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Assessment of Chemical Toxicity Based on Morphological and Physiological Responses of Plant Hairy Roots

2003

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Assessment of Chemical Toxicity Based on Morphological and Physiological Responses of Plant Hairy Roots

（植物毛状根の形態的および生理的応答に基づいた薬剤毒性の評価）

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Preface

  This study was conducted under the direction of Professors Masahito Taya and Setsuji Tone at Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University from 1998 to 2003.

  The objective of this thesis is to evaluate responses of plant hairy roots against chemical stimuli on the basis of the morphological and physiological characteristics of the roots in order to apply the responses to the bioassay for assessing toxicities of chemicals existing in environments. The author hopes that the findings obtained in this work would offer the engineering fundamentals to construct the strategy for toxicity assessment using plant root organ.

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Contents

General Introduction

Recently, diversified chemical substances such as industrial solvents, detergents, housekeeping chemicals, pesticides and, on rare occasion, unidentified compounds generated unintentionally are discharged to the fields at quite low concentration and accumulated all around the environment. Pollution caused by these kinds of substances has become one of the most serious problems in aquatic and terrestrial surroundings. Facing to these situations, anxieties toward the acute and chronic impacts of these toxicants have been aroused from a viewpoint of assuring the human health as well as protection of animals and plants in the field. Against these kinds of problems, it could be the most effective to minimize the harms by means of detecting the toxicological information in the field before the problem rises to the surfaces and, if necessary, taking proper countermeasures to redeem the environment, which acts a fail-safe. In this context, it is essential to establish the easy-handle detection methods that can facilitate the preemptive assessment of the widespread chemicals as latent toxicants or the successive check of environment to cope with accidental emergence of the pollutants.

Nowadays, physicochemical analyses such as a gas chromatograph-mass spectrometry are mainly employed to assay the toxic substance in the field, providing the sophisticated methods for identification and quantification of chemicals. However, these methods require the expensive equipments and materials that need the laborious procedures for preliminary condensation and extraction in order to detect low concentration of the chemicals mentioned above. Therefore, the analyses are inevitably limited just for the sources of pollutants which are predicted to exist, and practically unsuitable to tracing the dynamic transition of the toxicants. To supplement these physicochemical methods, the analytical method based on biological responses against chemicals (bioassay) has been applied as a screen to detect in advance chemical contamination in aquatic and terrestrial surroundings. The bioassays are relatively inexpensive and simple methods, being capable of screening the sample including new compounds for which the physicochemical methods have less developed yet, only if the

chemical compounds in the sample are biologically active (Fawell and Horth, 1998; Buikema Jr. *et al.*, 1982; Shoji *et al.*, 2000). Since the biological methods can assess gross toxicity resulted from the mixed chemicals, and therefore the bioassays possess a great potential in terms of the preventive warning for the existence of the toxicants in the environment.

Table 1 lists the examples of the bioassay systems. There have been attempts to use a wide range of biological devices, varying from simple cells such as bacteria to aquatic vertebrates as test organisms, based on simple measurements at endpoints of individual cell death and grasp of information on the genetic materials of cells depending on the purposes. Tissue and cell cultures of animal and human origins have already been used as alternative bioassay systems for toxicological testing of various classes of chemicals, since bioassays using the mammalian cells, especially human cells, are direct devices in order to evaluate toxicities to human. Some problems to be solved are, however, associated with the use of vertebrate cell and tissue cultures. These include the limited applicability of undifferentiated cultures of animal/human cells due to their different nature from *in vivo* responses, and the instability of *in vitro* cultures differentiated cells. Furthermore, the cultures of animal/human-derived cells and tissues require complex culture media, and it is relatively difficult to maintain their stability during long-term tests (Lowe *et al.*, 1995).

All these difficulties, which usually necessitate complicated procedures as well as time-consuming and relatively costly tests, can be avoided if suitable plants and plant-derived seeds, organs, tissues and cells are used as toxicity screens. In general, higher plants are exposed to various environmental stimuli from nutrients, water, toxic chemicals, light and temperature as well as biological interactions with viruses, microorganisms, insects and so on. These stimuli induce specific responses in cells including the adaptation and/or protection of plants without moving ability. Plant materials such as cultured cells and excised organs have been often used as detecting devices in bioassay, and responses of the growth and metabolic activities have been monitored as clues for understanding ecological interactions between chemicals and

Table 1 Selected studies of bioassays using various organisms

Endpoints for toxicity of interest	Response parameters		
	Bacteria	Plant materials	Animal cells
Cytotoxicity			
Viability and other Metabolic activity	Bioluminescence (Jennings et al., 2001)	Photosynthetic activities (Thiemann et al., 1989)	MTT reduction (Olabarrieta et al., 2001)
	ATP luminescence (Dalzell and Christoffi, 2002)	Chlorophyll florescence (Merz et al., 1996)	ATP luminescence (Cree and Andreotti, 1997)
Proliferation	Cell density and colony formation (Zhu et al., 2001)	Cell number and weight (Grossmann et al., 1992)	Cell number (Yang et al., 2002)
Morphology		Size of parts (Momena et al., 2002)	Motion (Baba et al., 1988)
Genotoxicity			
Genetic damage	Reporter gene based-luminescence (Rettberg et al., 2001)	DNA strand breaks (Stavreva et al., 1998)	DNA strand breaks (Poli et al., 1999)
		Micronuclei (Ma et al., 1984)	Sister chromatid exchange (Giri et al., 2002)
Phenotypical mutation	Chemical resistance (Békaert et al., 1999)	Variation in color (Rodrigues et al., 1996)	Variation in color and shape (Inoue et al., 2001)

Notes in the parentheses indicate references. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

organisms (Kristen, 1997; Pestemer and Günther, 1995). The *in vitro* cultures of plant materials have been used as materials to grasp the biological responses of higher plants under artificially controlled conditions (Sato, 1986). Though the suspension cultures of plant cells like calli can be the choice for those purposes, they do not necessarily reflect the physiological features in differentiated and compartmentalized tissues, as found in roots or leaves.

Plant roots are known as a growing organ endowed with various functions including the absorption of water and nutrients. Moreover, their growth is markedly sensitive to chemical or physical factors and, therefore, growth of the root system is expected to be an indicator of the presence of nutrients, pollutants or herbicides in the root environment, which permits them to be a candidate suitable for the prediction of acute toxicities of various chemicals against human health (Kristen, 1997). Especially in the case of the transformed clones of roots induced by *Agrobacterium rhizogenes* (so-called hairy roots), as compared with normal excised root tissues, stable and reproducible growth can be achieved by means of the infinite propagation keeping their phenotype in a form of differentiated cells as adventitious roots under controlled culture conditions. Therefore the hairy roots enable to be a tool for the bioassay as well as a

Table 2 Examples of response parameters in bioassays using plant roots.

Response parameters	Plant species	References
Viability of apical cells	*Hordeum vulgare*	Pan *et al.* (2001)
Elongation at apical meristems	*Cucumis sativus*	Kratky and Warren (1971)
	Lolium perenne	Wong and Bradshaw (1982)
	Lepidium sativum	Grossmann *et al.* (1992)
	Pinus pinea	Arduini *et al.* (1994)
Ultrastracture of apical meritems	*Phaseolus vulgaris*	Fayez and Kristen (1996)
	Allium cepa	Chauhen *et al.* (1998)
Chromasome aberrations or miclonuclei in apical cells	*Allium cepa*	Chauhen *et al.* (1986)
	Vicia faba	Degrassi and Rizzoni (1982)
	Vicia faba	Ma *et al.* (1995)

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model system for the grasp of the growth and physiological properties of plant root tissues (Sanità Di Toppi *et al.*, 1999; Mugnier, 1988; Flores *et al.*, 1987).

Different from undifferentiated cells such as callus tissues, the plant roots possess unique proliferative properties of elongating at each apical meristems (growing points, GPs) and budding of tips as lateral roots. Furthermore, the linear growth manner of the respective single roots results in cellular age distribution along the longitudinal direction of the roots, as illustrated in the upper part of **Fig. 1**. However, almost all researches of bioassay using plant roots have been conducted by focusing on the responses at the position of the root apex as listed in **Table 2**, and there are few attempts to evaluate root responses against chemicals considering these features of the plant roots described above. In the present study, responses of the hairy roots against chemical stimuli are examined on the basis of the growth and physiological characteristics of the roots in order to apply the responses to the bioassay for assessment of toxicity in environments. For establishment of the bioassay system based on the biological responses of the hairy roots, the scope of this study is designed to include research areas illustrated in the lower part of Fig. 1. It covers two stages, namely, understanding of growth and physiological features in the hairy roots as the fundamental study, and application of the hairy roots as the tool for the bioassay. Part I comprising the first two themes covers the characterization of the proliferative potentials unique to the root systems taking into account the longitudinal changes in cell maturity; elongation potential at apical meritematic cells of the hairy roots described in Chapter 1, and tip budding potential from the hairy roots in Chapter 2. Part II which contains the last three themes deals with the evaluation of the hairy root responses against chemical stimuli based on the features of the roots examined in Part I; evaluation of elongating responses against chemical stimuli described in Chapter 3, responses of primary and lateral roots against chemical stimuli in Chapter 4 and responses against chemical stimuli at various longitudinal positions of the roots in Chapter 5. A general view of this work will be briefly discussed in the following paragraphs.

Characteristics of hairy roots

| Root elongation at apical meritems | Longitudinal change in cell maturity | Tip budding as lateral roots |

Part I
Characterization of growth and physiological properties

Chapter 1

Characterization of root elongating potential considering longitudinal cell maturity

Chapter 2

Characterization of tip budding potential considering longitudinal cell maturity

Part II
Evaluation of responses to chemical stimuli and application of bioassay

Chapter 3

Evaluation of responses to chemical stimuli based on root elongation

Chapter 5

Evaluation of responses to chemical stimuli at different longitudinal positions

Chapter 4

Evaluation of responses to chemical stimuli in primary and lateral roots

Composition of this study

Fig. 1 Composition of this study for developing bioassay system based on characteristics of plant hairy roots

In the first chapter, changes in the root elongation are examined under various culture conditions using the heterotrophic and photoautotrophic hairy roots of pak-bung to understand the property of the root elongation at the tips. The elongating potentials of these hairy roots are discussed in terms of the ATP contents in the tip parts and carbon balance considering the gradient of chlorophyll content in the longitudinal direction of photoautotrophic roots.

Chapter 2 covers the changes in positional distribution in the potential of lateral root budding as well as protoplast size and oxygen uptake rate investigated using the segments prepared from the hairy roots of madder and horseradish. The kinetic behavior of lateral root budding is also described in terms of oxygen uptake rate of the segmented hairy roots.

The second part of the study, which extendeds the availability of the hairy roots as the sensing tool, begins at Chapter 3. The elongating responses of the heterotrophic and photoautotrophic hairy roots of pak-bung are examined against model chemicals with distinct herbicidal mechanisms. The elongating behavior of hairy roots is evaluated by automatic tracing of the root tip points with an observation system assisted by image analysis to achieve sensitive and prompt measurement.

Chapter 4 deals with the comparisons of hairy root responses against chemicals between the primary and lateral roots which are examined during successive cultures of the heterotrophic hairy roots of pak-bung. The varied responses of the lateral roots are discussed from the aspects of phenotypic and genotypic features.

In the last chapter, the responses of the local chlorophyll pigmentation in the photoautotrophic roots of pak-bung against herbicides with different modes of action are examined by non-destructive measurement employing a color image analysis. A methodology is also proposed to assess the toxicities of individual herbicides as well as field water as a model pollutant on the basis of both the responses of root elongation and local chlorophyll pigmentation of the roots.

PART I

CHARACTERIZATION OF PROLIFERATIVE POTENTIALS OF HAIRY ROOTS TAKING ACCOUNT OF PHYSIOLOGICAL PROPERTIES

The hairy roots possess unique proliferative properties of elongating at respective apical meristems and budding of tips as lateral roots, and their linear growth manner of the each single root results in cellular age distribution along the longitudinal direction of the roots. They can be clues to find out ecological interactions between chemicals and the hairy roots. For the usage of hairy roots as a measure for estimation of environmental stress, it is desirable to understand these growth and physiological features of the roots under varied culture conditions.

In this part, the characterization of the hairy root properties as the fundamental study was conducted for application of the roots as the tool of the bioassay. The first two chapters in this study were included in this part, describing both the potentials of root elongation and tip budding, respectively, considering changes in cell maturity along the single root. In Chapter 1, changes in root elongating potentials were examined under the various culture conditions, and discussed from a viewpoint of the ATP and carbon balance in the roots. In Chapter 2, changes in tip budding potentials were examined at various longitudinal positions of the single root, and correlated with the oxygen uptake rate of the roots.

Chapter 1

Characterization of Root Elongating Potentials
in Terms of ATP and Carbon Balances in Hairy Roots

1.1 Introduction

The *in vitro* cultures of isolated plant cells have been developed to investigate the responses in plants to the environmental stimuli under controlled conditions. Especially, photoautotrophic cell lines of plants have been induced in the cultures of calli or suspension cells (Yamada and Sato, 1978; Yasuda *et al.*, 1980), since they could be model materials for field-grown plants in biological approaches to understand the changes of photosynthetic functions in response to environmental factors (Sato, 1986).

Plant roots naturally proliferate underground as heterotrophic organs, depending on the shoots and leaves for their energy sources. In spite of the inherent property of roots, a photoautotrophic line of pak-bung hairy roots was derived from heterotrophic ones (Nagatome *et al.*, 2000a). The induced hairy roots possessed high photosynthetic potential as compared with those cultivated under both the heterotrophic and photomixotrophic culture conditions.

In the cultures of the hairy roots, their growth uniquely occurs by means of elongation at apical meristems, implying that proliferative potentials of hairy roots rely on the activities of cells exhibiting in the root tip parts. In this chapter, the root elongation and ATP transition in the tip parts were examined under various culture conditions using the heterotrophic and photoautotrophic hairy roots of pak-bung. The elongating potentials of these roots were then characterized in terms of the ATP contents, and cell yield of elongating roots and maintenance energy were discussed based on the changes in ATP contents. Especially for the photoautotrophic hairy roots, the changes in the elongating potential of the roots were argued with respect to carbon balance considering the gradient of chlorophyll content found in the direction of root length.

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1.2 Experimental

1.2.1 Plant materials

The heterotrophic (HT) and photoautotrophic (PT) hairy root lines of pak-bung (*Ipomoea aquatica*) were used for the experiments. These hairy roots have been established in the previous studies (Taya *et al.*, 1989b; Nagatome *et al.*, 2000a). The HT hairy roots were maintained by means of subculturing at 2 week's interval using a 200 cm^3-conical glass flask containing 100 cm^3 of Murashige-Skoog (MS) liquid medium (Murashige and Skoog, 1962) with 20 kg/m^3 sucrose. The PT hairy roots were maintained through subcultures at 4 week's interval using a 125 cm^3-conical glass flask containing 50 cm^3 of sugar-free MS liquid medium. The liquid cultures in flasks were conducted at 25 °C with 100 rpm shaking on a rotary shaker (Model NR-30, TAITEC Co., Saitama, Japan) kept in a dark room (HT hairy roots) or under an atmosphere of 5.0 % carbon dioxide and continuous light irradiation at 11 W/m^2 (PT hairy roots). The cultured masses of HT and PT hairy roots are shown in **Fig. 1.1**.
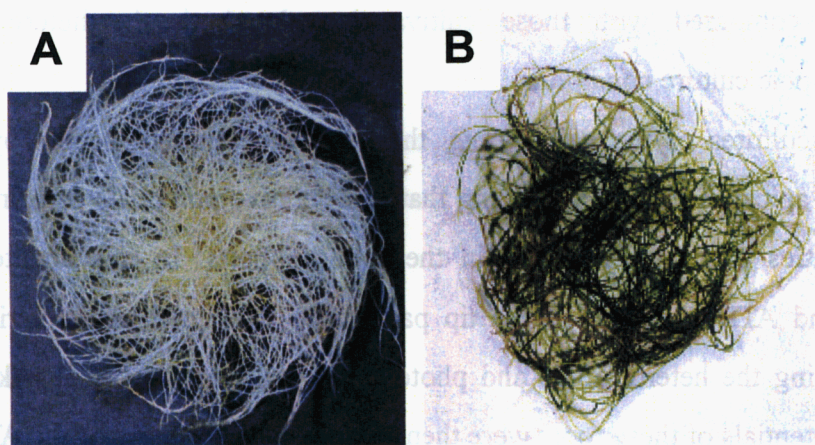

Fig. 1.1 Photographs of pak-bung hairy roots. (A) HT hairy roots and (B) PT hairy roots.

1.2.2 Preparation of root inocula for solid cultures

To prepare inocula of the HT roots with various ATP contents in the tip parts, the hairy roots were subjected to the conditioning culture for 48 h on the solid media with 2.5, 5, 10 or 20 kg/m^3 sucrose, followed by trimming of lateral roots and preparing the segment of single root with a fixed length from tip of $L_0 = 3.0 \times 10^{-2}$ m (see **Fig. 1.2**). In the case of the PT roots, the conditioning culture was conducted for 48 h on the sucrose-free solid medium kept under the same conditions as mentioned above, and the segment of single root with a prescribed length of L_0 was prepared as an inoculum. This operation for trimming and cutting was conducted in a laminar flow hood under sterile condition. ATP contents in the tip parts ($\hat{C}_{ATP}$) were $8.1 \times 10^{-5} - 3.6 \times 10^{-4}$ and 1.9×10^{-4} moles of ATP per unit length, respectively, for the segments prepared from the HT and PT roots.

1.2.3 Solid culture conditions

In the experiments, the prepared root segments were incubated at 25 °C on Petri dishes containing the MS medium solidified with 2 kg/m^3 Gelrite® (San-ei Gen F. F. I. Inc., Osaka, Japan). In both cultures of the HT and PT roots, sucrose as a carbon and energy source was omitted from the medium. The dish with the HT roots was kept in a usual dark chamber, and one with the PT roots was placed under an atmosphere with 5.0 % CO_2 in an incubator (Model CFH-305, Tomy Seiko Co., Ltd., Tokyo, Japan) while being irradiated by fluorescent lumps (FL 20SSW, Matsushita Electric Industrial Co., Ltd., Osaka, Japan). The incident light intensity (I) was adjusted at 1.0, 3.3 and 11 W/m^2 by changing the distance between lamps and dishes. To investigate the influence of photosynthesis inhibitor on the root elongation, 0.5 $\mu mol/dm^3$ of 3-(3,4-dichloro-phenyl)-1,1-dimethylurea (DCMU) was added to the medium according to the procedure described elsewhere (Horn *et al.*, 1983).

1.2.4 Analytical methods

Light intensity was measured with an illuminometer (DX-100, Takemura Electric Works Ltd., Tokyo, Japan) on the outer wall of flask at the level of the medium surface in the liquid culture or at the top of Petri dish in the solid culture.

