effects of gravity on plant growth, it is necessary to grow plants under various gravitational conditions for long periods of time. Moreover, while experiments in space are ideal for examination of the effects of microgravity on plants, opportunities for such experiments are limited. When a

single-axis clinostat is used, the effect of the unilateral gravity vector can be maintained at a low level, but the perpendicular gravity vector still acts on the samples. Furthermore, in addition to strong centrifugal forces, conditions of hypergravity, with a gravitational force close to unity are also useful for examination of the effects of gravity on plant growth.

From the reasons listed above, the author began by constructing new equipment, namely, a three-dimensional clinostat and a novel centrifugation system. The design and performance of the equipment are outlined below.

The clinostat

The three-dimensional clinostat was equipped with two frames that crossed each other at right angles. The two frames could be rotated independently and randomly. The directions and speeds of rotation were controlled by a micro-computer system. The rotation speeds of two frames were 0.5-2 rpm. At higher speeds, plants can perceive centrifugal forces as a gravitational siganal (Shen-Miller et al. 1968). And at lower speeds, an specific intracellular structure that is susceptible to gravity, such as amyloplasts, might be sedimented. Actually, behaviour of starch granules in root statocytes of garden cress grown in space (Volkmann et al. 1986) was reproduced in grown on the three-dimensional clinostat (Hoson et al. 1997, Y. Masuda and A. Sievers, S. Murakami and M. Yamada, personal communications). In this system, since the direction of the gravity vector for the plant samples could be changed simultaneously in more than one plane, elimination of the effect of the

gravity vector on the plant samples was more effective than in a clinostat with a single horizontal axis. The sample stage of the clinostat was sufficiently large (600 mm×340 mm) to allow a large number of plant samples to be treated simultaneously. Electrical power was supplied to the stages through slip-rings. Light was supplied by fluorescent tubes and plant samples could be grown, with illumination, for long periods of time. The simulation of a microgravity environment by a three-dimensional clinostat of this type was reported to be more effective than that by single-axis clinostats (Hoson et al. 1992).

The centrifugation equipment

The electrical power for the light source for the buckets was supplied via the rotating axis. Plant samples could be grown under hypergravity, with illumination, for at least one week without interruption. Since the maximum rotating radius was 300 mm, larger plant samples could be exposed to hypergravity than was possible with a commercial centrifuge. The centrifugal force on the plant samples could be regulated by changing the speed of rotation to yield forces up to 20×*g*. The equipment had two buckets in which plant samples were set. The directions of installation of the buckets on the arms of the equipment were reversible: one bucket could be fixed to expose plants to hypergravity from the shoot apex to the basal part (this direction of hypergravity was referred to as 'basipetal') and the other bucket could be fixed in the opposite direction (referred to as 'acropetal'). At the maximum speed of rotation (260 rpm), plant samples were exposed to basipetal hypergravity at Basi-20×*g* or acropetal

hypergravity at Acto-13×*g*.

The author constructed the three-dimensional clinostat in cooperation with Dr Y. Masuda's group at Osaka City University (Hoson et al. 1988, 1992; Kasahara et al. 1994) and the centrifugation system in cooperation with Dr M. Yamashita of the Institute of Space and Astronautical Science (Kasahara et al. 1995a, 1995b).

To examine the effects of various gravitational conditions on plant growth with the newly designed equipment, the author initially used *Adiantum capillus-veneris* L. as plant material. The growth of *Adiantum* is controlled by light (Furuya 1978, Raghavan 1989). Irradiation of imbibed spores with red light results in synchronous germination and the elongation of protonemata without cell division, and irradiation of protonemata with white light induces the synchronous division of cells (Wada and Furuya 1970).

Under simulated microgravity in the three-dimensional clinostat, protonemata of *Adiantum* were shorter than the controls (Chapter 1; Kasahara et al. 1994). Elongation of protonemata was inhibited by basipetal hypergravity at more than Basi-15 × *g* but was promoted by acropetal hypergravity from Acro-5 × *g* to Acro-8 × *g*. In addition, more than half of the protonemal cells grown under hypergravity at Basi-20 × *g* were abnormal in terms of shape (Chapter 2; Kasahara et al. 1995a). These results indicated the utility of the newly developed equipment in studies of the effects of various gravitational conditions on plant growth.

Seedlings of cucumber (*Cucumis sativus* L.) were used in subsequent experiments to examine biochemical changes in

plants exposed to various gravitational conditions. Cucumber was chosen because sets of cucumber seedlings of uniform height could easily be prepared and the seedlings were of suitable size for fixation on the stage of the clinostat or in the buckets of the centrifugation equipment.

Although simulated microgravity had no significant effects on the growth of cucumber seedlings, as compared with the stationary controls, the elongation growth of hypocotyls was inhibited by centrifugal hypergravity. Moreover, the reduction in elongation growth was accompanied by an increase in the thickness of hypocotyls (Chapter 3; Kasahara et al. 1995b). The role of evolution of ethylene, the level of 1-aminocyclopropane-1-carboxylic acid (ACC) and the activity of ACC synthase in the hypocotyls were higher under hypergravity than in the stationary controls. These results suggested the involvement of the enhanced production of ethylene in the thickening of hypocotyls of cucumber seedlings during exposure to hypergravity (Chapter 4).

In the present study, newly designed equipment and both *Adiantum* and cucumber were used to clarify the effects of simulated microgravity and centrifugal hypergravity on the growth of a unicellular system and a higher plant (Kasahara et al. 1994, 1995a, 1995b).

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CHAPTER 1

Effects of simulated microgravity on the germination and elongation of protonemata of *Adiantum capillus-veneris*

Abstract

Germination of spores and elongation of protonemata of *Adiantum capillus-veneris* L., which can be controlled by light irradiation, were examined under various gravitational conditions including microgravity simulated by a three-dimensional clinostat. The elongation of protonemata that had been irradiated from below and grew downward was greater than that of protonemata growing horizontally or upwards. Under microgravity, protonemata were shorter than the controls. Germination of spores, direction of growth, and cell division were not affected by gravitational conditions.

Introduction

Many studies on the effects of gravity on plant growth have been reported since Darwin's initial examination of the issue at the end of the last century (Darwin 1880). In higher plants, perception of a gravistimulus leads to differential growth of shoots and roots. This growth response, known as gravitropism, is thought to be caused by the asymmetrical distribution of endogenous plant hormones and/or calcium ions (Lee et al. 1983). Gravitropism has been studied in many species of higher plants (Halstead and Scott 1984), but there have been few reports dealing with algae, mosses and ferns.

Growth of *Adiantum capillus-veneris* L. is controlled by light (Furuya 1978, Raghavan 1989). Irradiation of imbibed spores with red light causes synchronous germination and elongation of protonemata without cell division, and irradiation of protonemata with white light induces synchronous division of the cells (Wada and Furuya 1970). Therefore, protonemata of *Adiantum capillus-veneris* L. should be a useful plant material to examine the effects of gravity on the growth of a unicellular system and on cell division.

Experiments on the effects of gravity on plant growth have been performed in space (Halstead and Dutcher 1987, Lorenzi and Perbal 1990, Volkmann et al. 1986). Experiments in space are ideal for the examination of the effects of gravistimuli on plants, but they are also accompanied by technical problems such as mechanical stress due to high levels of vibrations during take-off and landing. In attempt to cancel the effects of gravity on plant samples on the ground, I produced a new

three-dimensional clinostat in cooperation with Dr Y. Masuda's group of Osaka City University (Hoson et al. 1988, 1992). In the present study, I have now used this clinostat to examine the effects of gravity on the growth and cell division of protonemata of *Adiantum capillus-veneris*.

Materials and Methods

Plant material and growth conditions — The spores of *Adiantum capillus-veneris* L. were a gift from Dr M. Wada (Tokyo Metropolitan University). They were collected in greenhouses at Tokyo Metropolitan University in 1986 and stored at 4℃. The growth medium for germination of *Adiantum* spores consisted of 1/10 strength of the mineral salts of the medium of Murashige and Skoog (1962) solidified with 1.0% (w/v) agar. After sterilization with diluted Purelox™ (a solution of approximately 0.3% sodium hypochlorite; Oyalox Co., Tokyo), the spores were washed three times with sterile water, suspended in the above-mentioned medium without agar, and then inoculated onto the surface of agar-solidified medium in 30-mm-diameter Petri dishes. Three dishes were placed in a black plastic container (133 × 58 × 35 mm), the upper part of which was covered with 2 mm thick red plastic filter (Shinkolite, No. 102, Mitsubishi Rayon Co. Ltd., Tokyo). After imbibition for 4 days at 25℃ in darkness, germination was induced by irradiation with fluorescent light under three gravitational conditions (conditions A, B and C in Fig. 1). The number of spores that germinated was determined by light-microscopic observation.

In the experiments to examine elongation of protonemata and division of cells, sterilized spores were inoculated in four 15-mm lines on agar-solidified medium in a transparent plastic container (60×45×20 mm), as described by Ito (1969). The aligned spores were covered with a coverslip (0.13-0.16 mm thick, 6 × 8 mm), and two transparent plastic containers were

placed in a black plastic container with red plastic filter. The spores were germinated by exposure to light after 24-h imbibition in darkness. The direction of growth and the elongation of protonemata during 6 days at 25℃ in red light under four gravitational conditions (conditions D, E, F and G in Fig. 1) were determined from photomicrographs. The length of a protonema was measured as the distance from the border of the spore coat to the tip of the protonema filament, and the direction in which the protonema initially elongated was taken as the direction of growth. Directions were classified as directions 1 to 4, as shown in Fig. 3.

The statistical significance of differences was calculated by applying the *t*-test at the 1% level.

Three-dimensional clinostat — The three-dimensional clinostat was equipped with two frames that crossed each other at right angles (Fig. 2). The two frames could be rotated independently and randomly. The directions and speeds of rotation were controlled by a micro-computer system. Electric power was supplied to the two frames through slip-rings. The outer frame was rotated at 0.2 or 0.6 rpm, and the inner one at 0.3 or 1.2 rpm. Plant samples were placed on a stage attached to the inner frame. Light was supplied by a fluorescent tube (FL20SS-N/18; Matsushita Electric Industry Co. Ltd., Osaka) located 25 cm above the samples. Fluence rates were 1.88 μol m^{-2} s^{-1} for red light and 11.5 μmol m^{-2} s^{-1} for white light.

Results

Effects of gravity on the rate of germination — The rate of germination of *Adiantum* spores under simulated microgravity conditions (condition C in Fig. 1) was the same as that under earth conditions (condition A) and reverse-earth conditions (condition B) (Table 1). Even when the rate of germination was reduced to approximately 50% by shortening the duration of irradiation with red light to 10 min, there were no differences in germination rates among spores under the three gravitational conditions (Table 1).

Effects of gravity on the elongation of protonemata — The elongation of protonemata that had been irradiated from below and grew downward (condition F in Fig. 1) was greater than that of protonemata growing horizontally (controls, condition D) (Table 2). Under microgravity conditions (condition G), protonemata were shorter than the controls (Table 2), although the diameter of protonemata (about 20 μm) was not changed under any of the 4 experimental conditions.

In another experiment, protonemata were first grown under earth conditions and then under microgravity condition, with a total growth period of 6 days. The elongation of protonemata grown under microgravity for 4 days or more was significantly reduced (Table 3). At the same time, no differences in elongation were observed between protonemata grown under microgravity for 4 days and those for 6 days, suggesting that microgravity at the early stage of growth did not affect the growth of protonemata.

Most protonemata grew toward the light source (direction 1 in Fig. 3), and only few grew in directions 2 or 3. Under 4 of the gravity conditions (Fig. 1 D-G), no conspicuous differences were found among the proportions of the protonemata that grew in the 4 directions (Fig. 3), indicating that *Adiantum* protonemata did not exhibit gravitropism. In all directions, protonemata grown under microgravity (condition G) were shorter than those that had grown horizontally (condition D). In comparison with the controls, the elongation of protonemata under reverse-vertical conditions (condition F) was promoted, in directions 1, 2 and 4.

Effects of gravity on cell division — Cell division of protonemata grown under continuous red light was induced by irradiation with white light after removal of the red filter. The first cell division induced by white light was monitored at 6-h intervals by light microscopy (Fig. 4A). In another experiment, the protonemata were alternately irradiated with red light and white light to induce the first, second and third cell divisions (Fig. 4B).

During 30 h under white-light, no significant differences in timing of division were observed among the 4 gravitational conditions (Fig. 4A). Alternate irradiation of protonemata with red and white light induced synchronous second and third cell divisions (Fig. 4B). While at two time points the differences were significant, no conspicuous effects of gravitational conditions on the timing of divisions were observed as a whole.