HT hairy roots

Liquid culture of hairy roots

Preparation of root segments

Conditioning culture on plate containing 2.5, 5, 10 or 20 kg/m³ sucrose

Lateral root

Lateral root

GP →

$L_0 = 3.0 \times 10^{-2}$ m

Solid culture of root segments

Elongation at GP

Sucrose-free plate

PT hairy roots

$I = 11$ W/m²

$I = 11$ W/m²

Conditioning culture on sucrose-free plate

GP →

$L_0 = 1.0 \times 10^{-2} - 7.0 \times 10^{-2}$ m

$I = 1.0, 3.3$ and 11 W/m²

DCMU added when necessary
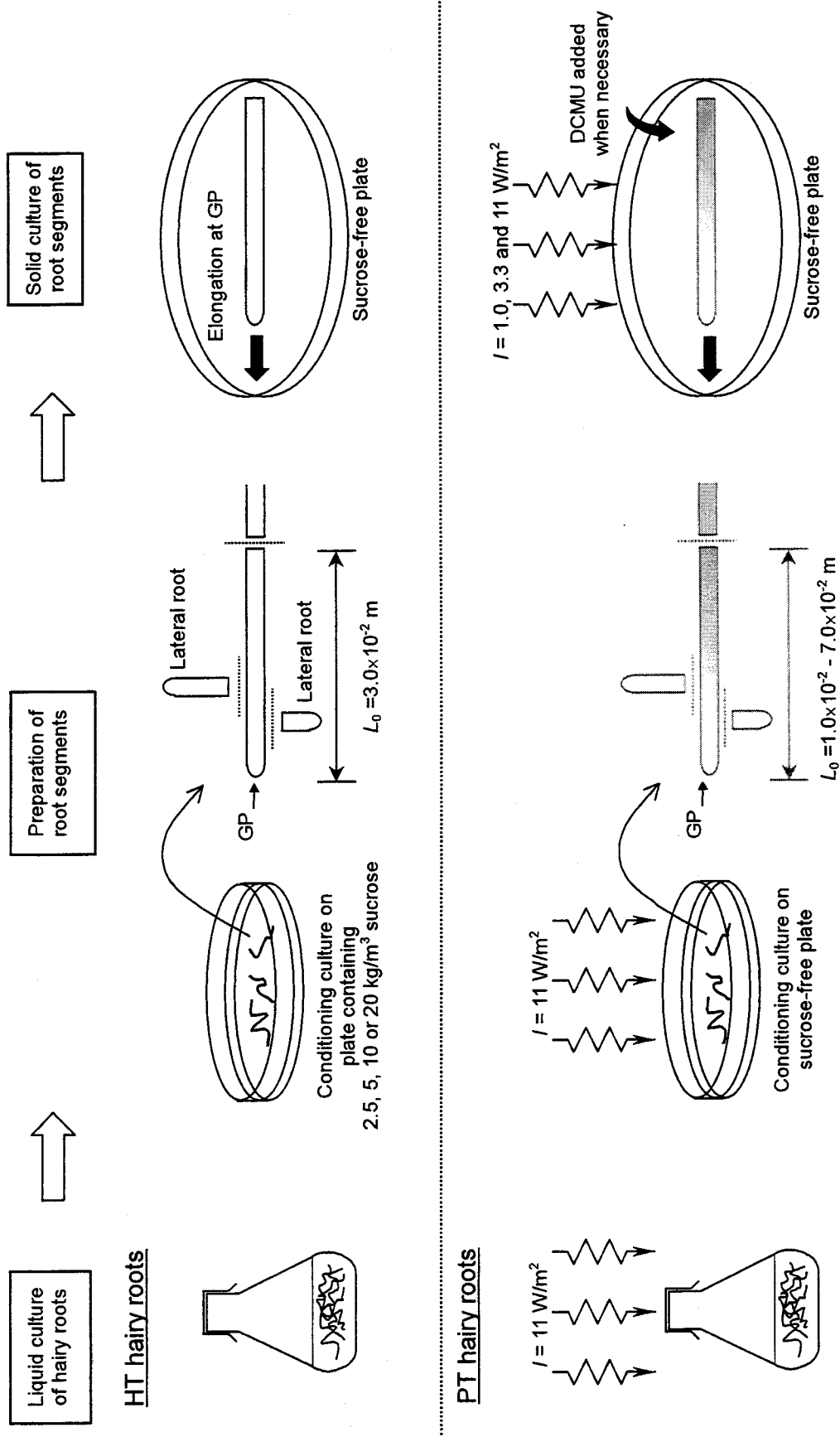
Sucrose-free plate

Fig. 1.2   Schematic drawing of culture operation of HT and PT roots.

In the course of the hairy root cultures, elongation length of each root tip of the segment ($\Delta L = L - L_0$) was measured by manually tracing the change in a position on the solid medium during indicated culture period, using the images of roots acquired through a lens (V6X16-1.9 Macro Lens, Canon Inc., Tokyo, Japan) connected to a charge-coupled device (CCD) camera (HCC-600, Flovel Co., Ltd., Tokyo, Japan) (see in **Fig. 1.3**). Root elongation rate at GPs (R_G) was recorded by dividing the ΔL value by culture time. Time of ceasing in root elongation (t_E) was determined as a point at which root elongation rate fell to $R_G = 5.0 \times 10^{-6}$ m/h. For evaluation of the t_E value, the elongation rate was recorded through continuous observation of the individual root segments on the plates at measurement interval of 0.2 h using an image-analyzing tool as described in following chapter.
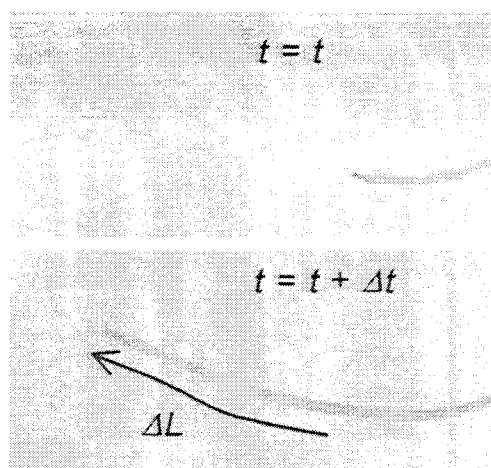
Fig. 1.3 Photographs of excised single root elongating at its tip during culture.

ATP content in the roots was measured by the combined use of an ATP Bioluminescence Assay kit (HS II, Roche Diagnostics GmbH, Mannheim, Germany) and a Micro-plate Reader (Wallac 1420 ARVOsx Multilabel counter, PerkinElmer Life Sciences Japan Co., Ltd., Kanagawa) as per to the suppliers' instructions. For the ATP assay, the tip parts with length of $\hat{L} = 2.0 \times 10^{-3}$ m were collected by excising from the

root segments cultivated on the plates. The data for ΔL, t_E and $\hat{C}_{ATP}$ were mean values of ten measurements made under the respective conditions.

The average CO_2 fixation rate and chlorophyll (Chl) content of the roots were measured according to the methods described elsewhere (Nagatome *et al.*, 2000a; Porra *et al.*, 1989), and recorded as a dry weight (DW) basis of the roots. Unless otherwise noted, gross amount of Chl in a single roots was measured spectrophotometrically, and the DW of the hairy roots was determined by means of gravimetric measurement after drying the roots at 80 °C for 72 h.

1.3 Results and Discussion

1.3.1 Changes in elongating potential of HT hairy roots under carbohydrate starvation

At first, the elongating potential of the HT roots was examined on the sucrose-free solid medium for 48 h using the HT root segments with various values of initial ATP contents ($\hat{C}_{ATP,0}$ = 8.1×10^{-5}, 1.5×10^{-4}, 2.8×10^{-4} and 3.6×10^{-4} mol-ATP/m). As shown in **Table 1.1**, the segments with $\hat{C}_{ATP,0}$ = 2.8×10^{-4} and 3.6×10^{-4} mol-ATP/m exhibited tip elongation, namely ΔL = 1.3×10^{-3} and 2.2×10^{-3} m for 48 h respectively, while no substantial elongation was observed in the cultures of root segments with $\hat{C}_{ATP,0}$ = 8.1×10^{-5} and 1.5×10^{-4} mol-ATP/m. These results imply that the tip elongating potential of the HT roots rests on the ATP content in the root tip parts

Figure 1.4A shows typical time profiles of the ΔL value during the cultures of the HT root segments with $\hat{C}_{ATP,0}$ = 2.8×10^{-4} and 3.6×10^{-4} mol-ATP/m. In each case, the tip elongating potential was found to stop substantially after given culture time of t_E, displaying the saturated profile of ΔL. As listed in Table 1.1, the values of t_E were determined as 16 and 24 h for the root segments with $\hat{C}_{ATP,0}$ = 2.8×10^{-4} and 3.6×10^{-4} mol-ATP/m, respectively (not determinable in the cases of $\hat{C}_{ATP,0}$ = 8.1×10^{-5} and 1.5×10^{-4} mol-ATP/m).

Table 1.1 Measured and estimated values during cultures of HT roots with different ATP contents in root parts

$\hat{C}_{ATP,0} \times 10^4$ [mol-ATP/m]	Measured value				Estimated value	
	$\Delta L \times 10^3$ [m]	t_E [h]	$\hat{C}_{ATP,E} \times 10^4$ [mol-ATP/m]	$\tilde{\mu} \times 10^2$ [h^{-1}]	$m_{ATP} \times 10^6$ [mol-ATP/(h·m)]	$Y_{ATP} \times 10^4$ [m/mol-ATP]
0.81 ± 0.43	nil	ND	ND	nil	2.4	ND
1.5 ± 0.69	nil	ND	ND	nil	2.4	ND
2.8 ± 0.23	1.3 ± 0.19	16 ± 3.5	1.8 ± 0.23	4.1	2.4	1.1
3.6 ± 0.46	2.2 ± 0.20	24 ± 3.1	2.0 ± 0.46	4.6	2.4	1.2

The values of ΔL and $\tilde{\mu}$ were determined for $t = 0$ to t_E. For the estimation of Y_{ATP}, the following values were used in Eq. (1.2): $\Delta \hat{C}_{ATP} / \Delta t$ = -6.2×10^{-6} and -6.3×10^{-6} mol-ATP/(h·m) for the root segments with $\hat{C}_{ATP,0}$ = 2.8×10^{-4} and 3.6×10^{-4} mol-ATP/m, respectively (see the dotted lines in Fig.1.4B). ND: Not determinable.

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Concerning the ATP content, as shown in Fig. 1.4B, the $\hat{C}_{ATP}$ value of each root segment gradually decreased with the progress of culture time, reaching almost zero within examined time up to $t = 120$ h. Moreover, it was found that the values of $\hat{C}_{ATP}$ at $t = t_E$ ($\hat{C}_{ATP,E}$) were 1.8×10^{-4} (for $\hat{C}_{ATP,0} = 2.8 \times 10^{-4}$ mol-ATP/m) and 2.0×10^{-4} mol-ATP/m (for $\hat{C}_{ATP,0} = 3.6 \times 10^{-4}$ mol-ATP/m), which were close to each other irrespective of the $\hat{C}_{ATP,0}$ values (see the closed keys in Fig. 1.4B). Together with the results that no root elongation occurred in the cases of $\hat{C}_{ATP,0} = 8.1 \times 10^{-5}$ and 1.5×10^{-4} mol-ATP/m, it was therefore considered that the $\hat{C}_{ATP}$ value of approximately 2.0×10^{-4} mol/m was a critical level for the HT roots to ensure the tip elongation.
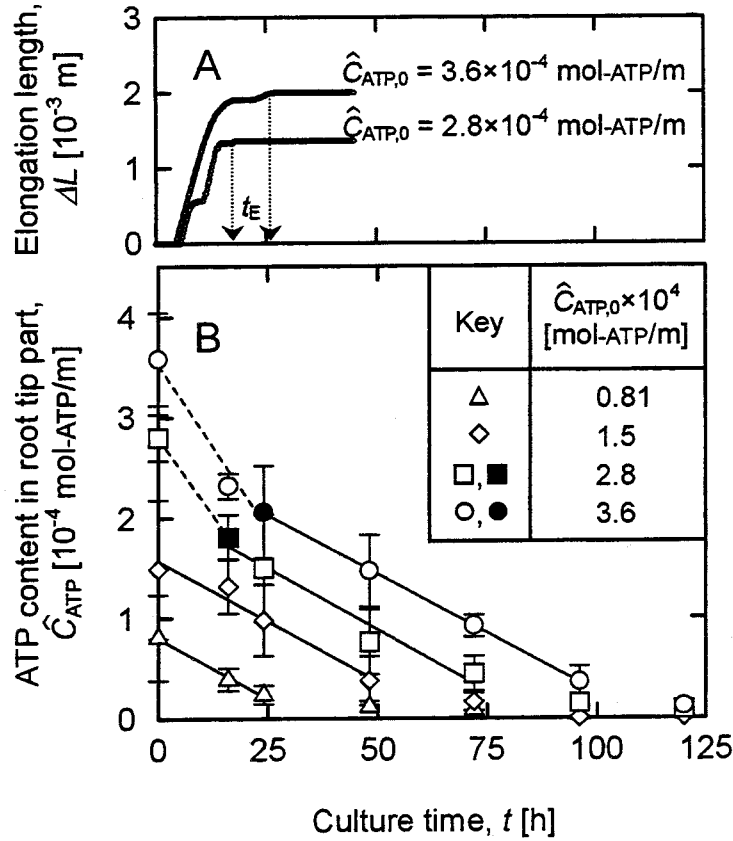

Fig. 1.4 Typical time profiles of root elongation (A) and changes of ATP contents in tip parts (B) during cultures of HT roots. The solid lines were fitted by Eq. (1.2) with $\tilde{\mu} = 0$. The closed keys show the $\hat{C}_{ATP}$ values at $t = t_E$. Vertical bar indicates standard deviation.

1.3.2 Changes in elongating potential of PT hairy roots under photoautotrophic conditions

In preliminary experiments, it was found that the PT hairy roots exhibited the dependencies in elongating potential not only on light intensity but also their lengths including root tip meristems. **Table 1.2** shows the ΔL values while the prepared root inocula with two lengths of $L_0 = 3.0 \times 10^{-2}$ and 7.0×10^{-2} m were cultivated for 120 h on the solid medium at light intensities of $I = 1.0$, 3.3 and 11 W/m^2. In the cultures of the single root with $L_0 = 3.0 \times 10^{-2}$ m, the substantial elongation of root tips was observed only at $I = 11$ W/m^2. In the case of $L_0 = 7.0 \times 10^{-2}$ m, on the other hand, the root tip elongation occurred under the three light conditions, and the ΔL values increased with an increase in light intensity. These results suggest that overall light energy captured by the cells limits the elongating potential of the PT hairy roots under the conditions examined in the present study.

The influence of the L_0 value on the root elongation was then investigated in detail. **Figure 1.5** shows the elongation rate in the cultures of the single roots at $I = 11$ W/m^2. A threshold of the R_G value was seen in terms of the L_0 value, the R_G value being zero below $L_0 = 2.0 \times 10^{-2}$ m. The R_G value increased gradually with increasing L_0 value over $L_0 = 2.0 \times 10^{-2}$ m and reached 8.4×10^{-5} m/h at $L_0 = 6.0 \times 10^{-2}$ m. The difference of R_G values at $L_0 = 5.0 \times 10^{-2}$ and 6.0×10^{-2} m was not significant, which may mean that the

Table 1.2 Elongation length of root segments in cultures of PT hairy roots under various light conditions

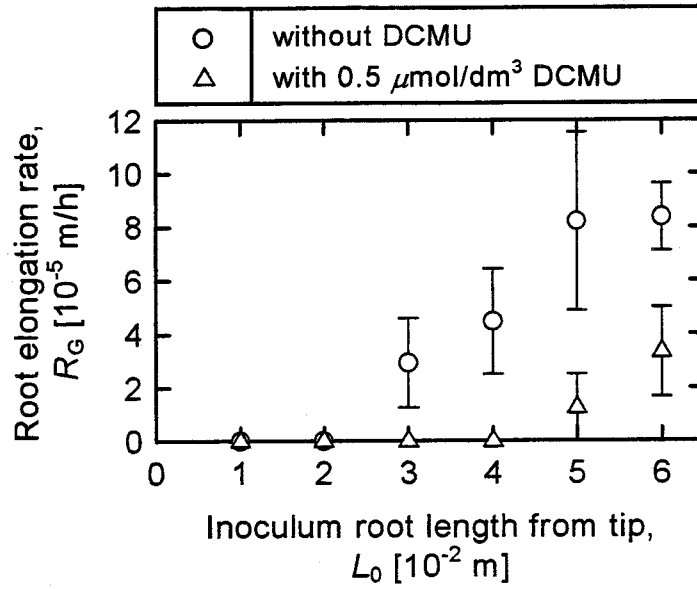| I [W/m^2]	ΔL [10^{-3} m]	
	$L_0 = 3.0 \times 10^{-2}$ m	$L_0 = 7.0 \times 10^{-2}$ m
1.0	nil	3.5 ± 0.9
3.3	nil	6.0 ± 1.6
11	2.9 ± 0.9	8.9 ± 1.2

| O | without DCMU |
| △ | with 0.5 μmol/dm^3 DCMU |

Fig. 1.5 Relationships of root elongation rate and inoculum root length in PT hairy roots. The cultures were conducted at $I = 11$ W/m^2. The vertical bar indicates standard deviation.

elongating potential of root segments was close to biological limitation of cell division in the apical meristems of the roots with these length. The value of $L_0 = 2.0 \times 10^{-2}$ m was regarded as an observed minimum root length for the root elongation ($L_{0,min}$) at $I = 11$ W/m^2. Similar relations between R_G and L_0 values were obtained in the cultures under the other light conditions (data not shown) and the values of $L_{0,min}$ were determined to be 5.0×10^{-2} and 4.0×10^{-2} m at $I = 1.0$ and 3.3 W/m^2, respectively.

As shown in Fig. 1.5, the cultures were also conducted on the solid medium containing 0.5 μmol/dm^3 DCMU under light condition of $I = 11$ W/m^2. As compared with the cultures without DCMU, the R_G values were appreciably lowered over the examined range of L_0 values (e.g., $R_G = 3.3 \times 10^{-5}$ m/h at $L_0 = 6.0 \times 10^{-2}$ m), and in addition the $L_{0,min}$ value shifted to a larger point of $L_{0,min} = 4.0 \times 10^{-2}$ m. It is thus apparent that there is a minimum length to permit the elongation of tip meristems of the PT hairy root cells, being dependent on the photosynthetic potential of the roots under a given condition.
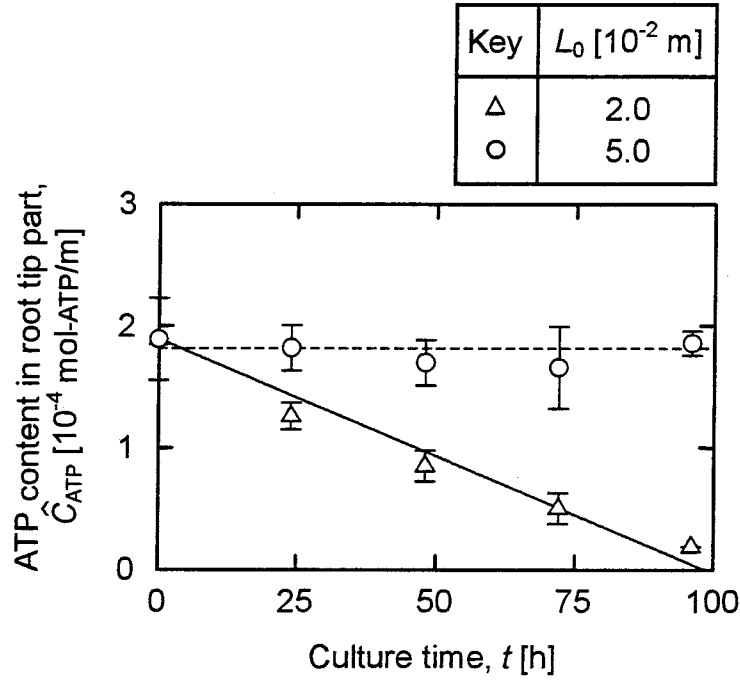
Key	L_0 $[10^{-2}$ m]
△	2.0
○	5.0

Fig. 1.6 Changes of ATP contents in tip parts during cultures of PT roots. The lines were fitted by Eq. (1.4) with $\tilde{\mu}$ = 0 (solid line) and $\Delta \hat{C}_{ATP} / \Delta t$ = 0 (dotted line). The cultures were conducted at I = 11 W/m^2. Vertical bar indicates standard deviation.