Discussion

In attempt to cancel the effects of gravity on the ground, many clinostat types have been used in studies of plant gravitropism. On single-axis clinostats, plants are fixed on an axis that is slowly rotated (Hoshizaki and Hamner 1962, Prasad and Cline 1987, Shen-Miller et al. 1968). The effect of the unilateral gravity vector can be controlled at low levels, but the right-angled gravity vector acts on the plant samples. To eliminate the effects of this gravity vector on plant samples, I produced the new three-dimensional clinostat. Since the direction of gravity vector for the plant samples changes simultaneously in more than one plane, cancellation of gravity vector for the plant samples is more effective than in the case of clinostats with a single horizontal axis. Actually, behaviour of starch granules in root statocytes of garden cress grown in space (Volkmann et al. 1986) was reproduced in grown on the three-dimensional clinostat (Y. Masuda and A. Sievers, S. Murakami and M. Yamada, personal communications). However, since the gravitational field is not eliminated even by three-dimensional clinostats, data from clinostat-experiments should, wherever possible, be complemented by the corresponding space-experiments; as shown for example by the difference between the circumnutations of sunflower hypocotyls grown in space and those grown on clinostats (Brown et al. 1990).

There have been few quantitative studies on the effects of gravity on the growth of ferns, except for that of Bussmann (1939), who examined the effects of gravity on the dorso-

ventrality of the prothallium of *Adiantum cuneatum*. The results of the present study suggest that elongation of the protonemata of *Adiantum capillus-veneris* is affected by gravity, even though there is no gravitropic response (Tables 2 and 3). It should be noted that a specific intracellular structure that might be susceptible to gravity, such as the $BaSO_4$-containing statolith reported in the rhizoids of *Chara* (Sievers and Schröter 1971), was not found in serial sections of the tip of *Adiantum* protonemata (Wada 1988). Protonemata of *Adiantum* extend by tip-growth, and both microtubules and microfibrils appear to be involved in this process (Murata and Wada 1989, Raghavan 1989). Accumulation of the components necessary for growth in the apical regions of protonemata is controlled by microtubules and/or microfibrils. Studies of the effects of gravity on the formation of such cytoskeletons in *Adiantum* protonemata may be useful, since participation of the cytoskeletons in the perception of gravity has been reported for the rhizoids of *Chara* (Bartnik and Sievers 1988).

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Table 1. Effects of gravity on the rate of germination of *Adiantum* spores. The spores were imbibed for 4 days in the dark and irradiated with continuous red light for 4 days; or they were irradiated with red light for 10 min and then placed in darkness for 4 days at 25℃. Growth conditions A-C are shown in Fig. 1. Each value represents the mean ± SD (n=6).

Red-light irradiation	Growth conditions	Germination rate(%)
4 days	A	78 ± 3
	B	81 ± 7
	C	79 ± 2
10 min	A	49 ± 5
	B	43 ± 5
	C	47 ± 7

Table 2. Effects of gravity on the elongation of *Adiantum* protonemata. The spores were imbibed for 24 h in darkness, and protonemata then irradiated with continuous red light for 6 days at 25℃. Growth conditions D-G are shown in Fig. 1. Each value represents the mean ± SD (n > 100). Asterisks denote statistically significant differences from control values (condition D) at $P<0.01$.

Growth conditions	Protonemal length (μm)
D	397 ± 143
E	372 ± 166
F	542 ± 154 *
G	288 ± 132 *

Table 3. Effects of the duration of exposure to various gravitational conditions on the elongation of *Adiantum* protonemata. The spores were imbibed for 24 h in darkness and the protonemata were allowed to grow for various periods of time under different gravitational conditions, under continuous red light, at 25℃. Growth conditions D and G are shown in Fig. 1. Each value represents the mean ± SD (n=100). Asterisks denote statistically significant differences from the control values (6 days in condition D) at $P<0.01$.

Duration (days)		Protonemal length (μm)
Condition D	Condition G	
6	0	446 ±128
5	1	421 ±104
4	2	435 ±101
3	3	401 ±100
2	4	381 ± 88 *
0	6	381 ±130 *

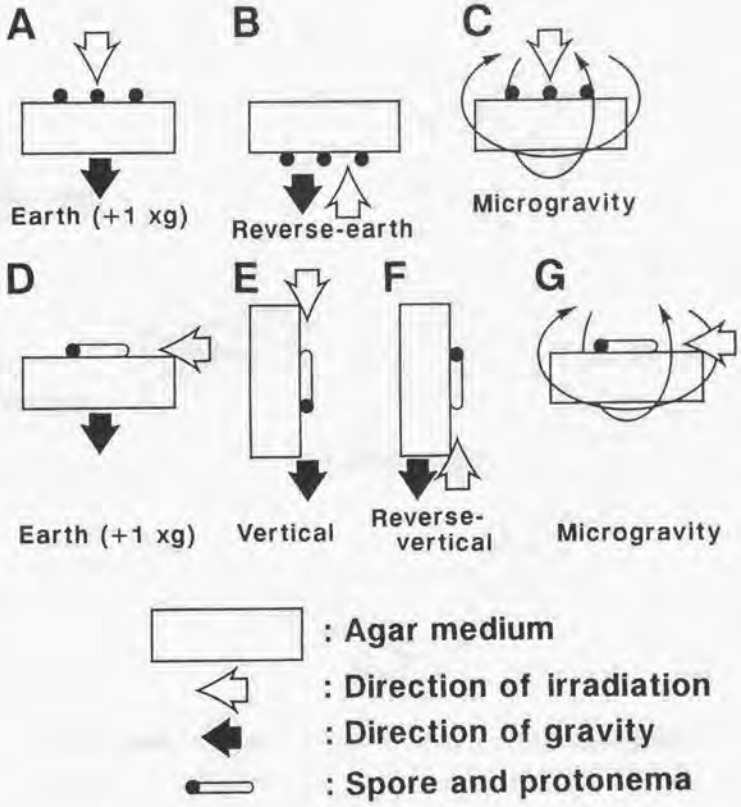

Fig. 1 Schematic illustrations of the directions of light irradiation and gravity relative to the spores and protonemata of *Adiantum* in the experiments for measurement of germination rates (A-C) and of elongation and cell divison of protonemata (D-G).

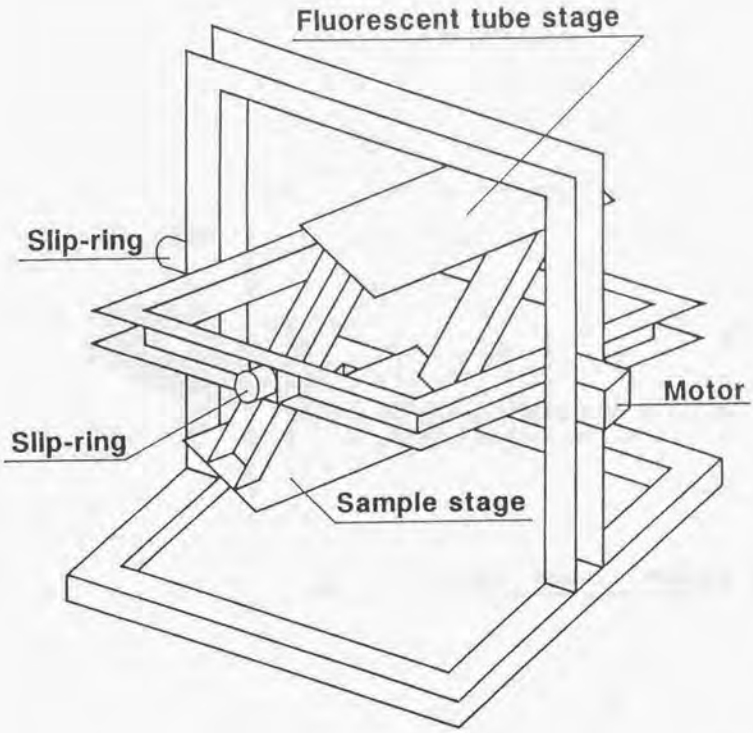

Fig. 2 The three-dimensional clinostat. The centrifugal force (F) which is generated by rotation of samples on the clinostat is calculated by the formula indicated below:

$$F = 11.18 \times (N/1000)^2 \times r$$

where N is rotating speed (rpm), r is turning radius (cm). The maximum centrifugal force in the clinostat is calculated to be $3.2 \times 10^{-4} \times g$ (N = 1.2 rpm, r = 20 cm), which is lower than the force that plant perceive as a gravitational signal (Shen-Miller et al. 1968). The detailed reliability of the clinostat was described by Hoson et al. (1997).

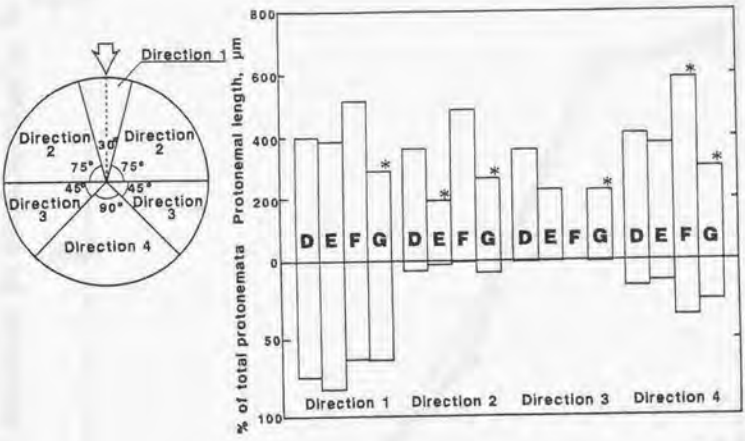

Fig. 3 Effects of the directions of light and gravity on the length and direction of growth of protonemata. Growth conditions D-G are shown in Fig. 1. More than 100 protonemata were counted for determinations of protonemal length and growth direction in each gravity condition. Asterisks denote statistically significant differences ($P<0.01$) in elongation as compared with the control (condition D). The directions 1 through 4 of protonemal growth are indicated on the left. No protonemata growing in direction 3 were observed under condition F.

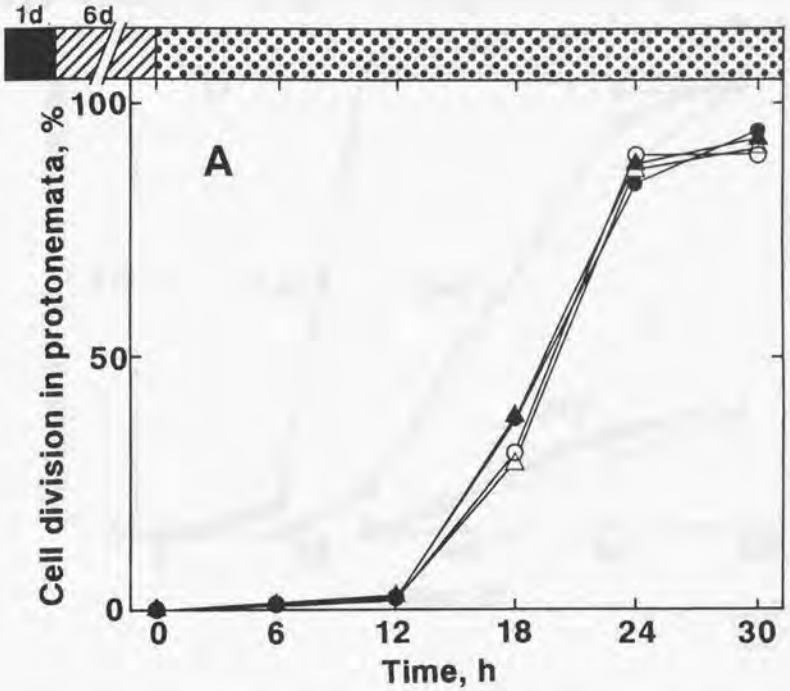

Fig. 4A Effects of the directions of light and gravity on the rates of cell division of protonemata. The 1st cell division induced by white light monitored at 6-h intervals. The schedules for light irradiation are indicated at the top: black solid bar, dark period; slashed bar, red light; dotted bar, white light. ●, Condition D; △, condition E; ▲, condition F; ○, condition G. Growth conditions D-G are shown in Fig. 1.

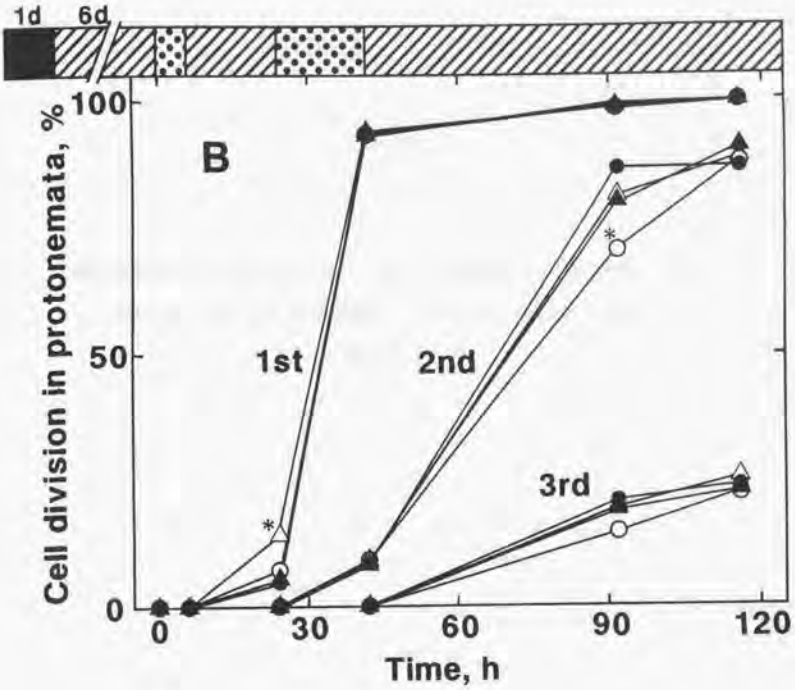

Fig. 4B Effects of the directions of light and gravity on the rates of cell division of protonemata. The 1st, 2nd and 3rd cell divisions induced alternate irradiation with red and white light. The schedules for light irradiation are indicated at the top: black solid bar, dark period; slashed bar, red light; dotted bar, white light. ●, Condition D; △, condition E; ▲, condition F; ○, condition G. Growth conditions D-G are shown in Fig. 1. Asterisks denote statistically significant differences ($P<0.01$) in elongation as compared with the control (condition D).

CHAPTER 2

Effects of hypergravity on the elongation and morphology of protonemata of *Adiantum capillus-veneris*

Abstract

Elongation growth of protonemata of *Adiantum capillus-veneris*, which can be controlled by light irradiation, was examined under acropetal and basipetal hypergravity conditions (from Acro-13 × *g* to Basi-20 × *g*) using a newly developed centrifugation equipment. Elongation of the protonemata under red light was inhibited by basipetal hypergravity at more than 15 × *g* but was promoted by acropetal hypergravity from 5 × *g* to 8 × *g*. Division of the protonemal cells that was induced by white light was inhibited under basipetal hypergravity at 20 × *g* but was unaffected under acropetal hypergravity at 15 × *g*. Upon exposure to continuous red light for 7 to 8 days, most of the protonemata grew as filamentous cells in the absence of a change in the normal gravitational force (control), but more than a half of the protonemal cells were abnormal in terms of shape when maintained under hypergravity at Basi-20 × *g*.

Introduction

Attempts to elucidate the effects of gravity on plant growth have been made in space (Halstead and Dutcher 1987, Lorenzi and Perbal 1990, Volkmann et al. 1986) and on land. In the latter case, clinostats have been used to counteract the effects of gravity (Hoshizaki and Hamner 1962, Shen-Miller et al. 1968).

Although there have been many studies of the effects of gravity on the growth of shoots and roots in higher plants, there have been few quantitative studies on the effects of gravity on the growth of ferns. An exception is the study by Bussmann (1939), who examined the effects of gravity on the dorsoventrality of the prothallium of *Adiantum cuneatum*. In chapter 1, I reported the effects of microgravity on the germination and elongation of *Adiantum capillus-veneris*, that were studied in a three-dimensional clinostat (chapter 1, Kasahara et al. 1994). In addition to microgravity, conditions of hypergravity, at more than Basi-1×*g*, are also useful for examination of the role of gravity on plant growth.

Ootaki (1963) reported that the developmental axis of a fern, *Pteris vittata*, is modified by centrifugal force. It has also been reported that hypergravity (Acro-3 to Basi-3×*g*) affects the germination, cell differentiation and height of fruiting bodies in the cellular slime mold *Dictyostelium discoideum* (Kawasaki et al. 1990). In this chapter, I examined the effects of basipetal and acropetal hypergravity on the elongation growth and cell division of protonemata of

Adiantum capillus-veneris using a newly developed centrifuge, which can be operated for more than one week without interruption.

Materials and Methods

Plant material and growth conditions — Spores of *Adiantum capillus-veneris* L. (a gift from Prof. M. Wada, Tokyo Metropolitan Univ.), were sterilized and inoculated into growth medium as described by Ito (1969). The growth medium consisted of 10-fold diluted mineral salts of Murashige and Skoog's medium (1962) and solidified with 1.0% (w/v) agar. The autoclaved medium was spread on an inner surface of a transparent plastic cuvette (10 mm × 10 mm × 45 mm). Spores, after sterilization with diluted Purelox (a solution of approximately 0.3% sodium hypochlorite; Oyalox Co., Tokyo, Japan) were inoculated in a 20-mm line parallel to the long axis of the cuvette on the solidified medium with a straight Nichrome wire (Fig. 1). The cuvette was filled with agar medium and the open end of the cuvette was sealed with Parafilm. All surfaces of the cuvette were covered with black vinyl tape except for one surface at right angles to that on which spores had been inoculated. Spores were irradiated with red or white light through this unobstructed wall of the cuvette.

Six to 10 cuvettes were placed in a black plastic container (133 mm×58 mm×35 mm) with a 2-mm-thick red plastic filter (Shinkolite, No. 102; Mitsubishi Rayon Co. Ltd., Tokyo, Japan) and were allowed to set horizontally in the dark at 25℃. After 24 h, the container was fixed in a bucket of the centrifuge and the spores were illuminated with continuous red light at 25℃ under hypergravity conditions (Fig. 2). For determination of the lengths of protonemata,

the cuvettes were taken off from the centrifugation equipment, and more than 25 protonemata were photomicrographed or recorded with a video system. The cuvettes that had been observed were discarded. The length of a protonema was measured as the distance from the border of the spore coat to the tip of the protonemal filament. The statistical significance of differences was calculated by applying Student's *t*-test at the 5% and 1% levels. Each experiment was repeated at least three times and similar results were obtained.

Hypergravity conditions — The centrifugation equipment shown in Fig. 2 was set in a temperature-controlled growth cabinet for hypergravity experiments. Cuvettes in the containers attached to a bucket were irradiated with a fluorescent tube at a distance of 100 mm. The centrifugal force on the cuvettes was regulated by changing the speed of rotation. At 260 rpm, protonemata were exposed to basipetal hypergravity at $20 \times g$ (this group is referred to as Basi-$20 \times g$) or to acropetal hypergravity at $13 \times g$ (Acro-$13 \times g$). The control was set near the centrifugation equipment on the floor of the growth cabinet and was irradiated from above (Basi-$1 \times g$) or from below (Acro-$1 \times g$). The fluorescent tube (FL4W, Matsushita Electric Industry Co. Ltd., Osaka, Japan), covered with a reflector, was fixed in the bucket of the centrifuge. Fluence rates were 1.89 μmol m^{-2} s^{-1} for red light and 11.5 μmol m^{-2} s^{-1} for white light at the level of protonemata in the cuvette.

Results and Discussion

Effects of hypergravity on the elongation of protonemata — The rates of germination of *Adiantum* spores were unaffected by the increased gravitational force that were examined in the present study (data not shown). Changes in the length of protonemata with time under various gravitational conditions are shown in Table 1. Basi-petal hypergravity at more than $10 \times g$ inhibited the elongation of protonemata, as compared to the control (Basi-$1 \times g$). Acropetal hypergravity had a different effect on protonemal elongation: force of Acro-$5 \times g$ and Acro-$8 \times g$ promoted elongation, as compared to the control (Acro-$1 \times g$), but at Acro-$13 \times g$ the promotive effect was not observed. The diameter (ca 20 μm) of protonemal filaments, as determined by light microscopy, was not affected by the basipetal or the acropetal hypergravitational forces examined in this study.

Mechanical stresses, such as vibration, are known to affect the growth of plants (Takahashi et al. 1991). It seems likely that any such effects on the elongation of *Adiantum* protonemata can be ignored, since hypergravity in the acropetal direction did not inhibit the growth of protonemata that were exposed to the same vibration as those subjected to basipetal hypergravity.

Inhibitory effects on protonemal elongation were also observed under the microgravity condition that was simulated by growth in a three-dimensional clinostat (chapter 1, Kasahara et al. 1994). Protonemata of *Adiantum* elongate by tip-growth and both microtubules and microfibrils appear to

be involved in this process (Murata and Wada 1989, Raghavan 1989). Since the accumulation of the components necessary for growth in the apical regions of protonemata must be controlled by microtubules and/or microfibrils, studies of the effects of hypergravity on the formation of such cytoskeletal components in *Adiantum* protonemata may be useful as part of an analysis of the effects of gravity on a unicellular system.

Effects of hypergravity on the cell division of protonemal cells — Division of protonemal cells that had been grown under continuous red light was induced by irradiation with white light after removal of the red filter. The first cell division was monitored at 12-h intervals by light microscopy (Fig. 3). The rate of cell division was reduced by basipetal hypergravity (20 × *g*) but not by acropetal hypergravity (Acro-13 × *g*) as compared to the control rates (Basi-1 × *g* and Acro-1 × *g*).

Effects of hypergravity on the shape of protonemata — Most protonemata grew as filamentous single cells (type A in Fig. 4) under the control conditions. After exposure to hypergravity at Basi-20 × *g*, various abnormally shaped protonemata were observed; these were classified into four types, types B-E, as shown in Fig. 4. Under hypergravity at Basi-20 × *g*, the proportion of abnormal types of protonemal cells increased with the duration of culture (Fig. 5), but no major effect of hypergravity at less than Basi-15 × *g* on the shape was apparent (Fig. 6). Hypergravity in the acropetal

direction did not markedly affect the proportion of abnormal protonemata (Figs 5 and 6). Basipetal and acropetal hypergravity affected both the length and the shape of the protonemata, but the nature of the effects on morphology was different from that on elongation. Basipetal hypergravity at 15 × *g* and acropetal hypergravity at Acro-5 × *g* and Acro-8 × *g* affected the length of protonemata but not the proportion of abnormally shaped protonemata significantly but not the proportion of abnormally shaped protonemata (Table 1). The results suggest that the formation of abnormal protonemata does not result from the changes in the rate of elongation of protonemata.

Under basipetal hypergravity at 20 × *g*, division of protonemal cells was inhibited (Fig. 3) and the proportion of abnormally shaped protonemata increased (Fig. 6). After irradiation with white light for 48 h at Basi-20×*g*, more than half of the abnormal protonemal cells had divided, suggesting that reduction in the rate of cell division was not due to the formation of abnormal protonemata.

The occurrence of branched protonemata, similar to those of type C in the present study, has been reported in *Pteris vittata* (Ootaki 1963). Basipetal hypergravity (5,000 *g* for 15 min or 15,000 × *g* for 1 min) resulted in branching of protonemata in *P. vittata*, but acropetal and lateral centrifugation did not have this effect. Although the force and duration of hypergravity differed between the experiments with *A. capillus-veneris* in this study and those with *P. vittata* (Ootaki 1963), the same dependence on the direction of hypergravity was noted with respect to the shape of

protonemata in the two species. Mineyuki and Furuya (1980) also reported a branched form of *Adiantum* protonemata. Basipetal centrifugation at 1,300 × *g* for 15 min induced the displacement of the nucleus with resultant branching of the protonema. Under my hypergravitational conditions (from Acro-13 × *g* to Basi-20 × *g*), no movement of intracellular components such as stratification of chloroplasts and nuclei (Ootaki 1963) or displacement of the nucleus (Mineyuki and Furuya 1980), was found during light microscopic observations. It is now seems important to examine the effects of hypergravity on the ultrastructure and, in particular, on the cytoskeleton of protonemal cells of *Adiantum*.

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Table 1. Effects of hypergravity on the elongation of protonemata of *Adiantum*. After imbibition of spores for 24 h in darkness, the protonemata from the spores were irradiated with continuous red light at 25℃ under hypergravity. Each value (μm) represents the mean±SD (n>25). Single and double asterisks denote statistically significant differences from control values (Basi-1×*g* or Acro-1×*g*) at $P<0.05$ and $P<0.01$, respectively. Gravity conditions are defined in the text.