With respect to the ATP content in the tip parts, the changes in the $\hat{C}_{ATP}$ value were traced during the cultures at light intensity of I = 11 W/m^2 for 96 h, using the PT root segments with L_0 = 2.0×10^{-2} and 5.0×10^{-2} m. In the case of L_0 = 5.0×10^{-2} m, as found in **Table 1.3** and **Fig. 1.6**, the ΔL value was 6.0×10^{-3} m for 96 h, and the $\hat{C}_{ATP}$ value was kept almost constant at 1.7×10^{-4} – 1.9×10^{-4} mol-ATP/m throughout the examined culture period. On the other hand, in the culture of the segment with L_0 = 2.0×10^{-2} m, the $\hat{C}_{ATP}$ value decreased linearly with elapsed culture time and no substantial root elongation was recognized during course of the culture. These results suggested that the amount of ATP supplied by photosynthesis balanced with that of ATP requirement for cell growth and maintenance in the root segment with L_0 = 5.0×10^{-2} m, while in the case of L_0 = 2.0×10^{-2} m the ATP supply did not meet the requirement for cell growth, being consumed only for maintenance energy.

Table 1.3 Measured and estimated values during cultures of PT roots with different segment lengths

$L_0 \times 10^2$ [m]	Measured value			Estimated value	
	$\Delta L \times 10^3$ [m]	$A_{Chl} \times 10^6$ [g]	$\tilde{\mu} \times 10^2$ [h^{-1}]	$m_{ATP} \times 10^6$ [mol-ATP/(h·m)]	$Y_{ATP} \times 10^4$ [m/mol-ATP]
2.0	nil	0.12	nil	2.2	ND
5.0	6.0 ± 1.8	2.9	3.6	ND	1.2

The values of ΔL and $\tilde{\mu}$ were determined for $t = 0$ to 96 h. For the estimation of m_{ATP} and Y_{ATP}, the following values were used in Eq. (1.4): $\Delta \hat{C}_{ATP} / \Delta t = -1.7 \times 10^{-6}$ mol-ATP/(h·m) for the root segments with $L_0 = 2.0 \times 10^{-2}$ (see the solid line in Fig. 1.6), and $\psi_{ATP} = 8.0 \times 10^{-2}$ mol-ATP/(h·g-Chl). ND: Not determinable.
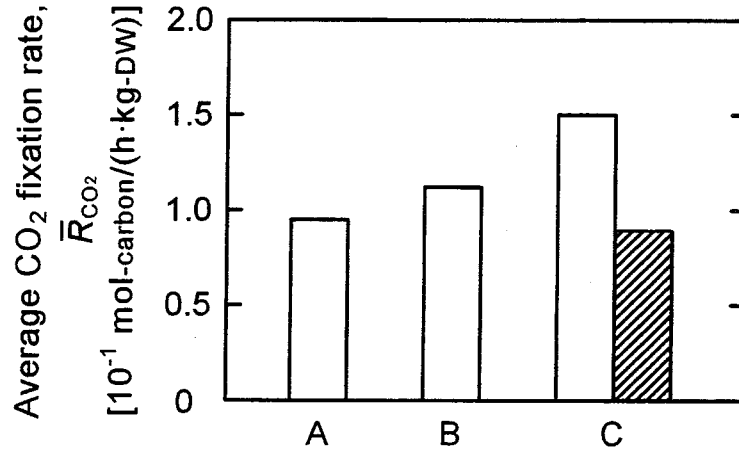
Fig. 1.7 Average CO_2 fixation rate of PT hairy roots under various conditions. (A) $I = 1.0$ W/m^2, (B) $I = 3.3$ W/m^2, (C) $I = 11$ W/m^2 (shaded bar: with 0.5 μmol/dm^3 DCMU).

To evaluate the photosynthetic activity of PT hairy roots of pak-bung, average CO_2 fixation rate ($\overline{R}_{CO_2}$) was measured under varied light conditions. In the present study, the $\overline{R}_{CO_2}$ value was expressed as the longitudinal mean of CO_2 fixation rate determined using the whole hairy roots. As shown in **Fig. 1.7**, the $\overline{R}_{CO_2}$ value was enhanced in response to an increase in light intensity, i.e., $\overline{R}_{CO_2}$ = 0.095, 0.11 and 0.15 mol-carbon/(h·kg-DW) at I = 1.0, 3.3 and 11 W/m^2, respectively. It was also demonstrated that the $\overline{R}_{CO_2}$ value measured at I = 11 W/m^2 fell to 8.9×10^{-2} mol-carbon/(h·kg-DW) in the presence of DCMU. These results are consistent with the retarded elongating potential of the hairy roots in the cultures at the lower I value or with the addition of DCMU as indicated in Table 1.2 and Fig. 1.5.

1.3.3 ATP balance in HT and PT hairy roots

To compare between the HT and PT roots in terms of ATP cost for catabolic and anabolic metabolisms, maintenance energy (m_{ATP}) and cell yield of elongating roots (Y_{ATP}) were estimated on an ATP basis. In the absence of a carbon and energy source in the culture of the HT roots (no sucrose), as illustrated in **Fig. 1.8**, mass balance for ATP at the tip part with length $\hat{L}$ yields the following equation.

$$-\frac{\Delta \hat{A}_{ATP}}{\Delta t} = \left(\frac{1}{Y_{ATP}}\right)\left(\frac{\Delta L}{\Delta t}\right) + m_{ATP} \cdot \hat{L} \qquad (1.1)$$

where $\hat{A}_{ATP}$ is ATP amount in the tip part with length $\hat{L}$. Based on Eq. (1.1), the change in the $\hat{C}_{ATP}$ value shown in Fig. 1.4B is correlated with the average value of specific elongation rate ($\tilde{\mu}$), as follows.

$$-\frac{\Delta \hat{C}_{ATP}}{\Delta t} = \frac{\tilde{\mu}}{Y_{ATP}} + m_{ATP} \qquad (1.2)$$

Hence, the $\tilde{\mu}$ value for the HT roots is given by the following equation, using root length at $t = t_E$ (L_E).

$$\tilde{\mu} = \frac{1}{\hat{L}} \cdot \frac{L_E - L_0}{t_E} \qquad (1.3)$$

where $\hat{L} = 2.0 \times 10^{-3}$ m in the present study.

For the HT roots, the m_{ATP} value was determined from the slopes of the $\hat{C}_{ATP}$ transition in the range of 2.0×10^{-5} mol-ATP/m $\leq \hat{C}_{ATP} \leq 2.0 \times 10^{-4}$ mol-ATP/m where the $\tilde{\mu}$ value was assumed to be zero, as depicted by the solid lines in Fig. 1.4B. The values of m_{ATP} for the HT roots were 2.4×10^{-6} mol-ATP/(h·m) regardless of the $\hat{C}_{ATP,0}$
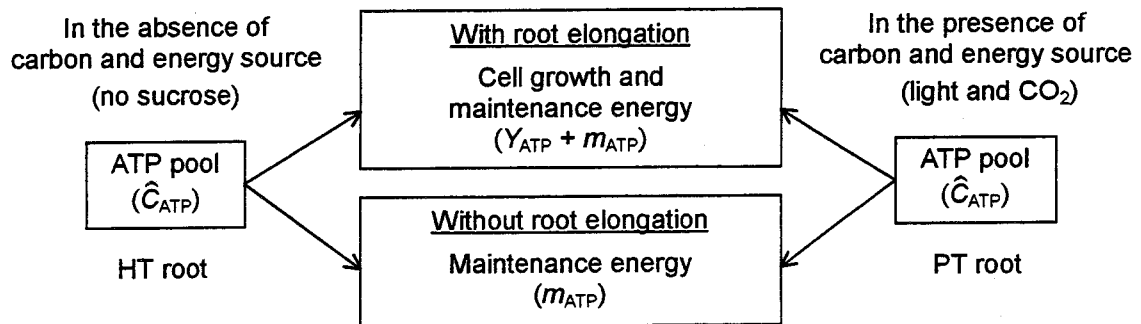

Fig. 1.8 ATP requirements for root elongation and maintenance energy in HT and PT roots under examined conditions

values examined in the present study. Moreover, the values of Y_{ATP} for the HT roots were determined as 1.1×10^4 and 1.2×10^4 m/mol-ATP in the cases of $\hat{C}_{ATP,0} = 2.8 \times 10^{-4}$ and 3.6×10^{-4} mol-ATP/m, respectively, based on Eq. (1.2) using the m_{ATP} value as well as the values of $\Delta \hat{C}_{ATP}/\Delta t$ (see the dotted lines in Fig. 1.4B) and $\tilde{\mu}$ obtained for $t = 0 - t_E$ (not determinable for $\hat{C}_{ATP,0} = 8.1 \times 10^{-5}$ and 1.5×10^{-4} mol-ATP/m). Table 1.1 summarizes the estimated values of m_{ATP} and Y_{ATP} together with the data used for the estimation in the case of the HT roots. It was found that the respective values of m_{ATP} and Y_{ATP} estimated for the HT roots were comparable with each other under the examined conditions.

To assess the change in the $\hat{C}_{ATP}$ value with respect to the PT roots, the ATP acquired through photosynthesis was considered, as illustrated in Fig. 1.8. In the present study, the rate of ATP supply to apical meristems from photosynthetic cells was assumed to be proportional to gross chlorophyll amount in the root segment (A_{Chl}) changing with the length from tip. Then, the change in the $\hat{C}_{ATP}$ value for the PT roots is expressed by the following equation.

$$\frac{\Delta \hat{C}_{ATP}}{\Delta t} = \left(\frac{\psi_{ATP} \cdot A_{Chl}}{L_0} \right) - \left(\frac{\tilde{\mu}}{Y_{ATP}} + m_{ATP} \right) \tag{1.4}$$

where the first and second terms in the right hand represent the rates of ATP formation and consumption, respectively. In the present study, the coefficient ψ_{ATP} was determined as 8.0×10^{-2} mol-ATP/(h·g-Chl) from separate experiments where the changes of ATP content under light and dark conditions were measured using the tip-excised PT root segments without root elongation.

In the case of the PT root segment with $L_0 = 2.0 \times 10^{-2}$ m, the m_{ATP} value was estimated as 2.2×10^{-6} mol-ATP/(h·m), employing Eq. (1.4) with the values of $A_{Chl} = 1.2 \times 10^{-7}$ g and $\tilde{\mu} = 0$ (see solid line in Fig. 1.6). With respect to $L_0 = 5.0 \times 10^{-2}$ m, moreover, the Y_{ATP} value was estimated as 1.2×10^4 m/mol-ATP from Eq. (1.4) using the values of $\tilde{\mu} = 3.6 \times 10^{-2}$ h^{-1} and $A_{Chl} = 2.9 \times 10^{-6}$ g in addition to the estimated m_{ATP} value of 2.2×10^{-6} mol-ATP/(h·m), where $\Delta \hat{C}_{ATP}/\Delta t$ was assumed to be zero as shown

by the dotted line in Fig. 1.6. In the cultures of the PT roots, the values of m_{ATP} and Y_{ATP} and the data used for the estimation are summarized in Table 1.3. From these results, it was found that the values of m_{ATP} and Y_{ATP} for the PT roots were closely coincident with those for the HT roots.

1.3.3 Carbon balance in PT hairy roots

In pak-bung hairy roots cultivated under light irradiation, the longitudinal change in sectional Chl content (C_{Chl}) is observed along with root length, owing to cell age distribution arising from a linear growth mode of the roots. With respect to the photoautotrophic hairy roots, in the present study, the value of C_{Chl} at the distance from root tip of l was given by the following equations based on the kinetic expression described in Appendix.

$$C_{Chl}(l) = \begin{cases} 0 & (0 \leq l \leq L_G) & (1.5) \\ C_{Chl,S}\,[1 - \exp\{-k(l - L_G)\}] & (L_G < l) & (1.6) \end{cases}$$

where k is the kinetic parameter concerning Chl formation and cell maturation. The A_{Chl} value in the single root with length L_0 was calculated by the following integral formula, assuming that a single root is cylindrical with diameter of D (Taya $et\ al.$, 1989a).

$$A_{Chl} = \frac{\pi\,\rho\,(1-w)D^2}{4} \int_0^{L_0} C_{Chl}(l)\,dl \qquad (1.7)$$

CO_2 fixation rate throughout the root segment with length L_0 (r_{CO_2}) was correlated with the value of A_{Chl} as follows.

$$r_{CO_2} = \psi_C\,A_{Chl} \qquad (1.8)$$

where the coefficient ψ_C denotes the photosynthetic activity on a Chl basis. In the present study, the ψ_C value was determined from the data shown in Fig. 1.7 using the mean value of Chl content in the whole hairy roots ($\overline{C}_{Chl} = 5.6$ g-Chl/kg-DW) used for the experiments. The values of ψ_C determined under the respective conditions are presented in **Table 1.4**.

Table 1.4 Values of parameters and constants used for calculation of carbon balance of PT roots

$C_C = 5.1 \times 10^2$ g-carbon/kg-DW

$C_{Chl,S} = 8.9$ g-Chl/kg-DW

$\overline{C}_{Chl} = 5.6$ g-Chl/kg-DW

$D = 1.0 \times 10^{-3}$ m

$k = 5.6 \times 10$ m^{-1}

$L_G = 5.0 \times 10^{-4}$ m

$w = 0.95$

$\rho = 1.01 \times 10^3$ kg-FW/m^3

$$\psi_C = \begin{cases} 1.7 \times 10^{-2} \text{ mol - carbon/(h·g - Chl)} & (I = 1.0 \text{ W/m}^2) \\ 2.0 \times 10^{-2} \text{ mol - carbon/(h·g - Chl)} & (I = 3.3 \text{ W/m}^2) \\ 2.7 \times 10^{-2} \text{ mol - carbon/(h·g - Chl)} & (I = 11 \text{ W/m}^2) \\ 1.6 \times 10^{-2} \text{ mol - carbon/(h·g - Chl)} & \\ \quad (I = 11 \text{ W/m}^2 \text{ with } 0.5 \text{ } \mu\text{mol/dm}^3 \text{ DCMU}) \end{cases}$$

To determine the $C_{Chl,S}$ and k values in Eq. (1.6), the longitudinal distribution of the Chl content in the PT hairy roots were measured by using the roots divided into several pieces cut at the different positions from root tips. As shown in **Fig. 1.9A**, the value of C_{Chl} increased with an increase in the l value, and Eqs. (1.5) and (1.6) were matched to these data by the non-linear squares method using $L_G = 5.0 \times 10^{-4}$ m. The solid line in Fig. 1.9A followed the experimental data with a correlation coefficient of 0.97 and the estimated values of $C_{Chl,S}$ and k are listed in Table 1.4.

The value of r_{CO_2} was regarded as the amount of carbon captured *via* photosynthesis per unit time in the single root with the length of L_0. The relations between r_{CO_2} and L_0 values were calculated by Eqs. (1.5) to (1.8) under the light conditions of $I = 1.0$, 3.3 and 11 W/m^2 using the parameter and constant values shown in Table 1.4. As seen in Fig. 1.9B, the calculated value of r_{CO_2} increased with increasing L_0 and I values, indicating that the changes of elongating potential observed
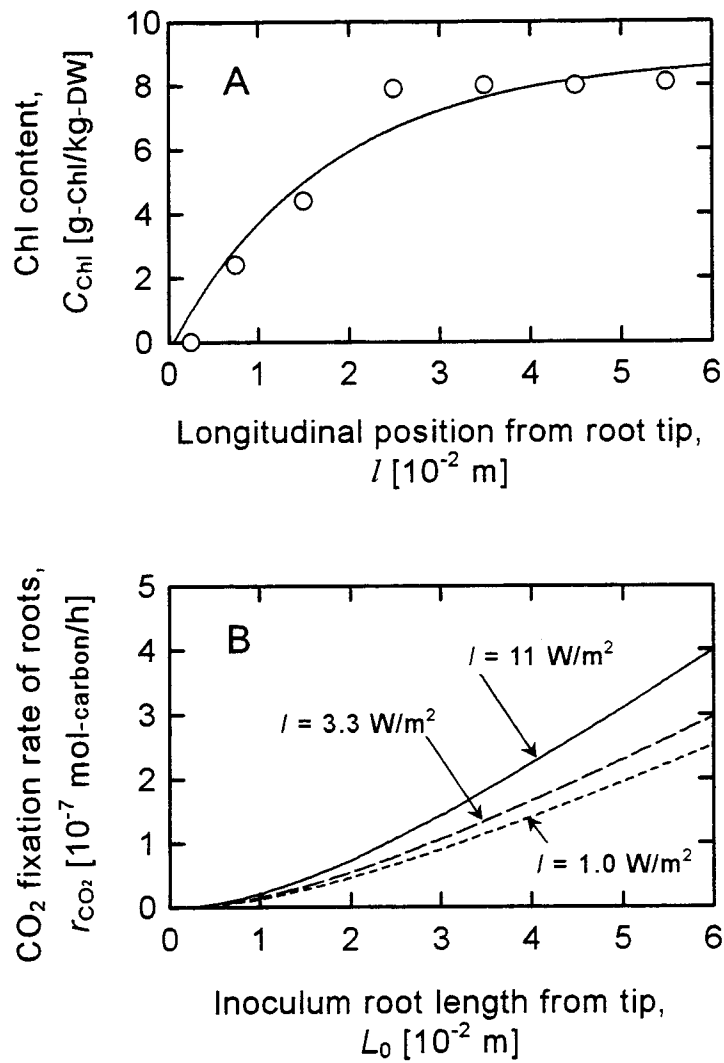
Fig. 1.9 (A) Longitudinal distribution of Chl content along PT roots. The line was drawn by matching Eqs. (1.5) and (1.6) to the data. (B) CO_2 fixation rates of PT roots with inoculum length of L_0. The lines were calculated values using Eqs. (1.5) to (1.8).

in Table 1.2 and Fig. 1.5 can be interpreted in terms of carbon amount captured by the root cells.

The carbon source captured by cells is generally utilized in both the anabolism for formation of cell mass components and the catabolism for generation of biochemical energy, that is,

$$r_{CO_2} = r_{ANA} + r_{CAT} \qquad\qquad (1.9)$$

In the present study, the rate of carbon consumption for anabolism (r_{ANA}) was estimated by the following equation, regarding the growth of the single root as extension of a cylindrical root at tip part.

$$r_{ANA} = \left(\frac{C_C}{12}\right)\left(\frac{\pi \rho \, (1-w)D^2}{4}\right)R_G \qquad\qquad (1.10)$$

Here, the value of C_C was determined to be 5.1×10^2 g-carbon/kg-DW from the elemental analysis of the hairy root mass (Table 1.4).