Gravity condition	Duration of irradiation		
	4 days	6 days	8 days
Basipetally			
1×*g*	45 ± 28	249 ± 77	422 ± 111
10×*g*	49 ± 35	219 ± 76	385 ± 120
15×*g*	34 ± 24	192 ± 59 **	345 ± 96 **
20×*g*	46 ± 29	148 ± 39 **	306 ± 76 **
Acropetally			
1×*g*	39 ± 24	235 ± 73	435 ± 98
5×*g*	53 ± 38 *	267 ± 63 *	487 ± 94 *
8×*g*	42 ± 20	275 ± 65 **	496 ± 97 **
13×*g*	48 ± 38	228 ± 82	459 ± 110

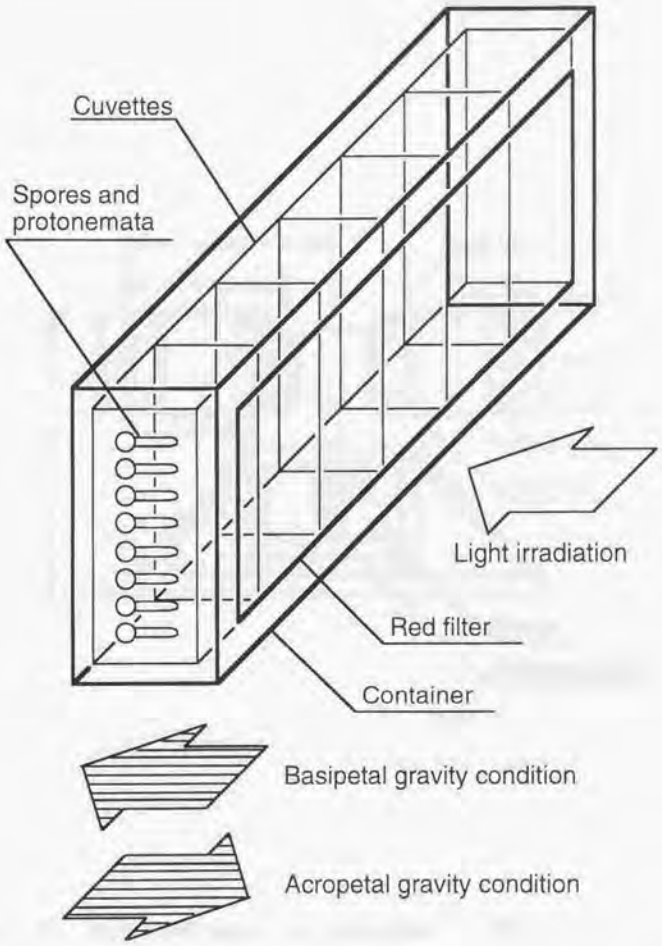

Fig. 1 Schematic illustration of the directions of light irradiation and hypergravity relative to the spores and protonemata of *Adiantum*.

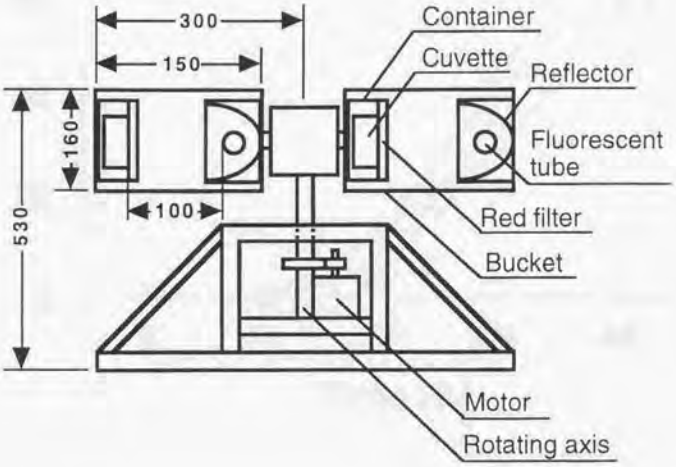

Fig. 2 The centrifugation equipment. The electric power for the light source for each bucket was supplied via the rotating axis. At maximum rotation (260 rpm), the protonemata were exposed to basipetal hypergravity at 20 × *g* (Basi-20×*g*) or acropetal hypergravity at 13×*g* (Acro-13×*g*). Numbers refer to distances in mm.

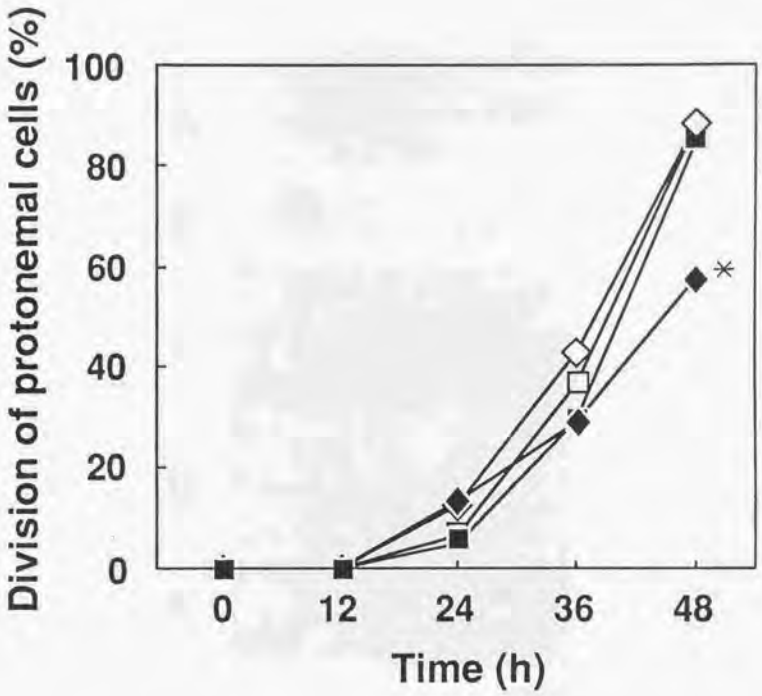

Fig. 3 Effects of hypergravity on the rates of division of protonemal cells. Division of *Adiantum* protonemal cells grown under continuous red light for 6 days was induced by irradiation with white light. The rate of cell division was obtained from more than 20 protonemata. The observations were repeated at least 10 times and the mean values are indicated in the figure. SE of each value was less than 5% of the mean. Asterisks denote statistically significant differences from control values (Basi-1 × *g*) at $P<0.01$. Conditions are defined in the text. ■, Basi-1×*g*; □, Acro-1 ×*g*; ◆, Basi-20×*g*; ◇, Acro-13×*g*;

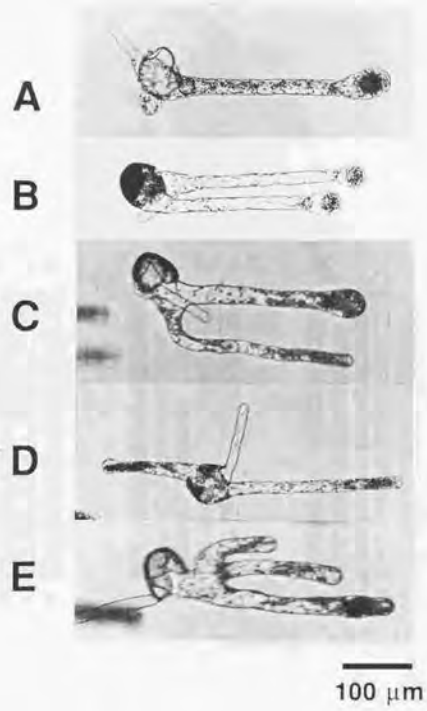

Fig. 4 Photomicrographs of various types of protonema obtained from spores of *Adiantum*. The protonemata were divided into 5 types by shape. Type A, a single filamentous protonema (normal type); type B, two protonemal filaments elongated from a single spore; type C, branched form; type D, two protonemal filaments that originated from a single spore and elongated in opposite directions; type E, an abnormal protonema which developed into a triple-branched filamentous protonema.

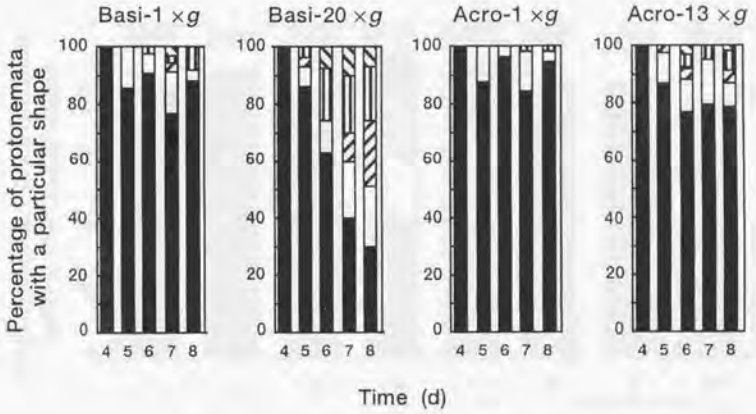

Fig. 5 Effects of hypergravity on the shape of protonemata. More than 100 protonemata were observed every day after day 4 of red-light irradiation. For explanation types of protonemal shape (A, ■; B, □; C, ▨; D, ▥; and E, ▧) see legend to Fig. 4.

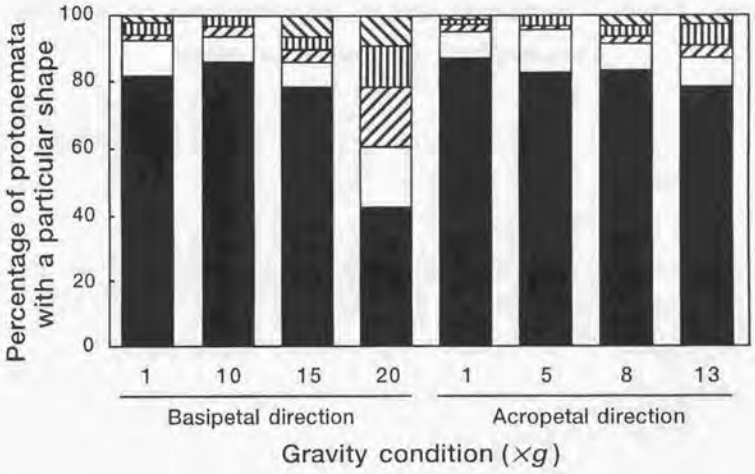

Fig. 6 Effects of various gravitational forces on the shape of protonemata. More than 100 protonemata were observed on day 8 of red-light irradiation. The various protonemal shapes are described in the legends to Figs 4 and 5.

CHAPTER 3

Effects of hypergravity on the elongation growth in radish and cucumber hypocotyls

Abstract

The elongation growth of the hypocotyls of radish and cucumber seedlings was examined under hypergravity in a newly developed centrifuge. The effects of hypergravity on elongation growth differed between the two species. The rate of elongation of radish hypocotyls was reduced under basipetal hypergravity (Basi-20 × *g*) but not under acropetal hypergravity (Acro-13 × *g*), as compared to growth under the control conditions (Basi-1 × *g* and Acro-1 × *g*). In cucumber hypocotyls, elongation growth was inhibited not only by basipetal but also by acropetal hypergravity. Under these conditions, the reduction in the elongation growth of both radish and cucumber hypocotyls was accompanied by an increase in their thickness. Although no distinct differences in relative composition of neutral sugars were found, the amounts of cell-wall components (pectic substances, hemicelluloses and cellulose) per unit length of hypocotyls were increased by exposure to hypergravity.

Introduction

Attempts to elucidate the effects of microgravity on plant growth have been made in space (Volkmann et al. 1986, Halstead and Dutcher 1987, Lorenzi and Perbal 1990) and on the ground. In the latter case, clinostats have been used to counteract the effects of gravity on plant samples (Hoshizaki and Hamner 1962, Shen-Miller et al. 1968).

Not only microgravity but also hypergravity at gravitational forces of more than 1×*g* is thought to be useful for studies of the role of gravity in plant growth. Centrifugal hypergravity has been used in some experiments designed to study gravitropism in roots (Lee et al. 1990), transport of auxin (Ouitrakul and Hertel 1969, Hild and Hertel 1972), graviperception (Wendt and Sievers 1986, Poff and Martin 1989) and the circumnutation of sunflower hypocotyls (Brown and Chapman 1976, Zachariassen et al. 1987). However, there have been a few reports of plant growth under hypergravity (Waldron and Brett 1990). One of the reasons for the paucity of research in this field has been the lack of apparatus in which plants can grow under hypergravity with light irradiation for long periods of time. In Chapter 2 , I reported a newly developed centrifugation system that allows growth of plants under hypergravity with illumination for at least one week (Kasahara et al. 1995).

In this chapter, I examine the effects of hypergravity on the elongation growth and the cell-wall composition in the hypocotyls of radish and cucumber seedlings, using this centrifugation system.

Materials and Methods

Plant materials and growth conditions — The centrifugation equipment was newly developed, as shown schematically in chapter 2. This equipment had two buckets in which plant samples were set. The directions installing the buckets to the arms of the equipment could be changed reversibly: one bucket was fixed to expose hypergravity from the shoot apex to the basal part of hypocotyl (this direction of hypergravity was referred to as 'basipetal') and another one was fixed in the opposite direction (referred to as 'acropetal'). At maximum rotating speed (260 rpm), plant samples were received basipetal hypergravity at 20×*g* (Basi-20 × *g*) or acropetal hypergravity (Acro-13 × *g*). Light was supplied by a fluorescent tube (FL4W; Matsushita Electric Industry Co. Ltd., Osaka) that was fixed in the bucket of the centrifugation equipment with an appropriately positioned reflector. PPFD at the surface of the container was 11.5 $\mu mol\ m^{-2}\ s^{-1}$.