Employing Eqs. (1.5) to (1.10) and the values of parameters and constants shown in Table 1.4, the carbon share for catabolism ($r_{CAT} = r_{CO_2} - r_{ANA}$) was estimated, assuming that the changes of Chl and carbon contents in the roots are negligible under
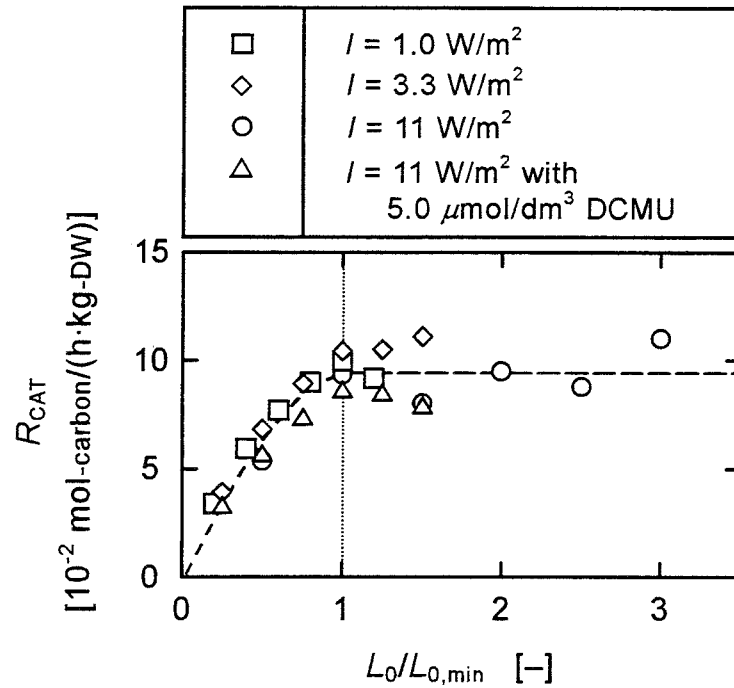

Fig. 1.10 Plot of R_{CAT} against $L_0/L_{0,min}$ in PT hairy roots under various conditions.

the conditions examined in the present study. **Figure 1.10** shows the plot of the calculated value of carbon consumption rate for catabolic metabolism per unit root mass (R_{CAT}) against the value of $L_0/L_{0,min}$, which was obtained in the cultures of the single root at $I = 1.0$, 3.3, 11 W/m^2 as well as $I = 11$ W/m^2 with the addition of 0.5 μmol/dm^3 DCMU. In this figure, the R_{CAT} value was calculated as follows.

$$R_{CAT} = r_{CAT} \left/ \left(\frac{\pi \rho (1-w)D^2}{4} \right) L_0 \right. \tag{1.11}$$

It was found that the data were laid to overlap each other irrespective of the culture conditions. The R_{CAT} value increased with an increase in the $L_0/L_{0,min}$ value up to $L_0/L_{0,min} = 1.0$, and afterwards reached the approximately constant value with an average of $R_{CAT} = 9.4 \times 10^{-2}$ mol-carbon/(h·kg-DW). As the $L_{0,min}$ value was defined as a minimum length required for the root elongation, it was considered that in the range of $L_0 > L_{0,min}$, the PT hairy roots could gain excessive carbon available for anabolism in addition to carbon for catabolism. It is thus possible that the estimated value of $R_{CAT} = 9.4 \times 10^{-2}$ mol-carbon/(h·kg-DW) is regarded as a minimum carbon requirement for allowing the PT hairy roots to proliferate. In other words, this value may be taken as carbon cost for anabolic metabolism to yield maintenance energy in the cells.

1.4 Summary

The elongating potentials of pak-bung hairy roots were examined using the HT and PT root segments with various $\hat{C}_{ATP,0}$ and L_0 values, and characterized in terms of ATP contents in root tip parts or carbon balance of the roots. The conclusions could be drawn from this chapter were as follows.

(1) When the HT roots with $\hat{C}_{ATP,0} = 8.1 \times 10^{-5} - 3.6 \times 10^{-4}$ mol/m were cultivated in sucrose-free medium, no root elongation occurred in the cases of $\hat{C}_{ATP,0} = 8.1 \times 10^{-5}$ and 1.5×10^{-4} mol-ATP/m. The values of $\hat{C}_{ATP,E}$ were 1.8×10^{-4} (for $\hat{C}_{ATP,0} = 2.8 \times 10^{-4}$ mol-ATP) and 2.0×10^{-4} mol-ATP/m (for $\hat{C}_{ATP,0} = 3.6 \times 10^{-4}$ mol-ATP/m). The $\hat{C}_{ATP}$

value of about 2.0×10^{-4} mol-ATP /m was therefore found to be a threshold for the HT roots to ensure the tip elongation.

(2) The PT roots exhibited the dependencies in elongating potential on their length as well as the culture conditions. There were minimum root lengths for the root elongation, $L_{0,min}$, and these $L_{0,min}$ values were 5.0×10^{-2}, 4.0×10^{-2} and 2.0×10^{-2} m at $I =$ 1.0, 3.3 and 11 W/m^2, respectively. When the root lengths were more than the $L_{0,min}$ values for the elongation, the root elongation rates increased with increasing root length and light intensity. It was found that the addition of photosynthesis inhibitor (DCMU) to the medium resulted in the lowering of root elongation rate and the increment of the $L_{0,min}$ value.

(3) From the time profiles of root elongation and ATP contents, the values of Y_{ATP} and m_{ATP} were estimated to be 1.1×10^4 – 1.2×10^4 m/mol-ATP and 2.4×10^{-6} mol-ATP/(h·m) for the HT roots, and 1.2×10^4 m/mol-ATP and 2.2×10^{-6} mol-ATP/(h·m) for the PT roots, respectively, coinciding closely with each other under the examined conditions.

(4) Carbon balance in the hairy roots was calculated from CO_2 fixation rate taking account into the gradient of Chl content along with root length and carbon consumption rates for both anabolic and catabolic metabolisms. As a result, the minimum carbon requirement was estimated to be about 9.4×10^{-2} mol-carbon/(h·kg-DW) for the proliferation of the hairy roots under the examined culture conditions.

Appendix 1

According to the previous paper (Kino-oka *et al.* 1996), the following assumptions were employed to formulate the Chl formation of pak-bung hairy roots cultivated under photoautotrophic condition. A single root segment is divided into two sections; growth section within GP $(0 \leq l \leq L_G)$ where root elongation occurs through cell division without Chl synthesis, and Chl formation section except GP $(L_G < l)$ where cell division does not occur but Chl is synthesized in individual cells under light

irradiation. Chl is accumulated inside chloroplast by means of permeation of the pigment through an envelope of chloroplast.

In the growth section, the elongation rate of a single root is defined as follows.

$$dL/dt = R_G \qquad (A1.1)$$

With respect to the pigment formation, the formation rate of Chl at a given distance from tip of a single root is expressed as follows.

$$\partial C_{Chl}^{i}(t, l)/\partial t = \begin{cases} 0 & (0 \le l \le L_G) & (A1.2) \\ k_{Chl}\ (C_{Chl}^{o} - C_{Chl}^{i}(t, l)) & (L_G < l) & (A1.3) \end{cases}$$

where k_{Chl} is apparent permeation rate constant through envelope of chloroplast.

Chl concentrations at inside and outside of chloroplast in the cells existing at distance of l, $C_{Chl}^{i}(t, l)$ and C_{Chl}^{o} in Eq. (A1.3), are correlated with $C_{Chl}(l)$ and $C_{Chl,S}$, respectively, by the following equations.

$$C_{Chl}(l) = \varepsilon\ C_{Chl}^{i}(t, l)/\rho\ (1 - w) \qquad (A1.4)$$

and

$$C_{Chl,S} = \varepsilon\ C_{Chl}^{o}/\rho\ (1 - w) \qquad (A1.5)$$

where, ε indicates volumetric ratio of chloroplast in a single cell.

According to Eq. (A1.1), longitudinal position along the roots, l, is correlated with t by the following equation.

$$\int_{L_G}^{l} dL = \int_{0}^{t} R_G\ dt \qquad (A1.6)$$

Combination of Eqs. (A1.2) to (A1.6) yields Eqs. (1.5) and (1.6) in the text under the condition of constant R_G value.

Chapter 2

Characterization of Tip Budding Potentials
Relating to Oxygen Uptake Rate of Hairy Roots

2.1 Introduction

In Chapter 1, root elongating potentials were examined under various culture conditions using the HT and PT hairy roots of pak-bung, and the changes in the potentials of these hairy roots were correlated with the ATP contents in the tip parts and carbon balance considering the gradient of Chl content in the longitudinal direction of the PT roots which was attributed to the cellular age distribution.

With respect to the changes in tip budding potentials, on the other hand, it was reported that in the cultures of horseradish hairy roots the potential of lateral root budding was stimulated in response to the addition of hormones such as 1-naphthaleneacetic acid (Nakashimada *et al.*, 1994; Repunte *et al.*, 1993). Moreover, when inocula of some hairy roots were prepared for subcultures in our laboratory, it was occasionally observed that roots cut with relatively short lengths underwent frequent ramification and lateral root budding. These findings suggest that the mode of lateral root budding can be a measure for the estimation of chemical and physical stimuli against the hairy roots.

The distribution of cell maturity is so far concerned with the positional variation of metabolite contents along the roots, as reported in the cultures of red beet hairy roots (Berzin *et al.*, 1999; Kino-oka *et al.*, 1995). In this chapter, the positional distribution in the potential of lateral root budding was investigated using the segments prepared from the hairy roots of madder and horseradish. Moreover, the process of lateral root budding was kinetically analyzed and discussed in terms of oxygen uptake rate of the segmented hairy roots.

2.2 Experimental

2.2.1 Plant materials

Hairy roots of madder (*Rubia tictorum*) and horseradish (*Armoracia rusticana*), which had been induced and established elsewhere (Kino-oka *et al.*, 1994; Noda *et al.*, 1987), were used for the experiments. The horseradish or madder hairy roots were maintained by means of subculturing at 2 week's interval in 80 cm^3 MS liquid medium containing 20 kg/m^3 sucrose or MS medium containing 20 kg/m^3 fructose without ammonium ion, respectively. The liquid cultures were conducted at 25°C in 200 cm^3 Erlenmeyer flasks shaken at 100 rpm on a rotary shaker (Model HRS-24, Shibata Scientific Technology Ltd., Tokyo, Japan) in the dark.

2.2.2 Culture conditions

To examine the potential of lateral root budding out of the hairy roots, cultures were conducted on the medium solidified with 2 kg/m^3 Gelrite® (San-ei Gen F. F. I. Inc., Osaka, Japan) in Petri dishes (90 mm in diameter) kept at 25°C in a dark room. The original roots obtained from the liquid cultures were subjected to trimming of lateral
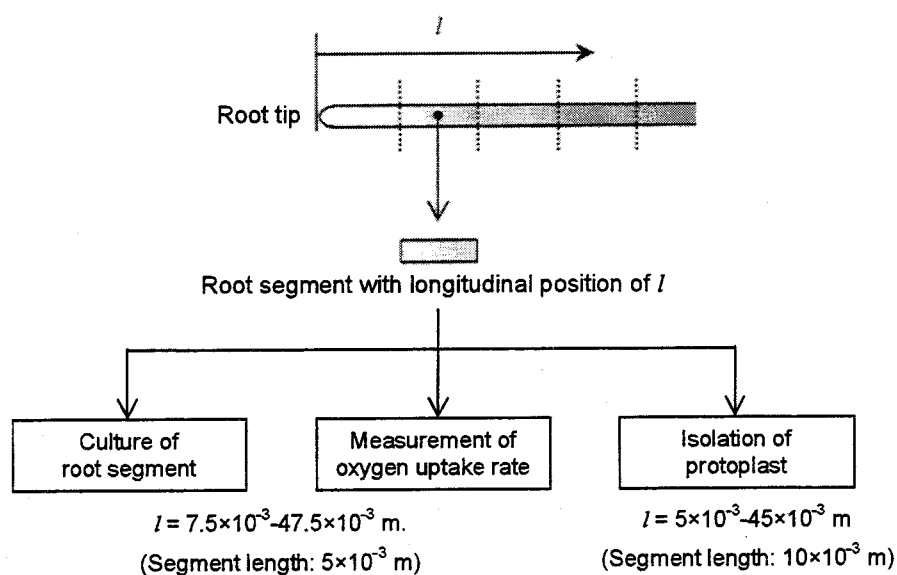

Fig. 2.1 Schematic drawing of experiments using root segments with various longitudinal positions

roots, and each single root was cut into segments of 5×10^{-3} or 10×10^{-3} m in length, giving various longitudinal positions from the root tips of l (see **Fig. 2.1**). About 25 root segments were prepared with regard to each longitudinal position of the hairy roots.

2.2.3 Analytical methods

The number of budding tips (BTs) generating on the segments (N_B) was evaluated by visually counting the BTs with length of more than 5.0×10^{-4} m on magnified images of the root segments acquired by the manner as described in Chapter 1. Oxygen uptake rate of the roots (Q) was measured at 25 °C with a chamber (O_2 Uptester 6C, TAITEC Co., Saitama, Japan) containing the prepared root segments in the liquid medium. The data of N_B and Q values were expressed on a basis of unit length of the root segments.

Protoplast isolation from the segments of madder hairy roots was conducted by means of incubating the segments in 5 ml of filter-sterilized enzyme solution at 35°C for 8 h. The enzyme solution contained 2 % (w/v) Meiserase (Meiji Seika Kaisha, Ltd., Tokyo, Japan) and 0.5 % (w/v) Macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan) in a mixture of 0.7 M mannitol and 0.5 % (w/v) potassium dextran sulfate. The isolated protoplasts were observed with a microscope (Model BX50-34, Olympus Optical Co., Ltd., Tokyo, Japan), and the diameter was measured on the image acquired using a CCD camera (HCC-600, Flovel Co., Ltd., Tokyo, Japan) attached to the microscope.

2.3 Results and Discussion

2.3.1 Changes in tip budding potential along with longitudinal position

Figure 2.2 shows photograph of segmented madder hairy roots with lateral roots, which were cultivated for 120 h. It was observed that frequency of lateral roots was varied depending on longitudinal position of the single hairy roots. To examine the time profiles of root budding, the cultures were conducted using the hairy roots segmented at various positions of l. **Figure 2.3** shows the changes in the N_B values during culture time of t = 0-240 h, using the segments of l = 7.5×10^{-3}, 22.5×10^{-3}, 32.5×10^{-3} and

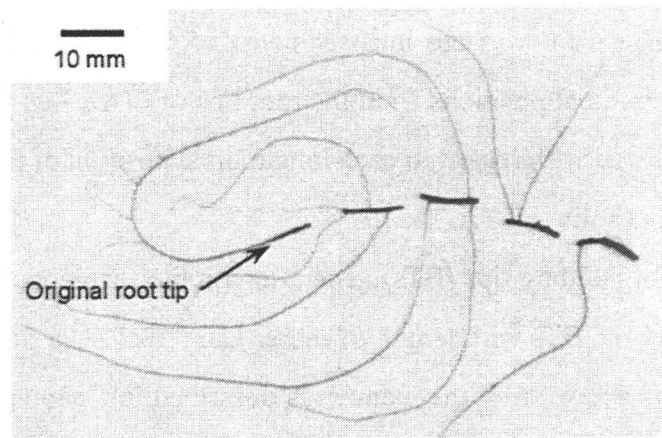**Fig. 2.2** Photograph showing lateral root budding from segments of madder hairy roots. The culture was conducted for 120 h.
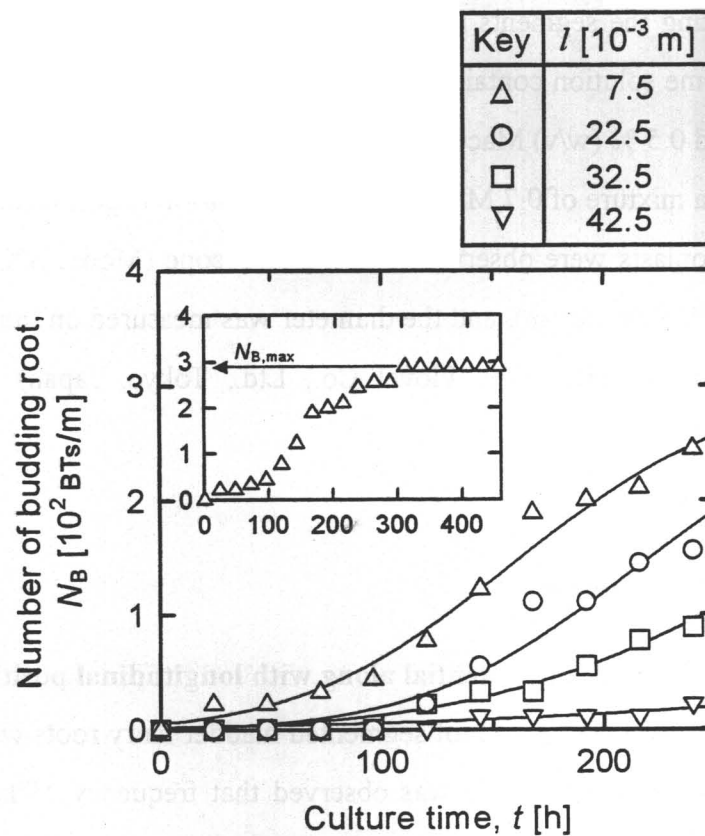

Fig. 2.3 Changes in the number of BTs during cultures of segments of madder hairy roots. The lines were drawn by matching the Eqs. (2.1) and (2.2) to the data. The inserted illustration shows the prolonged culture of the root segments of $l = 7.5 \times 10^{-3}$ m.

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42.5×10^{-3} m prepared from madder hairy roots. It was observed that, after appreciable lag periods, the N_B values increased gradually as culture time progressed, and higher values of N_B were obtained in the cultures of the segments with smaller l values during the examined culture period. The N_B values at $t = 240$ h were 2.4×10^2, 1.6×10^2, 8.8×10 and 2.2×10 BTs/m at $l = 7.5 \times 10^{-3}$, 22.5×10^{-3}, 32.5×10^{-3} and 42.5×10^{-3} m, respectively. A similar dependency in the N_B value on the longitudinal position was also recognized in the case of the segments prepared from horseradish hairy roots (data not shown). It was therefore considered that the potential of lateral root budding out of the segmented hairy roots reduced with increasing distance from the tips of original roots, which may be attributed to the lowered capacity of cell division caused by aging or maturation of the cells progressing along with longitudinal position.

2.3.2 Changes in protoplast size and oxygen uptake rate along with longitudinal position

Plant roots elongate linearly through the active cell divisions at root apical meristems and the subsequent cell enlargement (Cuadrado *et al.*, 1989). To examine the longitudinal change in size of the hairy root cells, protoplasts were prepared from the segmented madder hairy roots. As seen in **Fig. 2.4**, the protoplasts obtained from the root segment of $l = 45 \times 10^{-3}$ m appeared to be quite larger than those from $l = 5 \times 10^{-3}$ m.
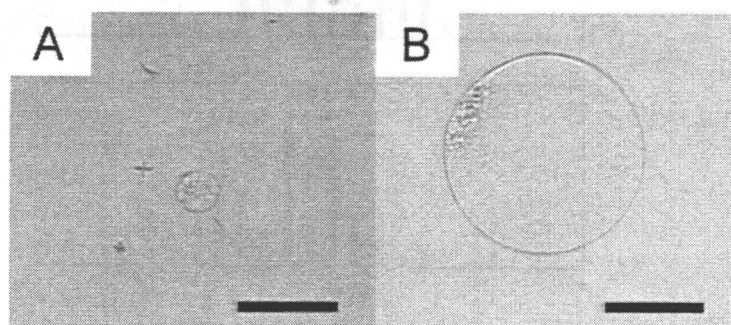
Fig. 2.4 Micrographs of protoplasts obtained from different longitudinal positions of madder hairy roots. (A) $l = 5 \times 10^{-3}$ m and (B) $l = 45 \times 10^{-3}$ m. The bars show 50 μm.

It was found that the frequency distribution of the protoplast diameter shifted to the range of the larger value with increasing the l value under the examined longitudinal positions of $l = 5 \times 10^{-3}\text{-}45 \times 10^{-3}$ m (**Fig. 2.5**), suggesting the cell maturation along with the longitudinal direction of the single root.
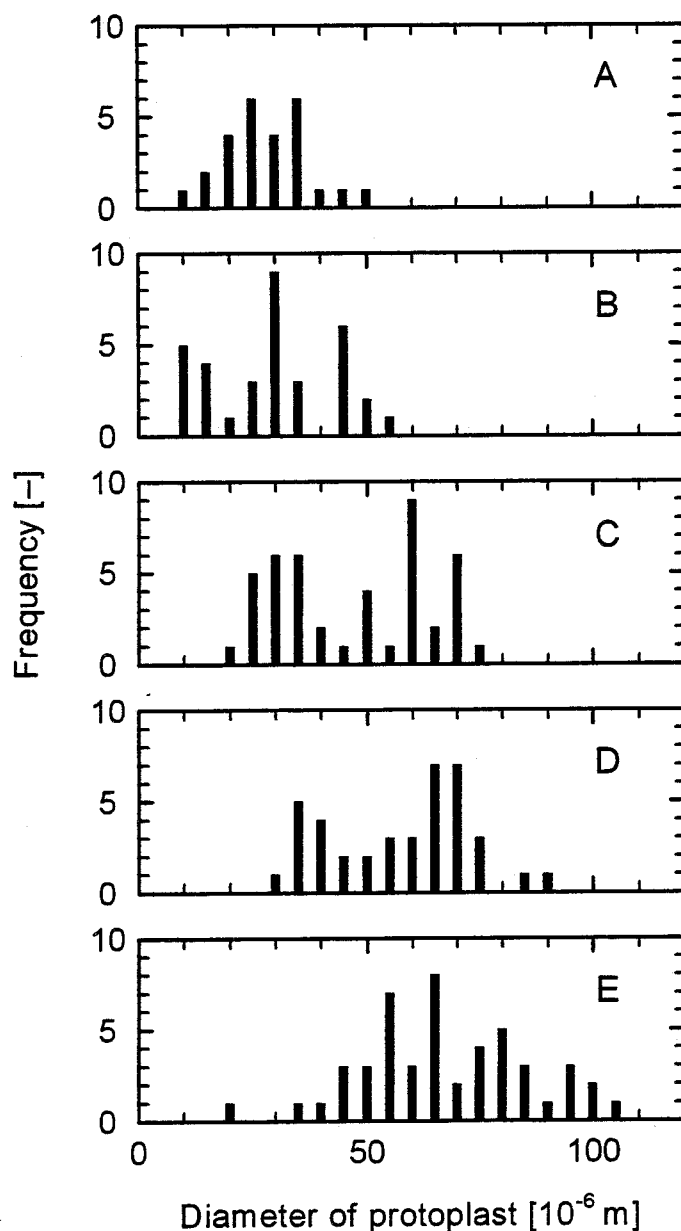
Fig. 2.5 Frequency distribution of diameter in protoplasts from madder hairy roots. (A) $l = 5 \times 10^{-3}$ m, (B) $l = 15 \times 10^{-3}$ m, (C) $l = 25 \times 10^{-3}$ m, (D) $l = 35 \times 10^{-3}$ m and (E) $l = 45 \times 10^{-3}$ m.

As an index to evaluate the variation in the cell activity along longitudinal position, oxygen uptake rate was measured using the segments of madder and horseradish hairy roots of l = 7.5×10^{-3}-47.5×10^{-3} m. As shown in **Fig. 2.6A**, it was found that the Q values of the root segments with smaller l values were relatively higher and gradually decreased with the increases in l value in both the cases of madder and horseradish hairy roots. The extent of the drop in the Q value of horseradish hairy roots was larger compared with that of madder hairy roots. It was considered that the segmental changes of oxygen uptake rate could also reflect the maturation degree of hairy root cells.
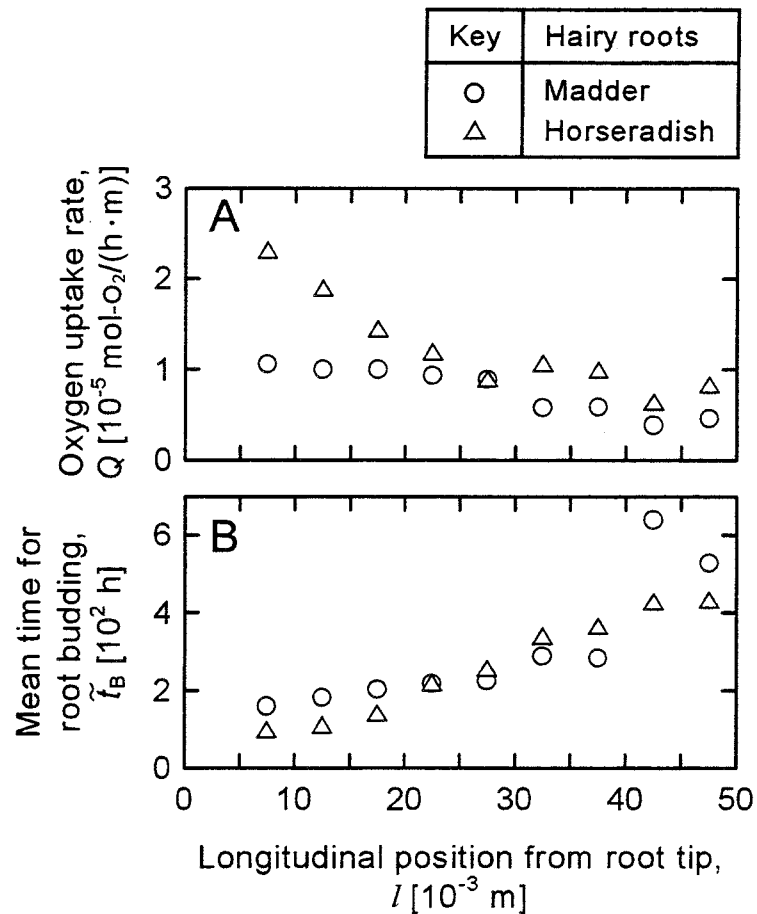
Fig. 2.6 Longitudinal distributions of oxygen uptake rate (A) and mean time for lateral root budding (B).

2.3.3 Correlation of root budding potential with oxygen uptake rate

In general, the budding of plant roots involves the development of endogenous root primordial cells and the subsequent outgrowths from cortices as root projections (Hinchee and Rost, 1986). The resuming ability of respective root primordia seems to decrease with an increase in cell ages, resulting in the prolongation of lag time for lateral root budding and the difference of N_B value along with l value as illustrated in Fig. 2.3.

To explain the segmental distribution of lateral root budding, in the present study, the time profiles of N_B values in the cultures of the segments with different l values (Fig. 1) were expressed by following formulation. Hence, it was assumed that a distribution for ΔN_B (changes in the N_B value for a given time interval Δt) follows the normal distribution probability function ($F_B(t)$) given by the following equation.

$$F_B(t) = \frac{1}{\sigma_B \sqrt{2\pi}} \exp\left\{ -\frac{(t - \tilde{t}_B)^2}{2\sigma_B^2} \right\}$$

(2.1)

where $\tilde{t}_B$ and σ_B are mean time of lateral root budding and standard deviation for the respective root budding, respectively. The changes in the N_B value with time were evaluated based on the integral form of Eq. (2.1), given as follows.

$$N_B = N_{B,max} \int_0^t F_B(t)\, dt$$

(2.2)

where $N_{B,max}$ is the maximum value of N_B regarded as the latent number of root primordial. In the present study, the $N_{B,max}$ values for the respective hairy roots were assumed to be independent of the distance from the root tip. The $N_{B,max}$ values were experimentally determined as the saturated N_B values in the prolonged cultures of root segments of $l = 7.5 \times 10^{-3}$ m for 456 h (see the insert in Fig. 2.3 for madder hairy roots). As a result, the $N_{B,max}$ values of 2.9×10^2 and 9.3×10^2 BTs/m were obtained for madder and horseradish hairy roots, respectively. Employing these $N_{B,max}$ values, the $\tilde{t}_B$ and σ_B values for the respective root segments were determined by fitting the experimental data to Eqs. (2.1) and (2.2) using the non-linear squares method, as depicted by the solid

lines in Fig. 2.3. The determined values of $\tilde{t}_{\mathrm{B}}$ were plotted against the l values of the respective segments from madder and horseradish hairy roots, as shown in Fig. 2.6B. The $\tilde{t}_{\mathrm{B}}$ values increased gradually with an increase in the l value for the both hairy roots in the range of $l = 7.5 \times 10^{-3}\text{-}47.5 \times 10^{-3}$ m.

Here, average rate of lateral root budding ($\tilde{R}_{\mathrm{B}}$) was defined as follows.

$$\tilde{R}_{\mathrm{B}} = \frac{N_{\mathrm{B,max}} \int_{0}^{\tilde{t}_{\mathrm{B}}} F_{\mathrm{B}}(t)\,\mathrm{d}t}{\tilde{t}_{\mathrm{B}}} \qquad (2.3)$$

The $\tilde{R}_{\mathrm{B}}$ value was regarded as a parameter for evaluating the positional activity of the hairy root cells. **Figure 2.7** shows the relationship between the $\tilde{R}_{\mathrm{B}}$ and Q values

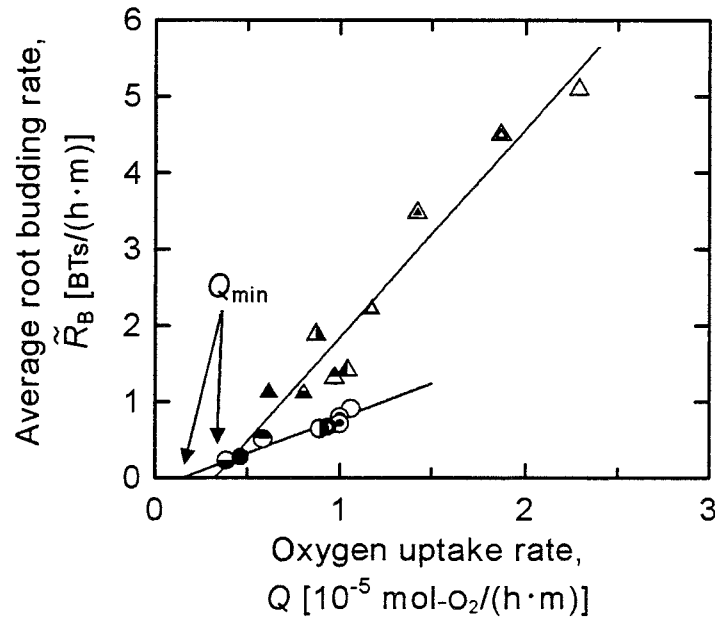| Madder	O	◉	○	⊙	◑	◐	⊖	⊜	●
Horseradish	△	▲	▲	◬	▲	▲	△	▲	▲
$l\ [10^{-3}$ m]	7.5	12.5	17.5	22.5	27.5	32.5	37.5	42.5	47.5

Fig. 2.7 Relationships between average rate of lateral root budding and oxygen uptake rate per length of root segment.

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determined for the segments from madder and horseradish hairy roots. Under the examined conditions, the positive correlations were seen between these values for both the hairy roots, suggesting that the respiratory activity can be a factor relating to the potential of lateral root budding. From the correlated lines, it was estimated that the R_B value reached zero at the values of $Q = 1.5 \times 10^{-6}$ and 3.2×10^{-6} mol-O_2/(h·m) for madder and horseradish hairy roots, respectively, indicating that substantial propagation of the hairy roots does not occur in the vicinity of these Q values.

In the present study, these values of Q were regarded as a minimum oxygen requirement (Q_{min}) for maintenance of hairy root cells existing in resting state without propagation. The Q_{min} values of 1.5×10^{-6} and 3.2×10^{-6} mol-O_2/(h·m) corresponded to 3.8×10^{-2} and 8.2×10^{-2} mol-O_2/(h·kg-DW) on a dry weight (DW) basis, employing an average conversion coefficient of 3.9×10^{-5} kg-DW/m.

Nagatome et $al.$ (2000b) demonstrated that under a lowered sucrose concentration (2.5 kg/m^3), the hairy roots of pak-bung and tobacco existed as resting cells with maintenance metabolism accompanied by no obvious growth. Under this condition, oxygen uptake rates per dry cell weight were reported to be 1.1×10^{-1} and 1.4×10^{-1} mol-O_2/(h·kg-DW) for pak-bung and tobacco hairy roots, respectively. According to the condition employed by Nagatome et $al.$, the oxygen uptake rates were determined to be 1.4×10^{-1} and 1.3×10^{-1} mol-O_2/(h·kg-DW) for madder and horseradish hairy roots, respectively (unpublished data). These values were somewhat higher than the minimum oxygen uptake rates of 3.8×10^{-2} -8.2×10^{-2} mol-O_2/(h·kg-DW) estimated in present study.

2.4   Summary

The segmental distribution in lateral root budding, protoplast diameter and oxygen uptake rate were examined using the hairy roots of madder and/or horseradish, and the following conclusions could be drawn.

(1) The higher values of N_B were obtained in the cultures of the roots segments with the smaller l values during the examined culture period of $t = 240$ h in both the

cases of madder and horseradish hairy roots. The N_B values at t = 240 h were 2.4×10^2, 1.6×10^2, 8.8×10 and 2.2×10 BTs/m at l = 7.5×10^{-3}, 22.5×10^{-3}, 32.5×10^{-3} and 42.5×10^{-3} m, respectively, for madder hairy roots.

(2) The diameter of protoplasts obtained from the segments of the madder hairy roots increased with an increase in the l values. The oxygen uptake rates of the root segments with smaller l values were relatively higher and gradually decreased with the increases in l value in both the cases of madder and horseradish hairy roots.

(3) Potential of lateral root budding from the segmented hairy roots of madder and horseradish could be correlated with the oxygen uptake rate of the root cells. The potential of lateral root budding was estimated to be suppressed at the oxygen uptake rates of 1.5×10^{-6} and 3.2×10^{-6} mol-O_2/(h·m) for madder and horseradish hairy roots, respectively.

PART II

EVALUATION OF HAIRY ROOT RESPONSES
TO CHEMICAL STIMULI AND
ITS APPLICATION TO BIOASSAY

In Part I, both the potentials of root elongation and tip budding were characterized considering changes in cell maturity along the single root to grasp the properties unique to the root systems. Based on the findings obtained in Part I, in this part, the hairy root responses against chemical stimuli were evaluated and applied to the bioassay for the assessment of toxicity of the chemicals. This part consisted of the latter three chapters in this study, and each of which covers the hairy root responses based on the root elongation at apical meristems, tip budding as lateral roots or cell maturity along the longitudinal direction. In Chapter 3, the root responses to herbicides were examined with respect to the root elongating potentials, and compared with those of other bioassays or an environmental criterion in terms of the sensitivity and rapidity for assessing the toxicities of test herbicides. In Chapter 4, variations in hairy root responses to chemicals between the primary and lateral roots were examined during successive cultures, and discussed from the aspects of phenotypic and genotypic features. In Chapter 5, the responses of the PT roots against herbicides with different modes of action were examined based on the local Chl pigment distributed along with the longitudinal direction of the roots, and applied to the classified assessment of toxicities of individual herbicides.

Chapter 3

Evaluation of Root Elongating Responses
against Herbicidal Stimuli

3.1 Introduction

Regarding the bioassay of herbicides, in particular, cultured plant cells as well as excised leaves and roots have been extensively used as detection tools since herbicides are inherent inhibitors or suppressors of metabolism in plant cells (Pestemer and Günther, 1995; Grossmann *et al.*, 1992; Sato *et al.*, 1987). From a viewpoint of engineering, the bioassay has often aimed at an on-line monitoring of the biological responses (*e.g.* respiratory and motional activities of test organisms), which can facilitate the simple and successive check of environment to cope with the accidental emergence of pollutants inviting acute toxicity. As for the monitoring of chemical toxicities based on the motional responses, computer-aided image analysis has been occasionally employed to quantify the behaviors of microorganisms or the morphological changes of cultured cells because the analysis enables time-saving and/or real-time measurement for the evaluation (Tahedl and Häder, 1998; Sakai *et al.*, 2000).

In this chapter, the root responses to herbicides with different modes of action were examined based on the elongating potentials of the HT and PT hairy roots of pak-bung as described in Chapter 1, and a quantitative parameter was presented as a measure to estimate the herbicidal stress using both types of hairy roots. Moreover, the elongating behavior of the plant hairy roots against herbicidal stimuli is evaluated by developing an observation system with computer-aided image analyzing technique, which can allow the sensitive and automatic tracing of the position of the root tip point. The comparison is, last of all, made between the elongating response of the roots evaluated by developed system and that of other bioassays in terms of the sensitivity and rapidity for assessing the toxicities of test herbicides.

3.2 Experimental

3.2.1 Plant materials

The HT and PT hairy root lines of pak-bung were used for the experiments. The HT and PT hairy roots were maintained by means of regular subcultures in MS liquid medium with and without 20 kg/m^3 sucrose, respectively, under the same conditions as described in Chapter 1.

3.2.2 Culture conditions and test chemicals

To investigate the influence of herbicides on root elongation, both types of hairy roots were cultivated at 25 ℃ on Petri dishes (90 mm in diameter) containing 20 cm^3 of the respective media solidified with 2 kg/m^3 Gelrite® (San-ei Gen F. F. I. Inc., Osaka, Japan). During culturing, the HT hairy roots were kept in a dark room, and the PT hairy roots were placed under a 5.0 % carbon dioxide atmosphere in an incubator (Model CFH-305, Tomy Seiko Co., Ltd., Tokyo, Japan) unless otherwise noted. The dishes for the PT hairy root cultures were illuminated with a bank of tubular fluorescent lumps (FL 20SSW, Matsushita Electric Industrial Co., Ltd., Osaka, Japan) at an incident light intensity of 11 W/m^2.

For the measurement of the root elongation by image analyzing system mentioned in the following section, the HT hairy roots was used. The single HT hairy root obtained from the liquid culture was cut into a root segment containing a root tip, and subjected to conditioning culture for 48 h on the solid MS medium with 20 kg/m^3 of sucrose. The prepared root was then transferred to the dish placed in a culture chamber (Model PCI-300, As One Co., Osaka, Japan) kept at 25 ℃ in the dark.

DCMU, 1-1'-dimethyl-4,4'-bipyridylium dichloride (paraquat), *O-3-tert*-butyl-phenyl 6-methoxy-2-pyridyl(methyl) thiocarbamate (pyributicarb) and 2,4-dichloro-phenoxyacetic acid (2,4-D) were used as test chemicals. These were employed as model chemicals with distinct herbicidal mechanisms, *i.e.*, inhibition of photosynthetic activity (DCMU), generation of oxidative stress (paraquat), inhibition of lipid metabolism (pyributicarb) and induction of hormonal imbalance (2,4-D). DCMU, paraqaut and

2,4-D were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, and pyributicarb was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Each medium was supplemented with various amounts of the herbicides, when necessary, according to the procedures found in the literature (Mugnier, 1988; Horn *et al.*, 1983).

3.2.3 Manual measurement of root elongation rate and analysis of ATP content

In the course of the hairy root cultures, the ΔL and R_G values were recorded according to the method described in Chapter 1. the R_G value was determined for 72-168 h after the start of culturing, to avoid the effect of fluctuation in the elongation rate observed in the early culturing period. The data were mean values of the measurements taken for about fifteen root tips under the respective conditions. The elongating response of hairy roots to the herbicides was estimated by the α value given as follows.

$$\alpha = \frac{R_G \text{ for herbicide - containing culture}}{R_G \text{ for control culture free from herbicide}} \times 100 \qquad (3.1)$$

ATP content in the roots was measured by the manner as described in Chapter 1. Root tips of 5×10^{-3} m in length were prepared from the solid cultures for the assay and the data were averaged for five tip specimens.