The attachment designed specifically for growth of seedlings is shown in Fig. 1. A plastic container (158×84×31 mm), was stuffed with rock wool (Nitto Boseki Co. Ltd., Tokyo) and then filled with a solution of Hyponex (0.1%, v/v; The Hyponex Co. Inc., Ohio, U.S.A.) solidified with 1.0% (w/v) agar.

After imbibition in running water for 6 h, seeds of cucumber (*Cucumis sativus* L. cv. Hokushin) and radish (*Raphanus sativus* L.) were allowed to germinate on wet paper towels for 42 h at 25℃ in darkness. The roots, which were

10-15 mm long, of germinated seeds were inserted into the agar in the plastic container through the holes bored in the lid. The container was covered with a transparent acrylic cover, fixed within a plastic holder, and set horizontally.

The centrifugation equipment was set in a temperature-controlled growth cabinet. As the controls, the containers in the bucket were set on the ground and irradiated from above (Basi-1 × *g*) or from below (Acro-1 × *g*). The controls were set near the centrifugation equipment on the floor of the growth cabinet.

After two days of growth under white light (PPFD; 61.8 μmol m^{-2} s^{-1}), the seedlings had grown to 5-15 mm in length, and the entire attachment was fixed in a bucket of the centrifugation equipment.

Measurements of growth — Growth rates of hypocotyls were determined from increases in lengths of hypocotyls. The buckets were taken off from the centrifuge and then the acrylic covers were removed. After the measurement of hypocotyl length with a ruler, the attachment was re-assembled and then the plant samples were re-set under four gravitational conditions to continue the experiment. Before the shoot apex reached the top of the acrylic cover, the hypocotyls were excised from the seedlings, and their lengths and fresh weights were measured. The statistical significance of differences was calculated by Student's *t*-test.

Analysis of cell-wall components — Cell walls were

isolated from hypocotyls by the method of Takeuchi and Komamine (1978). To remove the starch from the preparations of cell walls, the cell walls were treated with pancreatic α-amylase as described by Takeuchi and Komamine (1980) and fractionated into four components, namely pectic substances (PS), hemicellulose I (HC I), hemicellulose II (HC II) and cellulose, by the method described by Takeuchi and Komamine (1978). The amount of total carbohydrate in each fraction was determined by the phenol-H_2SO_4 method with Glc as the standard (Dubois et al. 1956). The concentration of uronic acid was determined by the modified carbazole method of Galambos (1967), with glucuronic acid as the standard. The cellulose fraction was hydrolysed with 72% (w/w) H_2SO_4 for 4 h at 20°C, and the reaction mixture was diluted to 3% H_2SO_4 with H_2O and was further hydrolysed for 2 h at 100°C. The other fractions of cell walls were hydrolysed with 2 M trifluoroacetic acid in sealed tubes at 120°C for 1 h. The neutral sugar composition of the hydrolysate was determined by gas-liquid chromatography (GLC) in terms of alditol acetate derivatives with myo-inositol as the internal standard, as described by Albersheim et al. (1967).

Results

Effects of hypergravity on the elongation of hypocotyls — The effects of hypergravity on the growth of radish and cucumber hypocotyls are shown in Tables 1 and 2, respectively. The growth rate of radish hypocotyls was significantly reduced by hypergravity at Basi-20×*g* but not by hypergravity at Acro-13×*g*. Fresh weights of hypocotyls were unchanged after growth under the four sets of gravitational conditions examined in this study, but the thickness (fresh weight per unit length) of the hypocotyls was increased as a result of exposure to hypergravity at Basi-20×*g* (Table 1).

Hypergravity affected the elongation growth of cucumber hypocotyls in a different manner from that of radish hypocotyls. Inhibition of elongation and thickening of the hypocotyls occurred as a result of exposure to either hypergravity at Basi-20×*g* or at Acro-13×*g* (Table 2).

The acropetal hypergravity treatment (Acro-13×*g*) resulted in considerable gravitropic bending of the hypocotyls of radish and cucumber. Although the magnitude of bending under Acro-13 × *g* was less than under Acro-1 × *g* condition, the direction of acropetal hypergravity became not parallel to the axes of hypocotyls during hypergravity treatment. As described above, the effects of acropetal hypergravity on elongation growth of hypocotyls were different between radish and cucumber, but the effects of bending on the growth rate of hypocotyls under acropetal hypergravity remain to be obscure.

Effects of hypergravity on the composition of cell walls in hypocotyls — In radish hypocotyls, relative amounts of PS, HC I and HC II were about 15-17%, 9-11% and 18-22% (w/w), respectively. Although the relative amount of each respective cell-wall component was the same under the four sets of gravitational conditions, total amounts of cell walls and the amounts of cell-wall components per unit length in radish hypocotyls were increased by hypergravity at Basi-20×*g* as compared with those under control conditions (Basi-1×*g* and Acro-1×*g*) and under hypergravity at Acro-13×*g* (Fig. 2). The neutral sugar composition of cell-wall components in radish hypocotyls is shown in Table 3. Rha, Ara and Gal were major constituents of the PS fraction, and Xyl and Glc were major constituents of both the HC I and HC II fractions. In the cellulose fraction, more than 94% of the total neutral sugars were Glc, indicating that most of this fraction was composed of α-cellulose. There were no distinct differences in neutral sugars in the cell-wall components from hypocotyls grown under the four sets of gravitational conditions.

In cucumber hypocotyls, more than 60% of total cell walls consisted of cellulose. Total amounts of cell walls and the amounts of cell-wall components per unit length of cucumber hypocotyls were increased both by at Basi-20 × *g* and by hypergravity at Acro-13 *g*, as compared with controls (Basi-1× *g* and Acro-1×*g*) (Fig. 3). The neutral sugar composition of cell-wall components in cucumber hypocotyls was similar to that in radish hypocotyls. In cucumber hypocotyls there were also no distinct differences in neutral sugar compositions of cell-wall components under the four sets of gravitational

conditions (Table 4).

Discussion

The effects of hypergravity on plant growth have been examined previously only in the case of hypergravitational force in the Basi-20×*g* direction, which was generated in a centrifugation equipment (Waldron and Brett 1990), but a study of hypergravity in the opposite direction seems also to be important for elucidation of the role of gravity in plant growth and morphogenesis. In our newly developed centrifugation equipment, the direction of hypergravity to the plant samples could be changed simply. Two buckets in which the samples were fixed were installed reversibly to the arm of the centrifugation equipment. In chapter 2, the hypergravity at Acro-13×*g* was shown to promote the elongation of protonemata of *Adiantum capillus-veneris*.

Exposure to hypergravity caused an increase in the ratio of fresh weight to length of hypocotyls, that is, thickening of the hypocotyls (Tables 1 and 2). Since the production of ethylene in response to stress leads to thickening of stems (Goeschl et al. 1966, Liebermann 1979), I attempted to measure the concentrations of ethylene in the atmosphere within the attachments to the centrifugation equipment under the four sets of gravitational conditions. Although the concentration of ethylene in the attachments was very low, our preliminary results suggested that the production of ethylene may be promoted under hypergravity. However, the different effects of both directions of hypergravity on the elongation growth of radish hypocotyls cannot be explained by the production of ethylene.

Growth of plant cells is controlled by the mechanical properties of the cell walls (Masuda 1978, Brett and Waldron 1990). In this chapter, the total amounts of cell walls and their components per unit length increased in hypocotyls of both radish and cucumber under conditions that induced the inhibition of elongation growth. Since there is a possibility that these increases resulted from the increase in cell number, it is important to elucidate the effect of hypergravity on cell division.

Waldron and Brett (1990) found differences in terms of the neutral sugar composition of non-cellulosic polysaccharides between pea epicotyls grown under Basi-120 $\times g$ and controls (Basi-1 $\times g$). In the present study, no distinct differences in relative compositions of cell-wall components were observed among the four sets of gravity conditions (Table 3 and 4). In our study, the maximum centrifugal force were equivalent to a force of Basi-20 $\times g$. It is now necessary to investigate the effects of greater gravitational force on structure of the cell walls.

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Table 1. Effects of hypergravity on the growth of radish hypocotyls. Four-day-old seedlings were grown under continuous light and the four sets of gravitational conditions at 25℃ for 24 h. Each value is the average of results from 20 hypocotyls ± SD. Asterisks denote statistically significant differences from the control value (Basi-1×*g*) at $p<0.01$.

Gravitational condition	Growth rate (mm/day) 4 - 5 day	Hypocotyl length (mm)	Hypocotyl fr wt. (mg)	Fr wt/length (mg/mm)
Basi-1×*g*	20.0 ± 7.6	46.9 ± 14.2	70.3 ± 26.9	1.49 ± 0.27
Acro-1×*g*	22.7 ± 7.5	49.6 ± 11.5	73.9 ± 16.6	1.50 ± 0.28
Basi-20×*g*	8.4 ± 2.9 *	35.7 ± 5.4 *	70.9 ± 15.1	1.99 ± 0.40 *
Acro-13×*g*	21.1 ± 4.8	49.3 ± 9.8	81.1 ± 20.7	1.64 ± 0.22

Table 2. Effects of hypergravity on the growth of cucumber hypocotyls. Three-day-old seedlings were grown under continuous light and the four sets of gravitational conditions at 25℃ for 48 h. Each value is the average of results from 20 hypocotyls ± SD. Single and double asterisks denote statistically significant differences from the control value (Basi-1×*g*) at $p<0.05$ and $p<0.01$, respectively.

Gravitational condition	Growth rate (mm/day)		Hypocotyl length (mm)	Hypocotyl fr wt. (mg)	Fr wt/length (mg/mm)
	3 - 4 day	4 - 5 day			
Basi-1×*g*	25.9 ± 5.7	22.0 ± 7.6	60.1 ± 8.0	145 ± 31	2.41 ± 0.33
Acro-1×*g*	32.9 ± 10.0	20.0 ± 6.2	61.9 ± 10.2	149 ± 22	2.37 ± 0.29
Basi-20×*g*	16.1 ± 3.5 **	11.9 ± 5.2 **	38.1 ± 2.9 **	143 ± 14	3.73 ± 0.33 **
Acro-13×*g*	22.0 ± 3.5 **	13.2 ± 3.3 **	45.4 ± 4.8 **	132 ± 18 *	2.90 ± 0.32 **

Table 3. Effects of hypergravity on the neutral sugar composition of cell-wall fractions from radish hypocotyls. Four-day-old seedlings were grown under continuous light and the four sets of gravitational conditions at 25℃ for 24 h.

Fraction	Gravitational condition	Neutral sugar composition (%)						
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc
PS	Basi-1×*g*	21.4	tr [a]	31.3	6.6	5.5	29.4	5.7
	Acro-1×*g*	17.2	tr	33.0	6.0	6.6	31.3	6.1
	Basi-20×*g*	19.6	tr	30.8	7.5	6.4	26.8	8.9
	Acro-13×*g*	17.1	tr	28.4	5.8	3.5	38.2	7.1
HC I	Basi-1×*g*	3.8	tr	8.7	43.3	7.0	11.4	25.8
	Acro-1×*g*	4.9	tr	11.6	34.6	7.7	17.0	24.3
	Basi-20×*g*	3.8	tr	8.2	43.3	8.0	11.9	24.9
	Acro-13×*g*	4.3	tr	10.2	38.0	8.9	14.8	23.7
HC II	Basi-1×*g*	1.4	3.0	2.0	22.3	12.6	13.7	45.1
	Acro-1×*g*	1.2	3.1	2.3	20.0	11.8	14.7	46.9
	Basi-20×*g*	1.2	3.1	1.9	21.4	13.6	13.2	45.7
	Acro-13×*g*	1.8	2.8	2.1	20.6	12.6	14.4	45.7
Cellulose	Basi-1×*g*	tr	tr	tr	1.7	1.9	1.1	95.4
	Acro-1×*g*	tr	tr	tr	1.6	2.3	1.6	94.6
	Basi-20×*g*	tr	tr	tr	2.1	2.9	1.2	93.8
	Acro-13×*g*	tr	tr	tr	2.5	2.8	tr	94.7

[a] tr: Trace (less than 0.5%)

Table 4. Effects of hypergravity on the neutral sugar compositions of cell-wall fractions from cucumber hypocotyls. Three-day-old seedlings were grown under continuous light and the four sets of gravitational conditions at 25℃ for 48 h.