3.3   Observation system for tracing elongation of single root tip

3.3.1 Experimental set-up

To evaluate the elongating behavior of the hairy roots, an observation system assisted by an image analyzing technique was constructed, as shown in **Fig. 3.1**, consisting of image-acquiring and image-analyzing units. For the image-acquiring unit, a light-emitting diode (LED) lamp (IDM-90/90RT, IMAC Co., Ltd., Siga, Japan) and an 8-bit CCD camera (STC-LS400, Sensor Technology Co., Kanagawa, Japan) attached with a zooming lens (PH6×8 MACRO, Canon Inc., Tokyo, Japan) were installed in a chamber kept at 25 ℃ to analyze the image of elongating roots at real time. The Petri
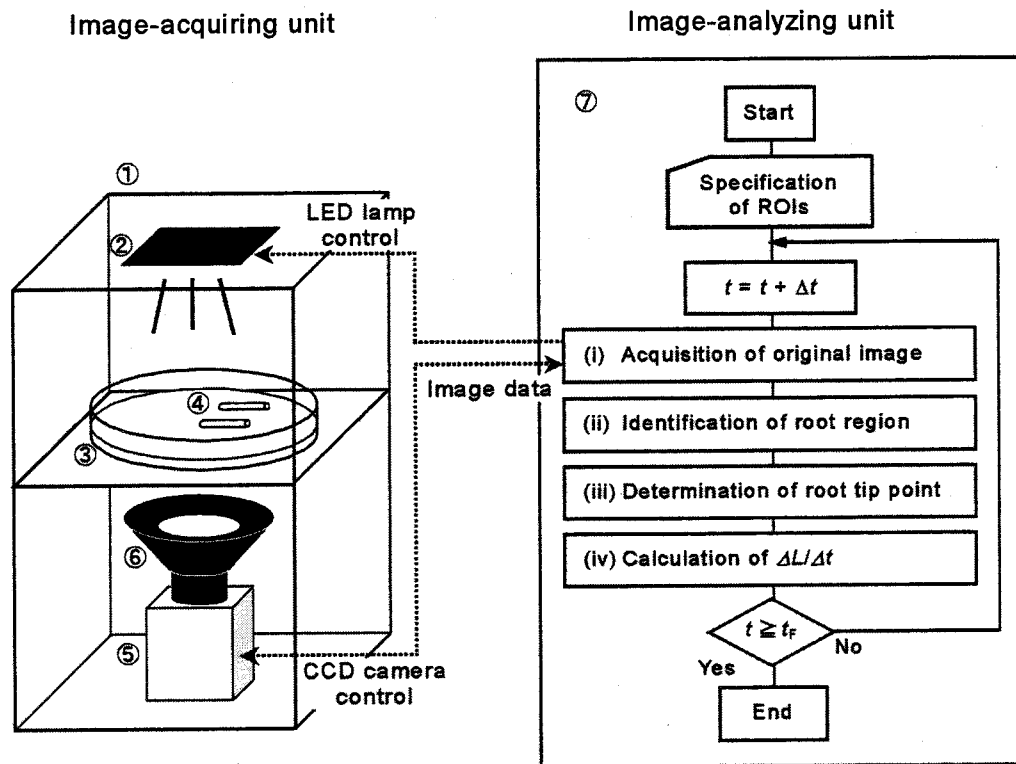
Fig. 3.1 Schematic diagram of image analyzing system. ① Culture chamber, ② LED lamp, ③ Petri dish, ④ hairy roots, ⑤ CCD camera, ⑥ zooming lens and ⑦ personal computer.

dish containing the prepared single root was positioned between the LED lamp and CCD camera, and the original image of root was acquired from the bottom of the dish while being illuminated with the LED lamp. The data of original image was inputted to a Pentium based computer with 128 MB RAM *via* an image acquisition board (IMAQ PCI-1408, National Instruments Co., Austin, TX, USA) as digitalized data of 480 × 640 pixels (acquired image area: 25.4 mm × 33.9 mm). Each pixel had 256 classified gray levels ranging from 0 (black) to 255 (white).

3.3.2 Procedure of image analysis

In the image-analyzing unit, as indicated in Fig. 3.1, the following diagram was included: (i) Acquisition of an original image, (ii) image processing for identification of

root region, (iii) image analysis for determination of a root-tip point, and (iv) calculation of elongation length and rate at the tip point. The procedure was repeated until final time of measurement (t_F) at an interval of measurement time of 0.2 h. The operations of equipments (LED lamp and CCD camera) for image acquisition and the image analysis were automatically performed employing a computer installed with a LabVIEW^{TM} software (National Instruments Co., Austin, TX, USA).
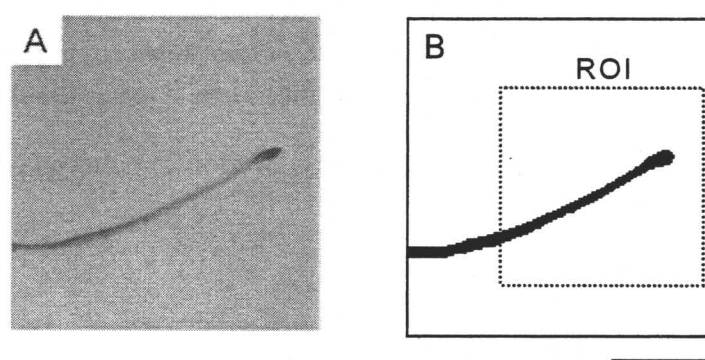
Fig. 3.2 Root tip in original gray image (A) and processed binary image (B). The bar corresponds to 10 mm.

For the identification of root region, a threshold value of gray-scale level was first determined so as to distinguish the root region from background on the original image shown in **Fig. 3.2A**. In the present study, the threshold was given as an empirically determined value of 191 in terms of the gray-scale level, and the root region was discriminated from the pixels with this threshold value or more. Figure 3.2B displayed the processed image (binary image) corresponding to the original one. The region of interest (ROI) was specified on the binary image for zoning the respective root regions including the tip points.

Figure 3.3 is the illustration for the definition of the root tip point on the binary image. In case of a curved locus of root tip elongation, the x- and y-coordinates of the root tip point at a given measurement time of t (P_t (x_t, y_t)) was determined according to

A: Root tip point at $t = t$, $P_t(x_t, y_t)$ ▨ Non-overlapped pixels in root region

B: Root tip point at $t = t - \Delta t$, $P_{t-\Delta t}(x_{t-\Delta t}, y_{t-\Delta t})$ ☐ Non-overlapped pixels on line j_t

C: Root tip point at $t = t - 2\Delta t$, $P_{t-2\Delta t}(x_{t-2\Delta t}, y_{t-2\Delta t})$ ■ Pixels in root region overlapped with line j_t

Fig. 3.3 Conceptual illustration for definition of root tip point on binary image.

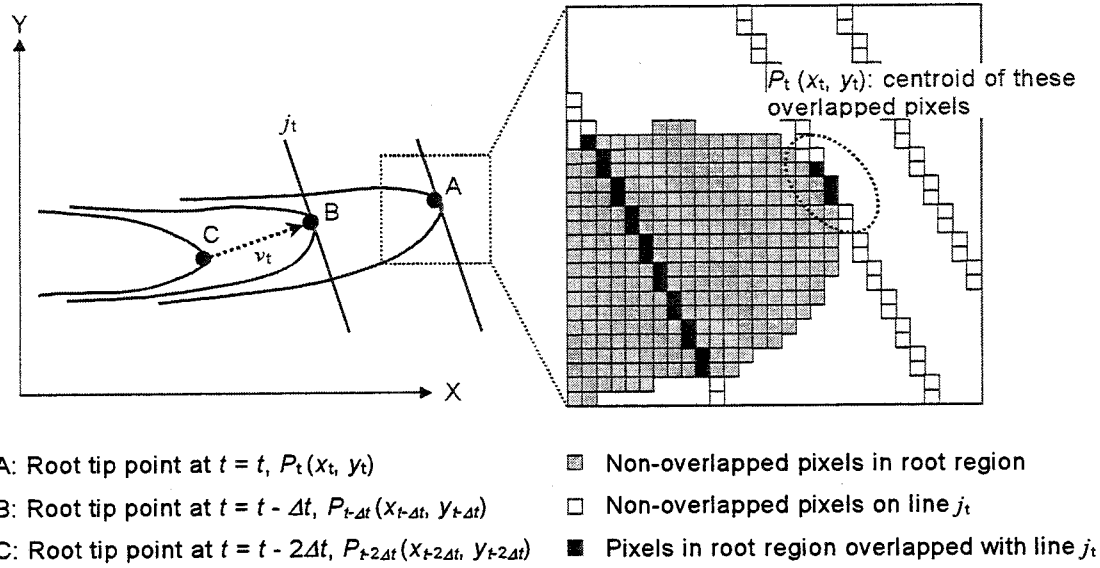the following procedure. (i) A vector for predicting an elongating direction at t (v_t) is defined as a line segment connecting between the $P_{t-\Delta t}$ and $P_{t-2\Delta t}$. (ii) A line perpendicular to v_t (j_t) is given. (iii) The number of the pixels overlapped with root region is counted on the line j_t. (iv) The line segment j_t is shifted along with the direction of v_t by one pixel. (v) These procedures (iii and iv) are repeated until the overlap of the pixel is not recognized in terms of the line j_t and root region. (vi) The point of P_t (x_t, y_t) is given as a centroid of pixels on j_t which is obtained from the shifted last line overlapped with root region. At the start of measurement, temporary points of P_{-1} (x_{-1}, y_{-1}) and P_{-2} (x_{-2}, y_{-2}) were given as centroids of the pixels in root region extracted by two separate lines drawn as to cross root region. An initial point of root tip (P_0 (x_0, y_0)) was then determined in the same manner as descried above.

Elongation length during the measurement interval was calculated as a distance between P_t and $P_{t-\Delta t}$, and the root elongation rate calculated by image analysis (R_G^C) was recorded by dividing the distance with the measurement interval after serial fifteen plots of the x- and y-coordinates of root tip position were subjected to smoothing. The data of the root tip point and elongation rate obtained through the measurement were logged in

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the computer for further analysis.

3.3.3 Evaluation of root elongation with constructed observation system

To judge the availability of measurement with the observation system, at first, a set of single root was cultured in the absence of herbicidal stress and the images of elongating roots were acquired every 0.2 h until the t_F values of 20, 30 and 40 h. The acquired images were subjected to the identification of root region and the determination of root tip point, and then the elongation length during the indicated measurement period of $t = 0 - t_F$ was estimated by the image analysis as mentioned above. **Figure 3.4** shows the relationship between the ΔL values obtained from the image analysis (ΔL^C) and those from the manual measurement. It was found that the

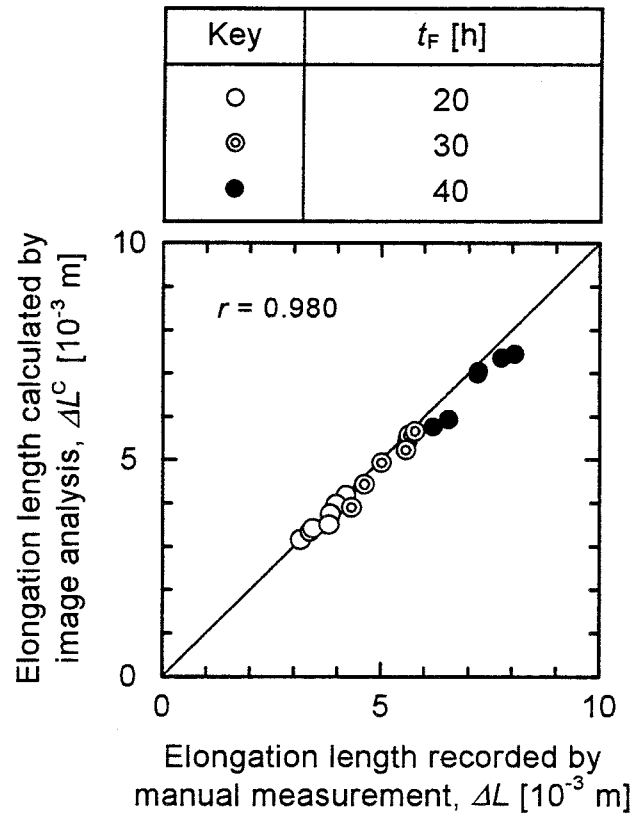| Key	t_F [h]
○	20
◎	30
●	40

Fig. 3.4 Relationship between root elongation lengths during $t = 0 - t_F$ estimated by image analysis and those by manual measurement.

data were plotted along with a diagonal, giving a correlation coefficient of 0.980. From this relationship, the image analysis system was judged to offer the successful accuracy for estimating the elongation of hairy root tip under the condition examined in the present study.

3.4 Results and Discussion

3.4.1 Elongating responses to herbicide for HT and PT hairy roots

As a measure of biological activity, the ATP contents in the tips of HT and PT hairy roots were first compared between the cultures treated with DCMU, parquaut and pyributicarb (10 μmol/dm^3) and control cultures without the herbicides. As shown in **Table 3.1**, exposure to the herbicides, in most cases, caused a reduction in ATP content to around 20 % of that of controls. An exception was the culture of HT hairy roots treated with DCMU, in which the ATP content was 97% of that of the control. These results indicated that the HT and PT hairy roots of pak-bung exhibited distinct responses to the test herbicides.

To understand the detailed responses of both types of hairy roots to the herbicides, the elongating potentials of root tip meristems were examined in the presence of DCMU, paraquat, pyributicarb or 2,4-D at concentrations of C_H = 0.001-10 μmol/dm^3, as shown

Table 3.1 ATP content in tips of hairy roots treated with DCMU, paraquat and pyributicarb

	ATP content in root tip [% of control]		
	DCMU	paraquat	pyributicarb
HT hairy roots	97±36	24±18	21±4.6
PT hairy roots	19±7.5	18±1.7	12±11

The roots were incubated for 48 h on the respective media to which the herbicides were added at 10 μmol/dm^3. ATP contents in the tips from the control cultures: 2.0 μmol/root tip (HT hairy roots) and 0.57 μmol/root tip (PT hairy roots).
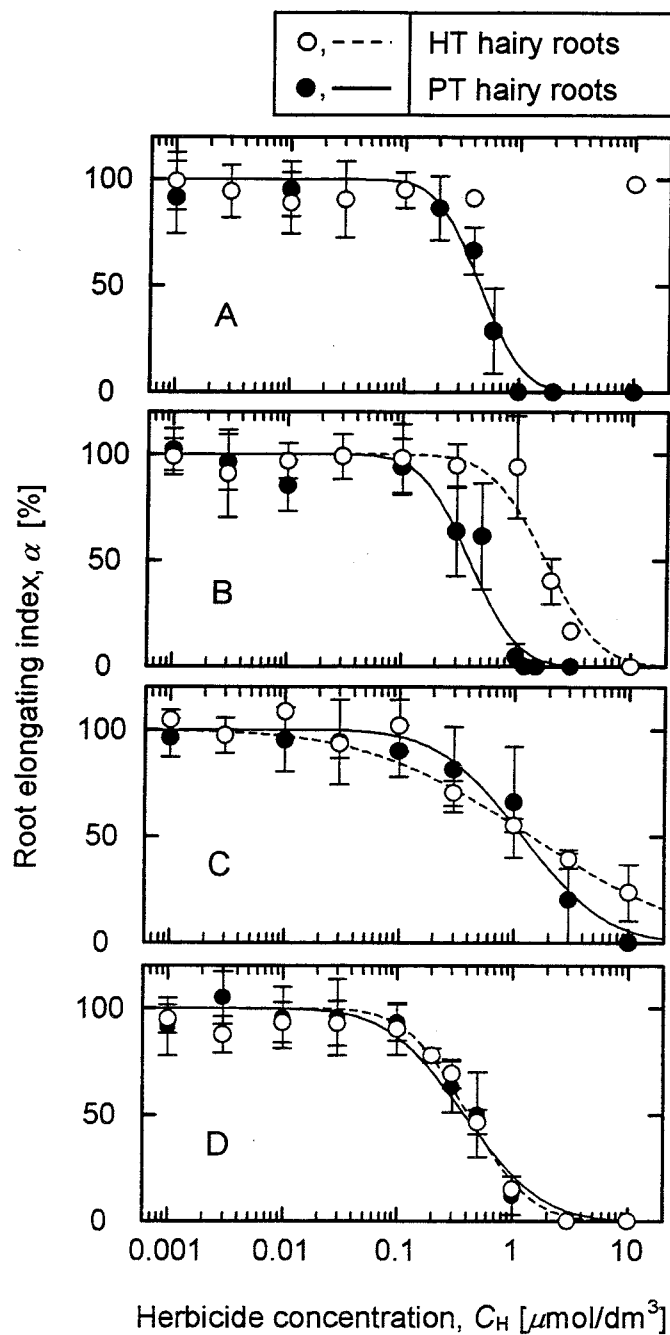
Fig. 3.5 Dose-response curves of the tip elongation of pak-bung hairy roots treated with various herbicides. (A) DCMU, (B) paraquat, (C) pyributicarb and (D) 2,4-D. The vertical bars show standard deviation. The lines were drawn by matching Eqs. (3.2) and (3.3) to the data.

in **Fig. 3.5**. Upon addition of DCMU, the elongating potential of HT hairy roots was not retarded, with the α value remaining above 90 % over the examined C_H region, while in the case of PT hairy roots, an appreciable decrease in the value was observed as the C_H value increased in the range of 0.1-1.0 μmol/dm^3, resulting in complete suppression of the elongating potential at C_H = 1.0 μmol/dm^3 (Fig. 3.5A). In the cultures treated with paraquat, the α values of both types of hairy roots followed sigmoidal profiles (Fig. 3.5B). In these cases, the α value of PT hairy roots was close to zero at C_H = 1.0 μmol/dm^3 and the C_H value giving complete inhibition shifted to 10 μmol/dm^3 for the HT hairy roots. A notable difference in the responses of HT and PT hairy roots to pyributicarb was not observed, the α values overlapping with each other over the examined C_H values (Fig. 3.5C). Overlapping profiles of the α values were also observed upon exposure of both types of hairy roots to 2,4-D (Fig.3.5D).

For the quantitative estimation of chemical hazards to organisms, the median effective concentration (EC$_{50}$) is commonly used. In the present study, the EC$_{50}$ value was defined as the herbicide concentration at which 50 % reduction of the control value is caused in terms of tip elongation rate, and the change in the α value with C_H was expressed on the basis of the normal distribution probability function (Hoekstra, 1987), as follows.

$$\alpha = \left\{ 1 - \int_{-\infty}^{z} \frac{1}{\sqrt{2\pi}} \exp\left(-\frac{z^2}{2} \right) dz \right\} \times 100 \tag{3.2}$$

Here, z is correlated with the C_H and EC$_{50}$ values, as well as the coefficient σ_H, using the following equation.

$$z = \frac{\log(C_H / EC_{50})}{\sigma_H} \tag{3.3}$$

By matching Eqs. (3.2) and (3.3) to the experimental data using the nonlinear least-squares method, as shown by the curves in Fig. 3.5, the EC$_{50}$ values for HT hairy roots were determined as 1.8, 1.4 and 0.47 μmol/dm^3 for paraquat, pyributicarb and

2,4-D, respectively (not determinable for DCMU). The EC_{50} values for PT hairy roots were likewise evaluated as 0.45, 0.37, 1.2 and 0.40 μmol/dm^3 for DCMU, paraquat, pyributicarb and 2,4-D, respectively.

DCMU is known to block the electron transport system involved in photosynthesis of plants and paraquat interferes with the redox reactions involved in photosynthesis and respiration and forms active oxygen species harmful to cells (Fuerst and Norman, 1991; Wakabayashi and Sato, 1992). It was therefore reasonable that the PT hairy roots were more sensitive than the HT hairy roots to these herbicides which are photosynthesis inhibitors. With respect to pyributicarb, on the other hand, the determined EC_{50} values for both types of hairy roots were approximately equivalent. The herbicidal mechanism of pyributicarb involves disturbance of lipid metabolism in cells (Morinaka et al., 1993), which supports the finding that the phytotoxic effect of this chemical on the HT and PT hairy roots is similar.

3.4.2 Trace of root elongating responses against herbicidal stimuli with observation system

To examine the kinetics of root elongating responses of the HT roots to herbicidal stimuli, the time courses of the R_G^C value were followed using the observation system constructed in the present study. The R_G^C value were recorded from $t = 5$ h onward to avoid the fluctuation in the R_G^C value observed just after the inoculation of the roots. In the control culture without herbicide, as shown in **Fig. 3.6A**, the R_G^C value lays an approximately constant level through the examined measurement time of 48 h and the average value of R_G^C was estimated as 1.9×10^{-4} m/h. On the other hand, as seen in Fig. 3.6B, the addition of 2,4-D (1.0 μmol/dm^3) to the culture caused the gradual deterioration of the R_G^C value with elapsed time. A similar profile of the R_G^C value was observed in the culture with pyributicarb (1.0 μmol/dm^3) as indicated in Fig. 3.6C.

To evaluate the inhibitory effect of the herbicidal stress on the elongating potential of the roots, in the present study, the time profiles of the R_G^C values obtained in the presence of herbicides were expressed by following empirical equation.
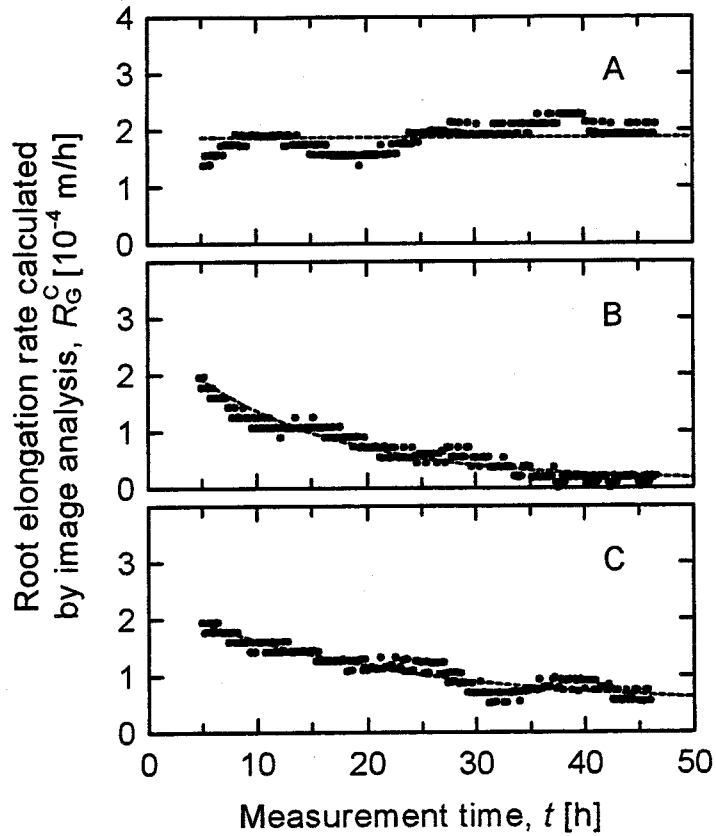
Fig. 3.6 Changes in root elongation rates determined by image analysis. (A) In culture without herbicide (control), (B) in cultures with 1.0 μmol/dm^3 2,4-D and (C) with 1.0 μmol/dm^3 pyributicarb. The lines were an average of the data (A) and drawn by matching Eq. (3.4) to the data (B and C).

$$R_G^C = (R_{G,5}^C - R_{G,S}^C) \exp \{-k' \, (t - 5)\} + R_{G,S}^C \tag{3.4}$$

where $R_{G,5}^C$ is the R_G^C value at $t = 5$ h as an initial value. Employing $R_G^{C,5}$ values obtained in the respective measurements, the parameter values of $R_{G,S}^C$ for the herbicide-containing cultures were determined by fitting Eq. (3.4) to the experimental data recorded from 5 h to t_F, using the non-linear squares method. In the control cultures, the $R_{G,S}^C$ values were averages of the data from 5 h to t_F. The results calculated for the data until $t_F = 48$ h are drawn as the dotted lines in Fig. 3.6A, B and C.

key	◎	◑	◐	●	▣	▣	◨	◧	■
Herbicides	2,4-D				pyributicarb				
C_H [μmol/dm^3]	0.2	0.5	0.7	1	0.1	0.3	1	3	10

Fig. 3.7 (A) Relationship between $R_{G,S}^{C}$ and R_G values obtained from cultures with various concentrations of 2,4-D or pyributicarb. The vertical and horizontal bars indicate standard deviation. (B) Changes in correlation coefficient with final time of measurement.
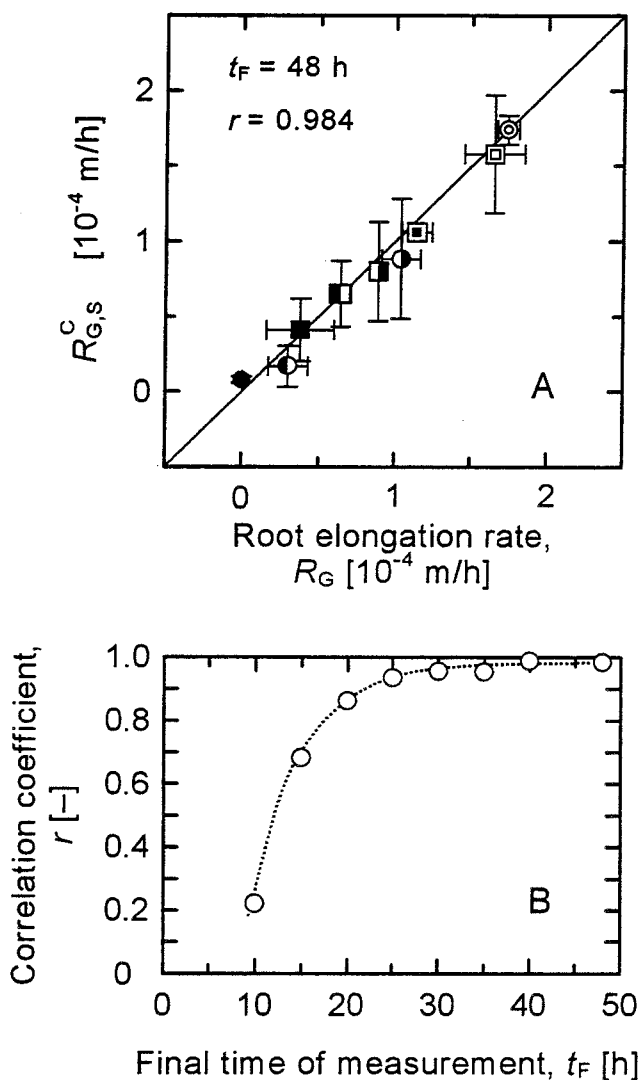
The comparison was then made between the $R_{G,S}^C$ value estimated from the data until $t_F = 48$ h and the R_G value manually evaluated in the separate cultures with longer exposure of the herbicide for 168 h. As shown in **Fig. 3.7A**, it was found that the values of $R_{G,S}^C$ and R_G exhibited a positive correlation with the correlation coefficient, $r = 0.984$ over the regions of 0.2-1.0 μmol/dm^3 2,4-D and 0.1-10 μmol/dm^3 pyributicarb. These results suggest that the $R_{G,S}^C$ value estimated from the data until $t_F = 48$ h, on the basis of the time profiles of the decreasing R_G^C value, could be a parameter to express the herbicidal toxicity against the hairy roots.

To judge the minimum measurement time required for the estimation of valid $R_{G,S}^C$ value by using the Eq. (3.4), the r value was calculated with respect to varied t_F values using the data of R_G^C with the indicated dose of 2,4-D or pyributicarb. As shown in Fig. 3.7B, the r value increased with an increase in the t_F value, giving $r = 0.935$ to 0.984 in the range of $t_F = 25\text{-}48$ h. It was considered that the measurement time of $t_F = 25$ h could offer the successful validity of the $R_{G,S}^C$ estimation using the observation system constructed in the present study.

Figure 3.8 shows the dose-response relationships in terms of tip elongation rate of the HT roots administered with pyributicarb or 2,4-D. In this figure, the α value for the observation system (closed keys) was calculated by the following equation using the $R_{G,S}^C$ value estimated from the data up to $t_F = 25$ h.

$$\alpha = \frac{R_{G,S}^C \text{ for herbicide - containing culture}}{R_{G,S}^C \text{ for control culture free from herbicide}} \times 100 \tag{3.5}$$

To determine the EC$_{50}$ value, the changes in the α value with C_H value were formulated based on Eqs. (3.2) and (3.3). The EC$_{50}$ values determined by using the $R_{G,S}^C$ values estimated from the data until $t_F = 25$ h were 0.40 and 1.1 μmol/dm^3 for 2,4-D and pyributicarb, respectively, under the examined conditions. These values were almost comparable to the EC$_{50}$ values determined by using the R_G value manually determined in prolonged cultures for 168 h, that is, 0.47 and 1.4 μmol/dm^3 for 2,4-D and pyributicarb, respectively.
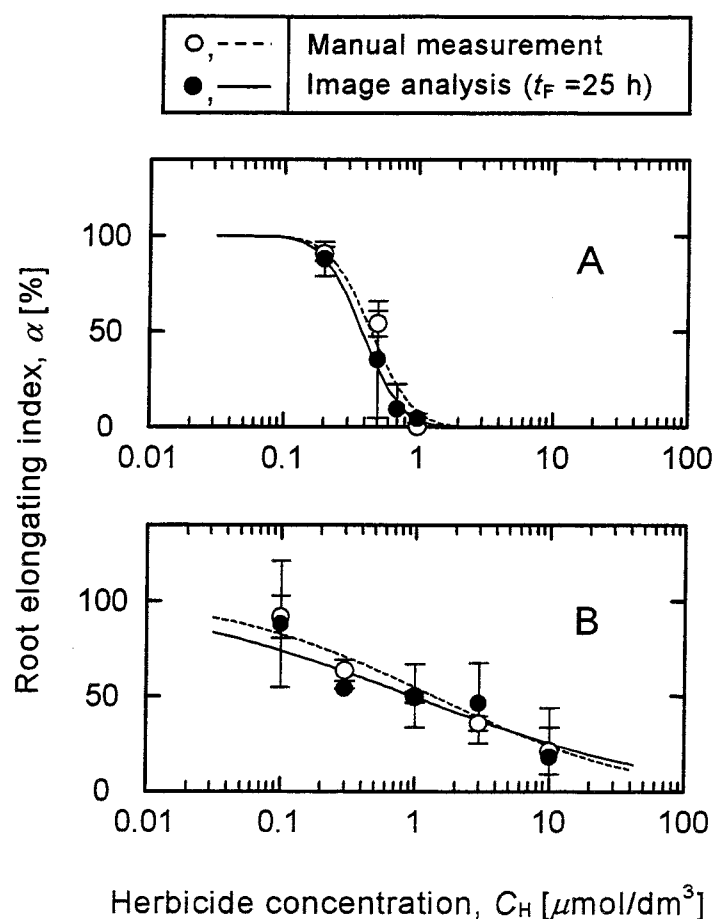
Fig. 3.8 Dose-response relationships of root elongation rate of HT hairy roots administered with herbicides. (A) 2,4-D and (B) pyributicarb. The lines were drawn by matching Eqs. (3.2) and (3.3) to the data.

3.4.3 Comparisons of root elongating responses with other bioassays

Table 3.2 lists EC_{50} values for the test chemicals reported in the literature using various plant materials. When compared with heterotrophic cultures, the hairy root system used in this work was found to have the relatively low EC_{50} values, *i.e.* high sensitivity, for the test herbicides. In the case of photoautotrophic cells, the EC_{50} values of PT hairy roots were comparable to or higher than those obtained using the reported assay system, although direct comparison is difficult because of the different conditions employed.

Table 3.2 EC$_{50}$ values determined in bioassays using plant materials

Plant species	EC$_{50}$ [μmol/dm^3]				References
	DCMU	Paraquat	Pyributicarb	2,4-D	
Heterotroph					
Maize[a]	65	65	NT	28	Grossmann et al. (1992)
Rape[a]	46	12	NT	17	Grossmann et al. (1992)
Tobacco[a]	200	20	NT	10	Sato et al. (1987)
Pak-bung[b]	>10	1.8	1.4	0.47	This work
Photoautotroph					
Spinach[c]	0.64	NT	NT	>1000	Laberge et al. (1999)
Scenedesmus acutus (Alga)[a]	0.057	30	NT	>100	Grossmann et al. (1992)
Duckweed[d]	0.047	0.034	NT	3.4	Grossmann et al. (1992)
Tobacco[a]	0.02	0.07	NT	2.0	Sato et al. (1987)
Pak-bung[b]	0.45	0.37	1.2	0.40	This work

The plant materials employed were suspended cells[a], hairy roots[b], thylakoids[c] and excised plants[d]. NT, Not tested.
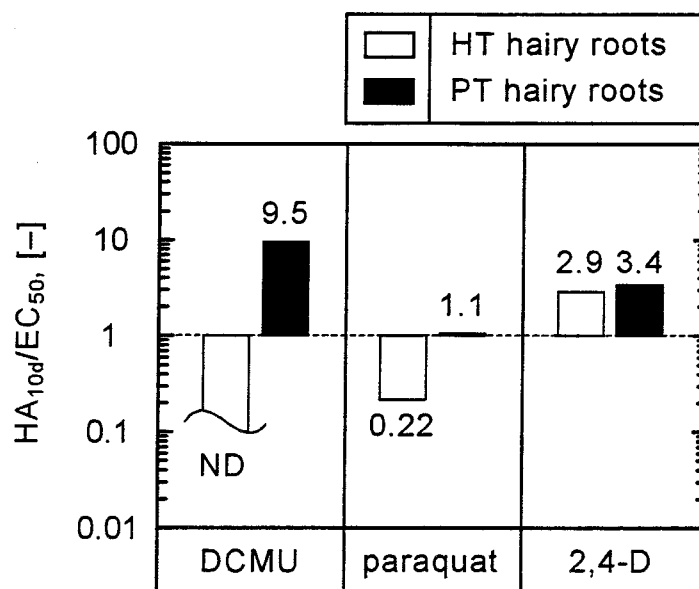
Fig. 3.9 Comparison between values of EC_{50} determined for pak-bung hairy roots and the HA_{10d} (10 d Human Health Advisory) with respect to DCMU, paraquat and 2,4-D. The HA_{10d} values are 4.3, 0.39 and 1.36 $\mu mol/dm^3$ for DCMU, paraquat and 2,4-D, respectively (Zavaleta et al., 1993). ND, Not determinable.

Shoji et al. (2000) determined the EC_{50} values of various organics and heavy metals in mammalian cell culture, and proposed an evaluation index for the acute toxicity of substances present in water, employing an environmental standard, the Human Health Advisory (HA). According to their proposal, in the present study, the EC_{50} values determined for the hairy root cultures were compared with the 10-day HA (HA_{10d}) value. Here, the HA_{10d} is an expected level of chemical at which no adverse effect on human health is produced through its intake for up to 10 days, as defined by the United States Environmental Protection Agency (Zavaleta et al., 1993). **Figure 3.9** illustrates the ratios of HA_{10d} to EC_{50} values (HA_{10d}/EC_{50}) with regard to DCMU and paraquat. In the case of the HT hairy roots, the values of HA_{10d}/EC_{50} for DCMU and paraquat were less than unity and the EC_{50} value for paraquat was about 4.5 times higher than the HA_{10d} value, indicating that the elongating response of HT hairy roots

was less effective for evaluating the acute toxicity of these chemicals. Regarding the PT hairy roots, on the other hand, the values of HA_{10d}/EC_{50} were 9.5 for DCMU and 1.1 for paraquat, which suggests that the elongating response of this hairy root line can be used for evaluating the acute toxicities of DCMU and paraquat.

When we apply the bioassay based on root elongating responses to the risk evaluation of the environmental samples or to the screening of the potent herbicidal chemicals, the rapidity and simplicity of the evaluation procedure would be important factors in addition to the sensitivity. Suzuki *et al.* (1997) summarized the relationship between the EC_{50} value as an index to express the sensitivity of organisms against the
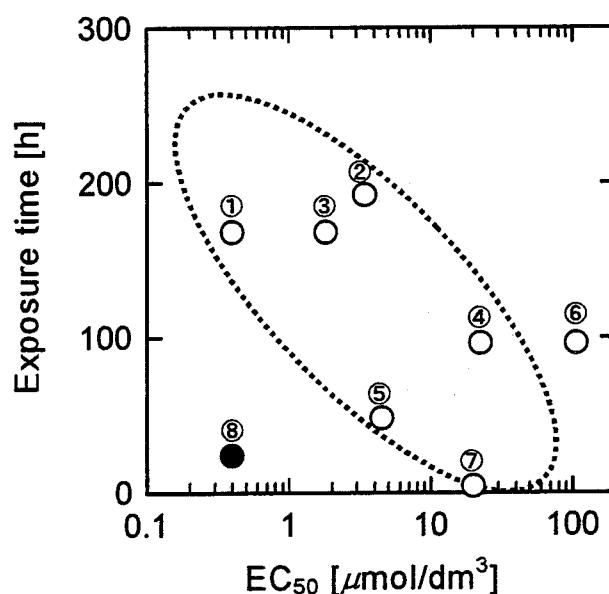
Fig. 3.10 Relationships between EC_{50} value and exposure time obtained from various bioassay systems against 2,4-D. ① HT hairy roots of pak-bung (This work; manual measurement), ② duck weed (Grossmann *et al.*, 1992), ③ cress seed (Grossmann *et al.*, 1992), ④ oat shoot (Kratky and Warren, 1971), ⑤ sorghum root (Kratky and Warren, 1971), ⑥ *Selenastrum caparicornutum* (alga) (St-Laurent *et al.*, 1992), ⑦ HepG2 (Shoji *et al.*, 1998) and ⑧ HT hairy roots of pak-bung (This work; image analysis).

toxic chemicals and the exposure time for determining the EC_{50} value for a given toxic chemical in the various kinds of bioassay systems, and pointed out that there was a tendency that long exposure time is generally demanded to attain high sensitivity in evaluating the toxicity of chemicals. A similar tendensy was seen betwee the EC_{50} value and exposure time (measurement time) in various kinds of bioassay systems for 2,4-D, as illustrated in **Fig. 3.10**, where our results are included using pak-bung hairy roots as assaying materials. Judging from the EC_{50} values, bioassay based on the elongating response of hairy roots was satisfactorily sensitive against 2,4-D, though the exposure time was relatively long in the case of the manual measurement. On the other hand, it was found that the predictive evaluation of elongating potential by the observation system constructed in the present study facilitated prompt evaluation of the toxicity, keeping the low value of EC_{50} (*i.e.* high sensitivity) for 2,4-D.

3.5 Summary

The elongating responses of the HT and PT hairy roots originated from pak-bung plant were examined against model chemicals with distinct herbicidal mechanisms, and following conclusions could be drawn.