Fraction	Gravitational condition	Neutral sugar composition (%)						
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc
PS	Basi-1×*g*	30.7	tr [a]	15.4	8.6	11.2	17.3	16.8
	Acro-1×*g*	26.9	tr	12.3	4.7	3.3	37.6	15.2
	Basi-20×*g*	33.4	tr	14.4	6.6	8.6	22.8	14.2
	Acro-13×*g*	24.2	tr	12.0	5.0	4.7	39.7	14.4
HC I	Basi-1×*g*	4.2	tr	5.6	52.0	9.6	8.6	20.1
	Acro-1×*g*	2.8	tr	5.0	55.6	7.8	10.1	18.7
	Basi-20×*g*	3.7	tr	4.8	54.0	7.1	10.3	20.1
	Acro-13×*g*	4.2	tr	4.8	50.2	8.4	13.1	19.4
HC II	Basi-1×*g*	1.4	3.3	0.8	18.1	15.4	12.4	48.7
	Acro-1×*g*	1.6	3.3	0.9	21.9	13.1	13.5	45.8
	Basi-20×*g*	1.7	3.1	0.9	24.4	13.2	12.0	45.0
	Acro-13×*g*	1.7	3.1	0.9	25.1	12.8	12.8	43.9
Cellulose	Basi-1×*g*	tr	tr	tr	3.6	6.9	4.6	84.8
	Acro-1×*g*	tr	tr	tr	1.3	5.5	0.9	92.2
	Basi-20×*g*	tr	tr	tr	1.3	4.6	1.1	93.1
	Acro-13×*g*	tr	tr	tr	1.2	4.6	1.3	93.1

[a] tr: Trace (less than 0.5%)

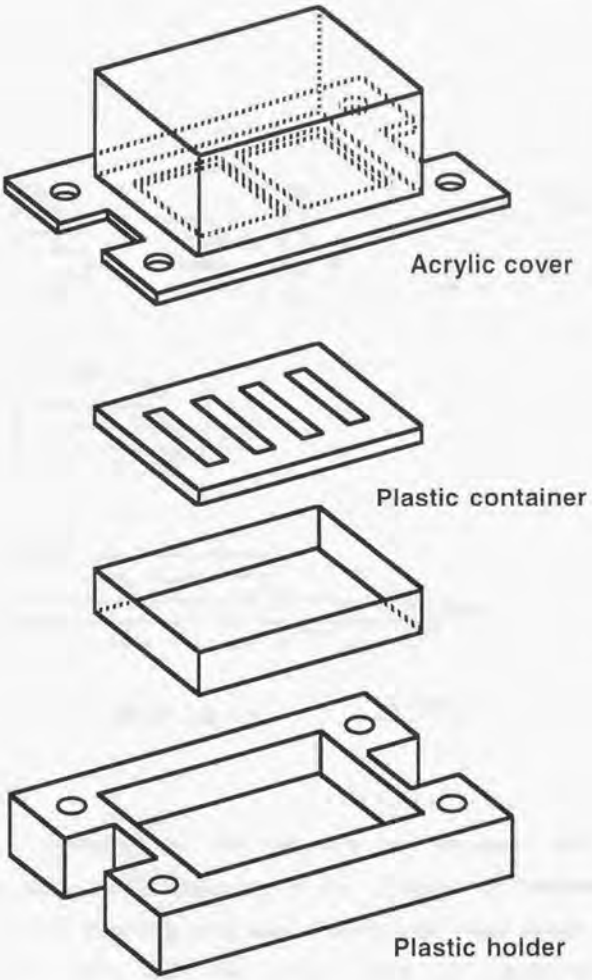

Fig. 1 The attachment for the centrifugation system. A plastic container was stuffed with rock wool and filled with agar medium. Assembled attachment was bolted to the bucket and centrifuged. White light was irradiated from above in this figure.

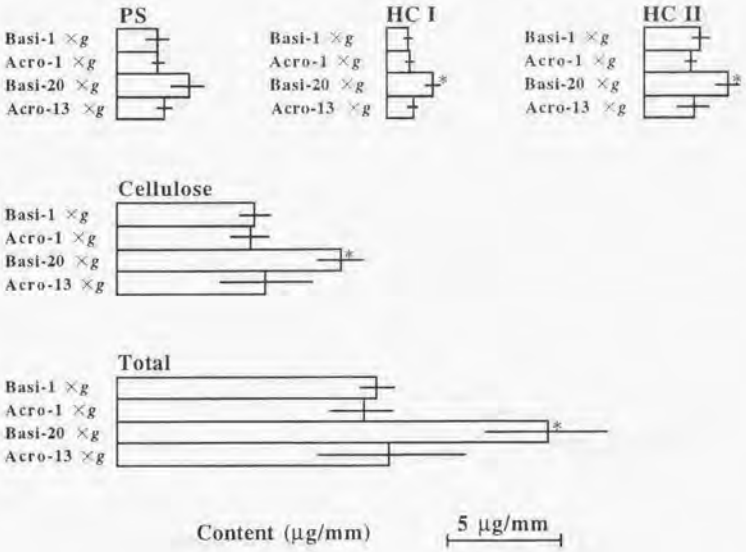

Fig. 2 Changes in the amounts of various cell-wall fractions from radish hypocotyls as a result of exposure to hypergravity. Four-day-old radish seedlings were grown under continuous light and the four sets of gravitational conditions at 25 ℃ for 24 h. The cell walls were then isolated from the hypocotyls and separated into four fractions. Each value is the average of results from four separate samples and the bars indicate SD. Asterisks denote statistically significant differences from the control value (Basi-1×g) at $p<0.01$.

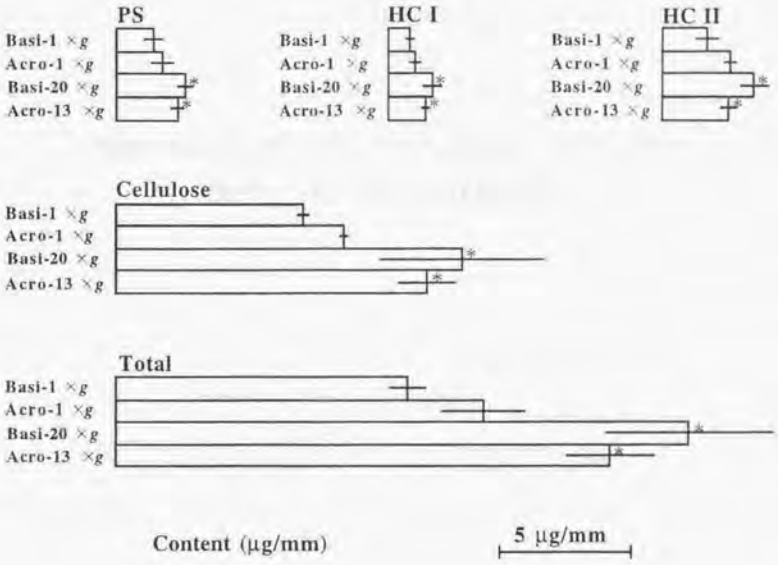

Fig. 3 Changes in the amounts of various cell-wall fractions from cucumber hypocotyls as a result of exposure to hypergravity. Three-day-old cucumber seedlings were grown under continuous light and the four sets of gravitational conditions at 25 ℃ for 48 h. The cell walls were then isolated from the hypocotyls and separated into four fractions. Each value is the average of results from four separate samples and the bars indicate SD. Asterisks denote statistically significant differences from the control value (Basi-1×*g*) at $p<0.01$.

CHAPTER 4

Enhanced Production of Ethylene by Cucumber Hypocotyls under Hypergravity

Abstract

Growth and production of ethylene by hypocotyls of cucumber seedlings were examined under various gravitational conditions using specially designed centrifugation equipment (Kasahara et al. 1995a, b). Exposure to basipetal hypergravity at 13 × *g* (Basi-13 × *g*) resulted in a significant increase in the thickness of hypocotyls as compared to the thickness under control conditions (Basi-1 × *g*), but further increases in gravitational force up to 20 × *g* (Basi-20 × *g*) did not have any additional effect on the thickening of hypocotyls. The thickness of hypocotyls also increased as the gravitational force was increased up to 13 × *g* (Acro-13 × *g*) in the acropetal direction. Thickening induced by exposure to hypergravity was suppressed by application of 2-aminoethoxyvinylglycine (AVG), a potent inhibitor of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase. The evolution of ethylene, the level of ACC and the activity of ACC synthase in the hypocotyls increased under hypergravity as compared to those in the stationary controls. These results indicate the involvement of the enhanced production of ethylene in the thickening of hypocotyls of cucumber seedlings during exposure to hypergravity.

Introduction

Investigations of the role of gravity in plant growth should involve studies not only of the effects of microgravity but also studies of the effects of hypergravity at gravitational forces above $1 \times g$. Chapter 3 describes how the effects of hypergravity on the growth of hypocotyls were examined using a centrifugation system, in which plant samples were exposed to basipetal hypergravity or acropetal hypergravity under continuous light irradiation. Under basipetal hypergravity at $20 \times g$ (Basi-$20 \times g$) and acropetal hypergravity at $13 \times g$ (Acro-$13 \times g$), elongation growth of cucumber hypocotyls was inhibited and the thickness of hypocotyls increased.

Ethylene (C_2H_4) is a volatile plant hormone that diffuses in the gas phase through the intercellular spaces and outside tissues, regulating many aspects of plant growth (Liebermann 1979, Yang and Hoffman 1984). Under non-stress conditions, ethylene is produced by plants at various development stages, such as fruit ripening and senescence (Adams and Yang 1977, McAfee and Morgan 1971, Yang and Hoffman 1984). There have been many reports of the increased production of ethylene under conditions of abiotic and biotic stress (Yang and Hoffman 1984), and the production of ethylene in response to stress has been shown to the lead to thickening of stems (Goeschl et al. 1966, Liebermann 1979).

This chapter describes an examination of the effects of various gravitational forces on the thickness of hypocotyls and the production of ethylene by cucumber seedlings.

Materials and Methods

Plant material and growth conditions — The system that was designed specifically to allow growth of seedlings under various gravitational conditions is shown in Chapter 3. A plastic container (158×84×31 mm) was stuffed with rock wool (Nitto Boseki Co. Ltd., Tokyo) and then filled with a solution of Hyponex (0.1%, v/v; The Hyponex Co. Inc., OH, U.S.A.) that was solidified with 1.0% (w/v) agar.

After imbibition in running water for 6 h, seeds of cucumber (*Cucumis sativus* L. cv. Hokushin) were germinated and allowed to develop on wet paper towels for 42 h at 25℃ in darkness. The roots, which were 10-15 mm long, of germinated seeds were inserted into the agar in the above-mentioned plastic container through holes bored in the lid. The container was covered with a transparent acrylic cover, fixed in a plastic holder, and set horizontally. During two days of subsequent growth under white light (PPFD, 61.8 μmol m^{-2} s^{-1}), the seedlings grew to 5-15 mm in length. Then the entire attachment was placed in a bucket of the centrifugation equipment.

Centrifugation equipment and measurements of growth — The equipment had two buckets and samples were set in each (Chapter 2). The direction of installation of buckets in the arms of the equipment was reversible: one bucket was fixed for exposure to hypergravity from the shoot apex to the basal part of hypocotyl (this direction of hypergravity is referred to as 'basipetal') and the other bucket was fixed in

the opposite direction ('acropetal'; Chapter 3). At the maximum speed of rotation (260 rpm), plant samples were exposed to basipetal hypergravity at 20 × *g* (Basi-20 × *g*) or acropetal hypergravity at 13 × *g* (Acro-13 × *g*). Light was supplied by a fluorescent tube (FL4W; Matsushita Electric Industry Co. Ltd., Osaka) that had been fixed in the bucket of the centrifuge. PPFD at the surface of the container was 11.5 μmol m^{-2} s^{-1}. The centrifugation equipment was set in a temperature-controlled growth cabinet. As controls, buckets were set on the floor of the growth cabinet, near the centrifugation equipment, and irradiated from above (Basi-1 × *g*) or from below (Acro-1×*g*).

The growth rate of hypocotyls was determined as described in Chapter 3. The statistical significance of differences was determined by Student's *t*-test.

Application of inhibitor — A solution of 2-amino-ethoxyvinylglycine (AVG; 1 × 10^{-5} M), which is the most effective and most commonly used inhibitor of the biosynthesis of ethylene (Amrhein and Wenker 1979, Lieberman 1979, Yu et al. 1979, Yang and Hoffman 1984), was sprayed on the terrestrial parts of seedlings with a spray. After 1 h in the horizontal position, plants were centrifuged.

Quantitation of ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) — A sample of gas (2 ml) was withdrawn from the head space of the attachment through a silicone septum with a gas-tight syringe. The ethylene in the sample was quantitated immediately after sampling. The sample was

injected into a gas chromatograph equipped with a flame ionization detector. Separation was performed on a glass column (0.3 cm i.d. × 100 cm) packed with Gasukuropack 54 (60-80 mesh; GL Sciences, Tokyo) at 50℃. The flow rate of the carrier gas (N_2) was 20 ml min^{-1}. The detector response was standardized by injection of known amounts of ethylene prepared by serial dilutions.

The ACC in hypocotyls was quantitated by the method Lizada and Yang (1979), as modified by Prasad and Cline (1987), with slight further modification. After determination of fresh weight, the excised hypocotyls (2.0-2.5 g fr. wt.) were homogenized in 2.5-4.0 ml of 5% (w/v) 5-sulfosalicylic acid (pH 1.8) with a glass homogenizer, and the homogenate was centrifuged at 20,000 × *g* for 15 min at 4℃. Each reaction mixture in a vial contained 0.4 ml of supernatant, 0.4 ml of 5% 5-sulfosalicylic acid, and 0.1 ml of 50 mM $HgCl_2$. The vial was sealed and then a syringe was used to inject 0.8 ml of NaOCl reagent (a mixture of equal volumes of 10 M NaOCl and 10 M NaOH) through a silicone septum. Vials were incubated in an ice bath for 15 min and then 1 ml of gaseous sample was withdrawn for quantitation of ethylene. The efficiency of oxidation of ACC, which averaged 81%, was estimated by analysis of replicate samples with internal standards of ACC. The data reported below were all corrected to account for the limited efficiency of oxidation.

Assay of ACC synthase — The excised hypocotyls (2.0-2.5 g fr. wt.) were homogenized with 1.5 ml of 200 mM Tris-HCl buffer (pH 7.5) that contained 20 mM EDTA, 10 μM pyridoxal

phosphate, 0.2% (v/v) 2-mercaptoethanol, 2% (w/v) sodium ascorbate, 1 mM phenylmethylsulfonyl fluoride and 1 M NaCl in a glass homogenizer. The homogenate was centrifuged at 20,000×*g* for 30 min at 4℃, and the supernatant was passed through a column of Sephadex G-25 (Pharmacia Biotech AB, Uppsala, Sweden) that had been equilibrated with 100 mM Bicine-KOH buffer (pH 8.5) plus 10 μM pyridoxal phosphate. The protein fraction was collected, concentrated by ultrafiltration (Ultrafree-CL; Nihon Millipore Ltd., Yonezawa), and used for assays of ACC synthase activity. The preparation of enzyme (0.8 ml) was mixed with 50 μl of 1.25 mM S-adenosylmethionine and incubated for 30 min at 30℃. The amount of ACC formed was determined, as described above, after termination of the reaction by addition of $HgCl_2$. The concentration of protein in each preparation of enzyme was measured as described by Bradford (1976) with bovine serum albumin as the standard.

Results

Effects of hypergravity on the thickness of cucumber hypocotyls — Chapter 3 described how exposure of cucumber seedlings to basipetal hypergravity at 20 × *g* (Basi-20 × *g*) or to acropetal hypergravity at 13 × *g* (Acro-13 × *g*) caused an increase in the ratio of fresh weight to length of hypocotyls, in other words, thickening of the hypocotyls, but the dose response and the time course of the effects of hypergravity on the thickness of hypocotyls remained to be investigated. This Chapter describes an examination of the effects of exposure to various gravitational forces for 24 or 48 h on the thickness of hypocotyls (Fig. 1). Basipetal hypergravity at 13 × *g* (Basi-13 × *g*) resulted in a significant increase in the thickness of hypocotyls as compared to that of hypocotyls under control conditions (Basi-1 × *g*). However, a farther increase in gravitational force up to 20 × *g* (Basi-20 × *g*) did not increase the thickness of hypocotyls. Exposure to acropetal hypergravity resulted in considerable gravitropic bending of the hypocotyls, but the extent of bending under acropetal hypergravity was smaller than under Acro-1 × *g* condition. Inversion of seedlings (Acro-1 × *g*) increased the thickness of hypocotyls. The thickness of hypocotyls increased as the gravitational force increased up to 13 × *g* (Acro-13 × *g*) in the acropetal direction. There was no significant difference between the thickness of hypocotyls grown under Basi-13 × *g* and that of hypocotyls grown under Acro-13 × *g*. The thickening of hypocotyls increased as the duration of exposure to hypergravity was extended from 24 h

to 48 h.

Table 1 shows the effects of AVG on the growth of hypocotyls. AVG had no significant effect on either elongation growth or the fresh weight of hypocotyls under hypergravity (Basi-20 × *g*) and under control (Basi-1 × *g*) conditions. Thickening of hypocotyls induced by exposure to hypergravity was partially suppressed by the application of AVG.

Effects of hypergravity on the production of ethylene — In view of the results described above, an attempt was made to examine the effects of hypergravity on the production of ethylene. Cucumber seedlings were grown for 24 h under four different conditions (Basi-1 × *g*, Acro-1 × *g*, Basi-20 × *g* and Acro-13 × *g*), and the concentration of ethylene generated by seedlings was measured by gas chromatography (Table 2). The concentrations of ethylene were very low and, therefore, only semi-quantitative results were obtained. Although absolute values varied between experiments, the concentrations of ethylene under hypergravity (Basi-20 × *g* and Acro-13 × *g*) were higher than those under control conditions (Basi-1 × *g* and Acro-1 × *g*), suggesting that the production of ethylene had been promoted by hypergravity.

Effects of hypergravity on the level of ACC and the activity of ACC synthase — In many plant tissues, the rate of production of ethylene is correlated directly with the endogenous level of ACC (Liebermann 1979, Yang and Hoffman 1984). The effects of exposure to various gravitational

conditions on the levels of ACC in cucumber hypocotyls are shown in Figure 2. Inversion of the seedlings and exposure to either basipetal or acropetal hypergravity up to 13 × *g* increased levels of ACC, but levels in hypocotyls grown under basipetal hypergravity at 20 × *g* (Basi-20 × *g*) were similar to those at 13 × *g* (Basi-13 × *g*). Thus, the effects of the various gravitational forces on the levels of ACC clearly reflected the effects on the thickness of hypocotyls (Fig. 1), suggesting that the enhanced production of ethylene might be involved in the thickening of hypocotyls under hypergravity.

Table 2 shows the effects of hypergravity on the activity of ACC synthase. Resembling the effects on the production of ethylene and the level of ACC, the effect of exposure of the seedlings to basipetal or acropetal hypergravity was to increase the activity of ACC synthase in the hypocotyls, as compared to the controls (Basi-1 × *g*). These results indicate that hypergravity stimulates the production of ethylene by increasing the activity of ACC synthase.

Discussion

Ethylene has numerous effects on plant growth and influences the response of plants to gravity (Liebermann 1979). Several authors have reported an increase in the production of ethylene by plants upon exposure to microgravity (Leather et al. 1972, Hensel and Iversen 1980, Driss-Ecole et al. 1994). Hilaire et al. (1996) observed recently that soybean seedlings grown under clinorotation produced twice as much ethylene as stationary controls. In *Pharbitis nil*, Prasad and Cline (1987) reported that inversion of shoots induced the release from apical dominance, radial expansion of stems and enhanced production of ethylene, and these effects were prevented by clinorotation in a horizontal clinostat. The results presented in this chapter demonstrate that the production of ethylene, the level of ACC and the activity of ACC synthase in cucumber hypocotyls were enhanced under hypergravity as compared to those under the control conditions. Thus, it appears that not only microgravity, achieved by rotation in a clinostat, but also hypergravity affect plant growth *via* the biosynthesis of ethylene.

Control of plant growth by an interaction between the effects of ethylene and those of auxin has been suggested in various plant species (Hanson and Kende 1976, Hoson et al. 1990, Lee et al. 1990, Sargent et al. 1973, Yoshii et al. 1980), and transport of auxin has been reported to be affected by hypergravity (Hild and Hertel 1972, Ouitrakul and Hertel 1969). Therefore, it is now necessary to investigate

the effects of the transport of auxin on the production of ethylene under hypergravity.

Many reports have shown that the ACC synthase is coded by a multigene family and that its various members are differentially expressed in response to many factors (Huang et al. 1991, Nakagawa et al. 1991, Olson et al. 1991, Rottmann et al. 1991, Liang et al. 1992, Zarembinski and Theologis 1993, Destefano-Beltran et al. 1995).

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Table 1. Effects of hypergravity and AVG on the growth of cucumber hypocotyls. Four-day-old seedlings were sprayed with 1×10^{-5} M AVG and then grown with continuous illumination under hypergravity (Basi-20×*g*) or under control conditions (Basi-1×*g*) at 25℃ for 2 days. Each value is the average of results from 20 hypocotyls ± SD.

Gravitational condition	Application	Growth rate (mm/2 days) 4 - 6 day	Length of hypocotyl (mm)	Fr.wt.of hypocotyl (mg)	Fr.wt./length (mg/mm)
Basi-1×*g*	None	31.6 ± 7.5 [b]	48.5 ± 8.1 [b]	121.5 ± 18.6	2.54 ± 0.39 [b]
Acro-1×*g*	AVG	29.0 ± 5.8 [b]	45.1 ± 7.6 [b]	104.9 ± 17.7	2.35 ± 0.30 [b]
Basi-20×*g*	None	19.0 ± 3.5 [a]	34.0 ± 4.0 [a]	125.5 ± 25.1	3.68 ± 0.46 [a]
Acro-20×*g*	AVG	16.7 ± 4.2 [a]	32.5 ± 3.1 [a]	105.1 ± 23.1	3.21 ± 0.51 [a,b]

[a], Statistically significant difference from the value obtained of C+1×*g* without application of AVG.

[b], Statistically significant difference from the value obtained of H+20×*g* without application of AVG.

Table 2. Effects of hypergravity on the production of ethylene. Four-day-old seedlings were grown with continuous irradiation under various gravitational conditions at 25℃ for 24 h. The concentrations of ethylene in the head space of attachments to the centrifugation equipment were determined by gas chromatography.

Gravitational condition	Concentration of C_2H_4 ($\mu l\ l^{-1}$)	
	Exp. I	Exp. II
Basi-1×*g*	0.098	0.104
Acro-1×*g*	0.062	0.079
Basi-20×*g*	0.113	0.323
Acro-13×*g*	0.260	0.185

Table 3. Effects of hypergravity on the activity of ACC synthase. Four-day-old seedlings were grown with continuous illumination under various gravitational conditions at 25 ℃ for 48 hr.

Gravitational condition	ACC synthase activity (nmol h^{-1} mg $protein^{-1}$)	
	Exp. I	Exp. II
Basi-1×*g*	0.25	0.49
Acro-1×*g*	0.33	0.80
Basi-20×*g*	0.46	0.90
Acro-13×*g*	0.55	0.70

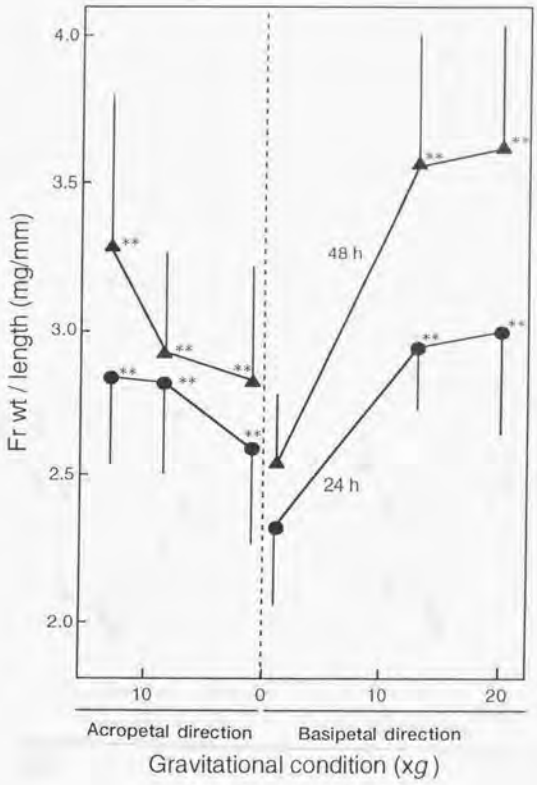

Fig. 1. Effects of various gravitational forces on the thickness (fresh weight per unit length) of cucumber hypocotyls. Four-day-old seedlings were grown with continuous illumination under various gravitational conditions, as indicated, for 24 h (●) or 48 h (▲) at 25℃. Each value and bar represent the average of results from 20 hypocotyls and the SD. Asterisks denote statistically significant differences ($p<0.01$) from the control value (Basi-1×g).

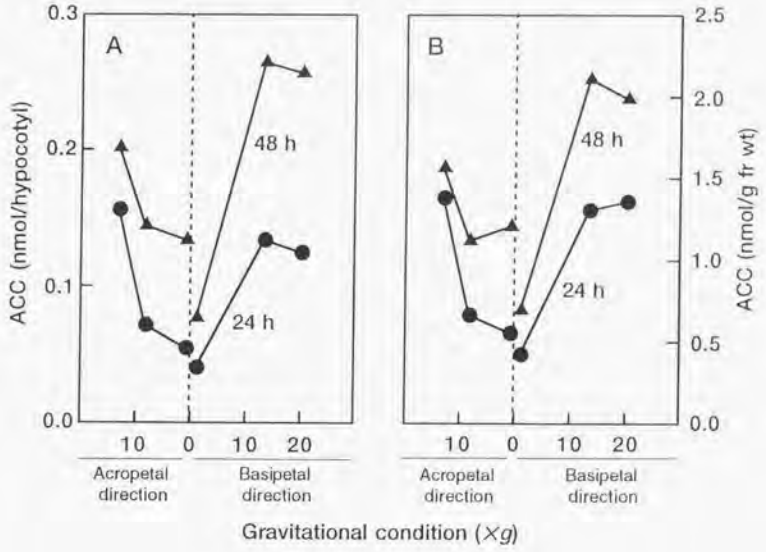

Fig. 2. Effects of various gravitational conditions on the level of ACC in cucumber hypocotyls. Four-day-old seedlings were grown with continuous illumination under various gravitational conditions for 24 h (●) or 48 h (▲) at 25℃. Levels are expressed on the basis of individual hypocotyls (A) and on the basis of the fresh weight of hypocotyls (B).

Concluding remarks

Concluding remarks

This thesis describes an examination of the effects of microgravity and hypergravity on the growth of lower and higher plants, as investigated with a three-dimensional clinostat and a centrifugation system that were newly developed to allow such studies to be performed.

In the experiments detailed in Chapter 1, germination of spores and elongation of protonemata of *Adiantum capillus-veneris* L. were examined under various gravitational conditions, which included microgravity that was achieved by use of the three-dimensional clinostat. Germination of spores, the direction of growth of protonemata, and cell division were unaffected by the various gravitational conditions examined, but the elongation growth of protonemata was inhibited under microgravity as compared to the growth of stationary controls. Elongation growth of protonemata was also examined under acropetal and basipetal hypergravity (from Acro-13 × *g* to Basi-20 × *g*) in the newly developed centrifugation system (Chapter 2). Elongation of protonemata was inhibited by basipetal hypergravity at more than Basi-15 ×*g* but it was promoted by acropetal hypergravity from Acro-5 × *g* to Acro-8 × *g*. Protonemata of *Adiantum* extend by tip-growth, and both microtubules and microfibrils appear to be involved in this process (Murata and Wada 1989, Raghavan 1989). Accumulation of the components necessary for growth in the apical regions of protonemata must be controlled by microtubules and/or microfibrils. An attempt was made to observe changes in the ultrastructure of protonemata by

electron microscopy using serial sections and by immunofluorescence microscopy using tubulin-specific antibodies. However, no distinct differences in ultrastructure and in the distribution of microtubules were observed between the protonemata grown under hypergravity and those grown under control conditions. However, since participation of the cytoskeleton in the perception of gravity has been reported in the rhizoids of *Chara* (Bartnik and Sievers 1988), more detailed studies of the effects of gravity on the formation of the cytoskeleton in *Adiantum* protonemata should be considered.

Under control conditions, most of protonemata of *Adiantum* grew as filamentous cells, but more than half of the protonemal cells had an abnormal shape when they were cultured under hypergravity at Basi-20×*g* (Chapter 2). It is unclear why shaped protonemata appeared during exposure to hypergravity. It remains to be determined whether or not the nucleus is present in the second protonemal filaments (Type B in Chapter 2) or in the branches of protonemata (Type C in Chapter 2). The presence of numerous chloroplasts in the second protonemal filaments and in branches hampered microscopic observations of nuclei. Improvements in the methods used for microscopy should overcome this problem. It is also necessary to investigate the effects of hypergravity on cell division of protonemata of the early stage of growth (immediately after irradiation with white light). Microscopic observations of spores of *Adiantum* of the early stage of growth are very difficult because of the thick outer layers of spores. In the few samples that were successfully

examined, no cell plate was observed in abnormally shaped protonemata.

Chapters 3 and 4 provide descriptions of the effects of hypergravity on the elongation growth of hypocotyls of higher plants. In the case of both radish and cucumber seedlings, basipetal hypergravity (Basi-20 $\times g$) inhibited the elongation growth of hypocotyls, with an accompanying increase in the thickness of these tissues. However, the thickening of cucumber hypocotyls induced by hypergravity was suppressed by application of AVG, a potent inhibitor of ACC synthase (Lieberman 1979, Yang and Hoffman 1984; Chapter 4). The evolution of ethylene, the level of ACC, which is the endogenous precursor of ethylene, and the activity of ACC synthase in the hypocotyls all increased under hypergravity as compared to those in the stationary control. These results suggest the involvement of the enhanced production of ethylene in the thickening of hypocotyls of cucumber seedlings upon exposure to hypergravity.

Limited information is available about the production of ethylene in lower plants (Osborne 1989). Tittle (1987) reported that auxin stimulated the production of ethylene in three species of fern. The possible involvement of ethylene in the inhibition of growth of protonemata of *Adiantum* upon exposure to hypergravity remains to be clarified. In preliminary experiments, addition of ACC (10^{-3} M) to the medium significantly ($P<0.01$) inhibited the elongation of protonemata and increased the proportion of abnormally shaped protonemata. Addition of AVG to the medium also strongly inhibited the germination of spores and the elongation of

protonemata. AVG at 10^{-3} M completely inhibited germination. Elongation growth was inhibited by AVG of 10^{-5}-10^{-4} M, and the inhibitory effect of hypergravity on protonemal length was negated by 10^{-4} M AVG. These results indicate the possibility that ethylene might participate in the inhibitory effects of hypergravity on the growth of *Adiantum* protonemata as well as in the effects of hypergravity on the hypocotyls of cucumber. However, recently some evidences were produced to show that a wide range of lower plants lacks the ability to convert ACC into ethylene. Osborne et al. (1996) showed that a semi-aquatic fern (*Regnellidium diphyllum*) and a liverwort (*Riella helicophylla*) did not generate [^{14}C]-ethylene from added [^{14}C]-ACC. Furthermore, John (1997) reviewed the distribution of ACC oxidase, the enzyme responsible for the final stage in the biosynthesis of ethylene, in land plants, and he concluded that angiosperms and other seed-bearing plants synthesize ethylene via ACC pathway, but ferns and other lower non-flowering plants use a different, unknown pathway. From these facts, it now seems important to investigate the biosynthetic pathway of ethylene in *Adiantum* to clarify the role of ethylene in growth regulation of protonemata.

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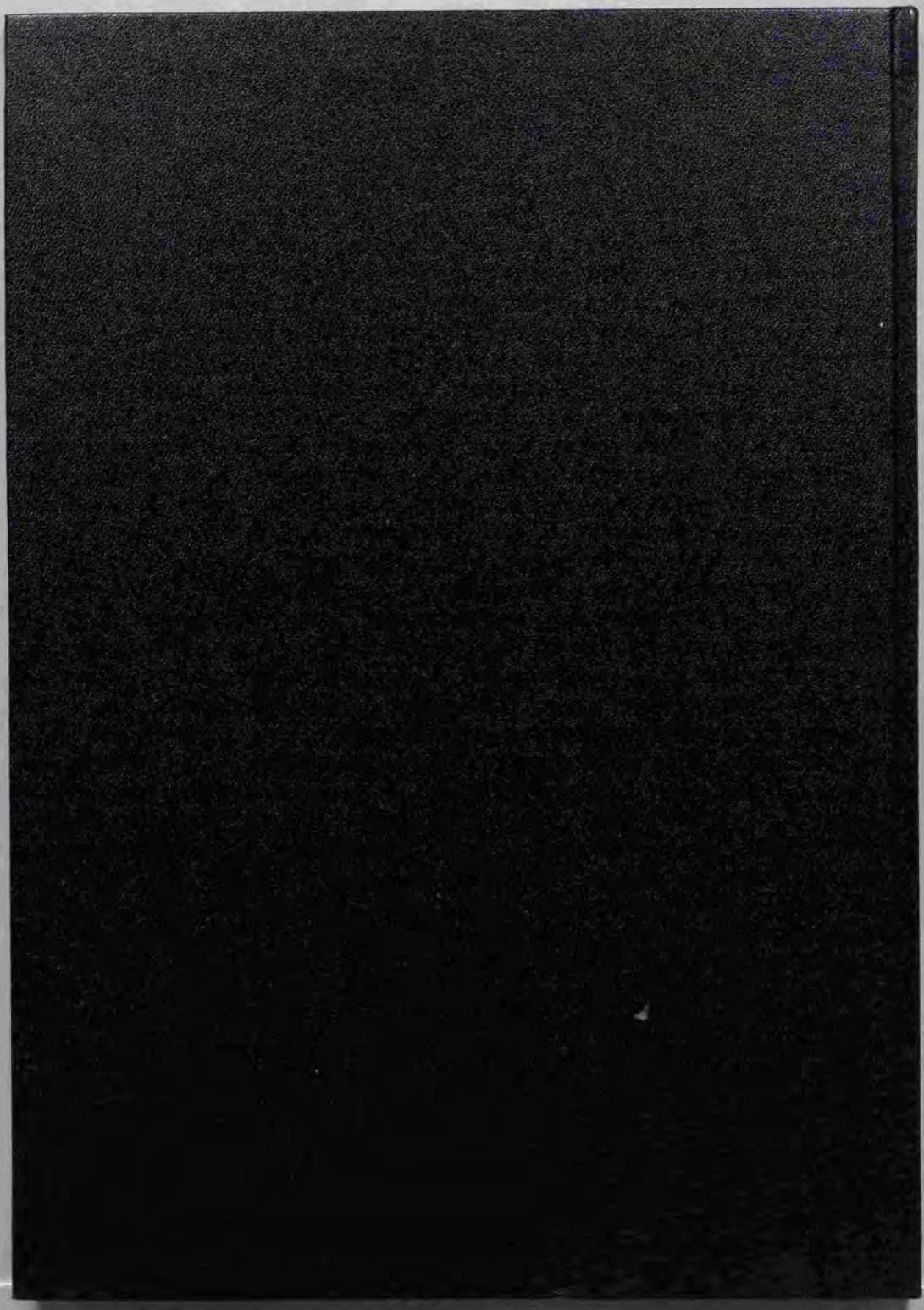

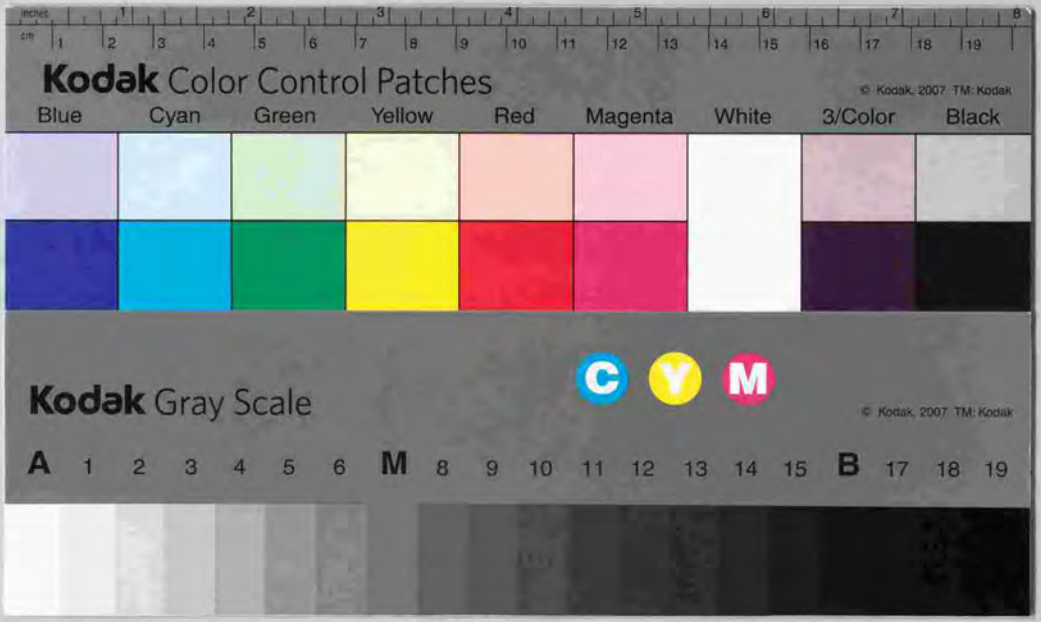
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