(1) The HT and PT hairy roots were found to exhibit different elongating responses depending on the modes of actions of test herbicides. The EC_{50} values, defined as the herbicide concentration at which the R_G value is decreased to 50 % that of the herbicide-free control, were determined as follows. For the HT hairy roots: EC_{50} = 1.8 (paraquat), 1.4 μmol/dm^3 (pyributicarb) and 0.47 μmol/dm^3 (2,4-D), and for the PT hairy roots: EC_{50} = 0.45 (DCMU), 0.37 (paraquat), 1.2 μmol/dm^3 (pyributicarb) and 0.40 μmol/dm^3 (2,4-D).

(2) The elongating behavior of HT hairy roots was evaluated by automatic tracing of the root tip point with an observation system assisted by image analyzing technique. The elongation rate, R_G^C, followed by the established system at the interval of 0.2 h exhibited an approximately constant value of 1.9×10^{-4} m/h throughout the examined

measurement time of 48 h in the control culture without herbicide, while the addition of 1.0 μmol/dm^3 2,4-D or pyributicarb to the culture caused the gradual deterioration of the rate with measurement time until 48 h.

(3) At the examined concentrations of 2,4-D (0.2-1.0 μmol/dm^3) and pyributicarb (0.1-10 μmol/dm^3), the correlation coefficient between the values of $R_{G,S}^C$ and R_G was increased with an increase in the measurement time in the observation system, giving r = 0.935 - 0.984 when employed the data of R_G^C in the range of t_F = 25-48 h. This means that the measurement time required for the successful estimation of the saturated values of R_G^C can be shortened to 25 h by using the constructed observation system.

(4) The EC$_{50}$ values were determined to be 0.40 and 1.1 μmol/dm^3 for 2,4-D and pyributicarb, respectively, based on the $R_{G,S}^C$ values estimated from the data until t_F = 25 h. It was considered that the observation system could be a predictive tool to permit the prompt evaluation of herbicidal toxicities with high sensitivity.

Chapter 4

Difference in Hairy Root Responses to Chemicals
Compared Between Primary Roots and Lateral Roots

4.1 Introduction

In the preceding chapter, the hairy root responses to chemicals were examined on the basis of the elongating potentials at the existing root tips, and the availability of the root responses was discussed on the assessment of the acute toxicity of test herbicides. On the other hand, in this chapter, the responses of the hairy roots against chemical stimuli were examined considering tip budding as lateral roots.

Bioassay has been used for assessing the genotoxicity as well as the cytotoxicity of the chemicals for human and ecosystems. Among the environmental pollution caused by the chemicals, chronic or heritable effects have to be seriously concerned and can be assessed only by employing biological responses. In the root system, primary main roots produce many lateral roots and each of which will yields more lateral roots. From a viewpoint of organogenesis, the lateral root budding is an interesting development process, because it involves the production of an entire organ from a quite small number of differentiated cells (Malamy and Benfey, 1997). Especially, the hairy roots were reported to display active tip budding as lateral roots compared with normal roots (Toivonen, 1993), and therefore the hairy roots might be available as the model system to investigate the biological responses against environmental stimuli as an analogy to generations of organisms.

In this chapter, difference of hairy root responses against chemicals between the primary and lateral roots was examined taking account of the lateral root orders in the cultures of pak-bung HT hairy roots. The variations in hairy root responses through the tip budding of the lateral roots are discussed in terms of phenotypic and genotypic indices.

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4.2 Experimental

4.2.1 Plant materials

The HT hairy root line of pak-bung was used for the experiments. The hairy roots were maintained by means of regular subcultures in MS liquid medium with 20 kg/m^3 sucrose, under the same conditions as described in Chapter 1. Prior to the experiments, the hairy roots were subjected to the conditioning culture for a week on the solid MS plate and the lateral roots generated from main roots were used as the inoculum of the 1st lateral order of the exposure in a series of successive cultures.

4.2.2 Culture conditions and test chemicals

Throughout the experiments, the roots were incubated on the medium solidified with 2 kg/m^3 Gelrite® (San-ei Gen F. F. I. Inc., Osaka, Japan) in Petri dishes (90 mm in diameter) kept at 25°C in a dark room. The medium was supplemented with various amounts of chemicals when necessary. For inoculation, root tip part with about 10 mm was used. In the course of culture, as shown in **Fig. 4.1A**, the cultures of the primary roots were conducted at 7 days' interval, followed by replanting the tip parts with 5-10 mm onto a new solid medium, to keep the constant levels of the nutrients and added chemicals. For the cultures of the lateral roots, on the contrary, lateral roots outgrown from the primary roots were excised at 10 mm from the budding tip and replanted anew every 7 days. Then the second lateral order of exposure was initiated, followed by the subsequent lateral order of exposures in a similar manner (see Fig 4.1B).

2,4-D and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were used as test chemicals. 2,4-D was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan, and MNNG was from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

4.2.3 Measurement of root elongation rate

The R_G value was recorded by manual measurement according to the method described in Chapter 1. The R_G value was determined during 72-168 h after replanting of each roots in the course of hairy root culture. The elongating response of the roots to the chemicals was evaluated as the α value given by Eq. (3.1) in Chapter 3.

Culture of primary roots   Culture of lateral roots

t = 0 day

        1st order   1st order
        ⊂⊐   ⊂⊐

          ⇩   ⇩

t = 7 day

  *R*G   *R*G
  ←   ←

        1st order   2nd order
        ⊂⊐   ⊂⊐

          ⇩   ⇩

t = 14 day
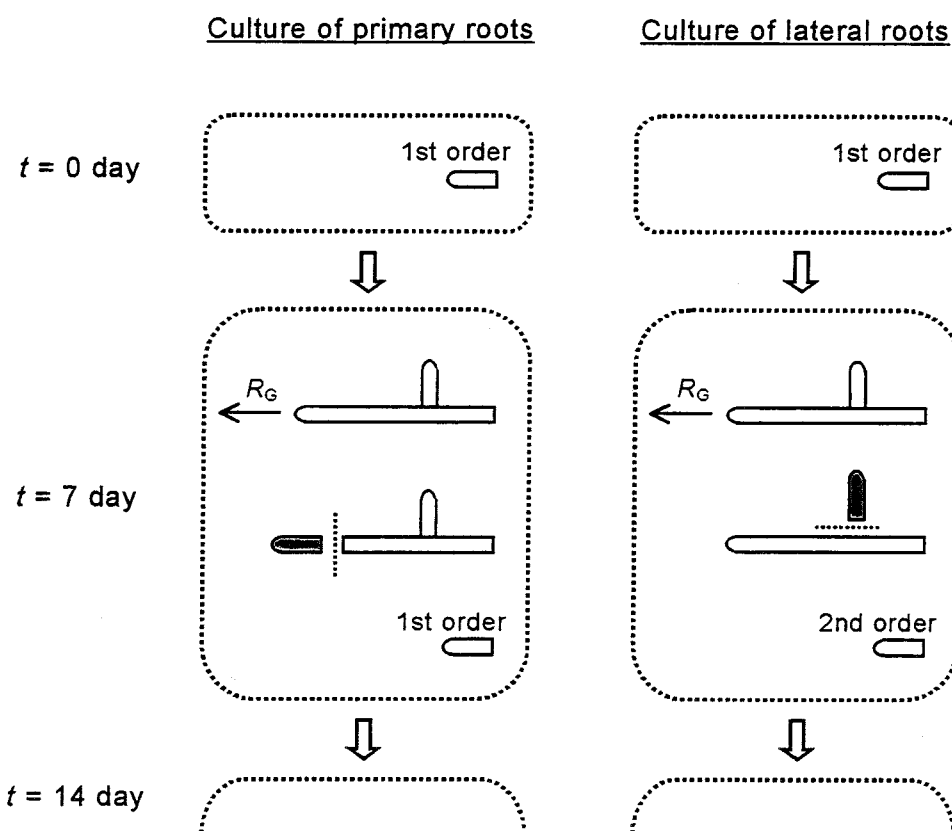
Fig. 4.1   Outline of culture operations of HT hairy roots of pak-bung

4.2.4 Random amplified polympholic DNA (RAPD) analysis

The root specimen obtained from the solid culture at the indicated exposure time was frozen in liquid nitrogen and subjected to fine pulverization. Genomic DNA was extracted and purified for RAPD analysis, by using a DNeasy Plant Mini kit (QIAGEN K. K., Tokyo, Japan) according to the suppliers' instruction.

Approximately 10 ng of DNA template was subjected to RAPD amplification performed in a reaction mixture of 20 μl, which contained 0.5 units of KOD Dash® DNA polymerase, 2 μl of 10× KOD Dash® buffer, 10 pmoles of decamer primer and 0.5 mM of four deoxynucleotide triphosphates (dNTPs). The primers used for the RAPD analysis are listed in **Table 4.1**. They were obtained from Espec Oligo Service Corp., Tsukuba, Japan. The other chemicals for PCR were purchased from Toyobo Co. Ltd.,

Osaka, Japan. The DNA amplification was performed with a DNA thermal cycler (GeneAmp® PCR system 2400, Perkin Elmer, Foster, USA). The reaction cycle was 30 during which each cycle consisted of denaturation for 30 seconds at 94 °C, annealing for 15 seconds at 54 °C and extension for 30 seconds at 74 °C, followed by the final extension step for 5 minutes at 74 °C. Reaction mixtures were stored at 4 °C prior to use.

Table 4.1 10-mer primers used for amplification

No.	Sequence (5'–3')	CG content [%]	T_m [°C]
A01	CAG GCC CTT C	70	42.7
A03	AGT CAG CCA C	60	38.5
A07	GAA ACG GGT G	60	38.5

The reaction products were separated by electrophoresis on a 1.5 % agarose gel in TAE buffer (40 mM Tris-acetate buffer (pH 7.6) with 1 mM EDTA). The electrophoresis was performed with a horizontal gel system (Mupid-21, Advance Co., Ltd., Tokyo, Japan) at constant voltage of 100 V for 40 minutes. The resultant gel were subsequently stained with ethidium bromide and the separated DNA bands were visualized on a UV transilluminator (Model TFS-20, UVP Inc., Upland, USA). λ DNA digested with *Hin* dIII was run for each agarose gel as a DNA molecular marker, and sizes of the bands visualized were 23130, 9416, 6557, 4361, 2322, 2027 and 564 bp from top to bottom. Image of gel was captured under UV illumination, using a digital camera (Model DC260 Zoom, Eastman Kodak Co., Tokyo, Japan).

4.3   Results and Discussion

4.3.1 Difference in phenotypic response to chemicals of primary roots and lateral

roots

To understand manner of lateral root budding as the hairy roots response to chemical stimuli, the tip elongating potentials were examined for both the primary and lateral root tips of HT hairy roots in the presence of 2,4-D or MNNG at concentrations of 0.01-10 μmol/dm^3. With addition of 2,4-D, differences were recognized in elongating responses between the primary and lateral root tips from the dose-response relationships shown in **Fig. 4.2**. The elongating potentials of lateral root tips were less than those of primary root tips over the examined concentrations. Moreover, it was found that the elongating potentials of lateral root tips upon the additions of respective 2,4-D concentrations decreased with increasing in the lateral root orders up to fifth within the examined exposure periods (Fig. 4.2B), while in the cultures of the primary roots, the sigmoidal profiles of the α values against 2,4-D did not significantly changed throughout the exposure time during 35 days (Fig. 4.2A). These results indicated that the deterioration of elongating potentials in the presence of 2,4-D was aggravated through the tip budding of lateral roots.

Figure 4.3 shows the dose-response relationships in the presence of MNNG. For the primary root tips, as shown in Fig. 4.3A, root elongating potentials were deteriorated with an increase in MNNG concentration, being suppressed with addition of MNNG at 1.0 μmol/dm^3. Notable changes in the sigmoidal profile with respect to the primary root tips were not seen throughout the exposure time during 35 days. On the other hand, tip budding and subsequent elongation as lateral roots were observed at the concentration of 10 μmol/dm^3 where the tip elongating potentials of the primary roots were completely suppressed (Fig. 4.3B). Moreover, it was found that, upon the prescribed concentration of MNNG, the lateral root tips exhibited the elongating potentials up to the fifth lateral root orders keeping 80 % of the elongation rate in the MNNG-free cultures of lateral root tips. This also implied the variation of elongating responses to the chemicals through the budding of lateral roots.

Key	○	◎	◉	○	●
t [day]	7	14	21	28	35
Root order	1	1	1	1	1

Key	○	◎	◉	○	●
t [day]	7	14	21	28	35
Root order	1	2	3	4	5

Fig. 4.2   Dose-response relationships of tip elongation of pak-bung HT hairy roots treated with 2,4-D. (A) Primary roots and (B) lateral roots. The vertical bars show standard deviation.

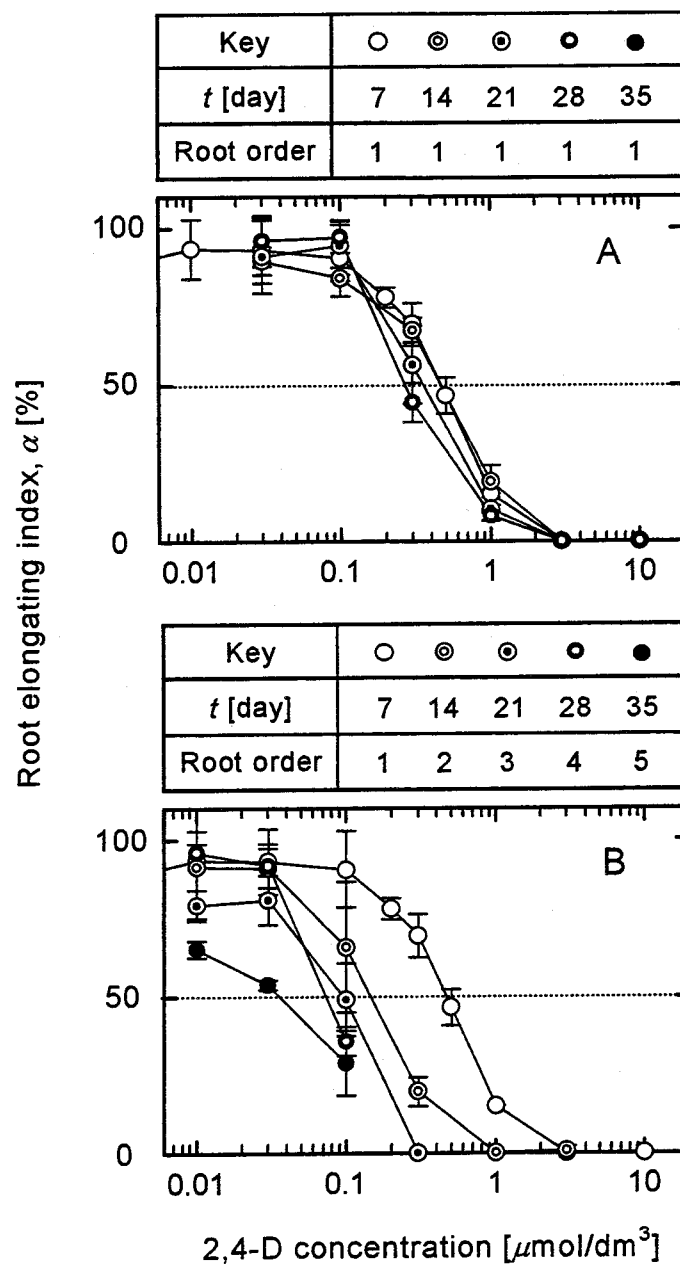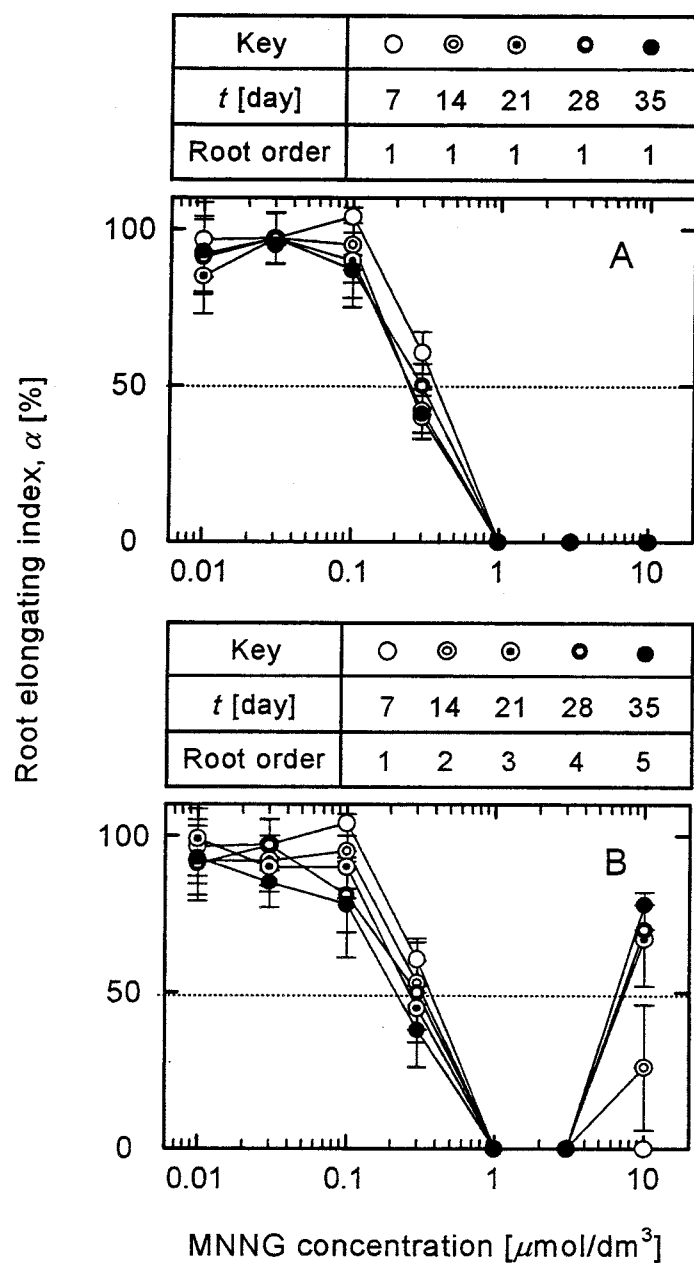**Fig. 4.3** Dose-response relationships of tip elongation of pak-bung HT hairy roots treated with MNNG. (A) Primary roots and (B) lateral roots. The vertical bars show standard deviation.

4.3.2 Difference in genotypic response to chemicals of primary roots and lateral roots

The exposure to genotoxic chemicals, such as mutagens, invites the formation of covalently bound adducts between the genotoxin and DNA, and the secondary effect on DNA, e.g., micronuclei and chromosome aberrations, resulting in phenotypic and genotypic mutations. For the test chemicals used in the present study, MNNG is a methylating agent which is known as one of potent mutagens and carcinogens. As for the mutagenicity of 2,4-D, Khalatkar and Bhargava (1985) demonstrated that the chemical caused phenotypic and genotypic changes in the higher plants, i.e., chlorophyll deficiency and chromosomal aberrations, suggesting that 2,4-D has mutagenic potential although the mutation frequency was lower than that produced by chemical mutagens such as ethyl methane sulphonate (EMS) and MNNG. Moreover, with respect to the phenotypic mutation through successive generation of the individuals, it has been often recognized that the mutation frequency was different in M1 and M2 generation of higher plant, and considered that the induced mutations are conserved over time in the somatic tissues, passing to the gametophytes. (Conte et al., 1998; Khalatkar and Bhargava, 1985; Walbot and Cullis, 1985) Considering these findings as well as the view that organogenetic process of the lateral root budding from primordium cells might be a model of plant germination via seeds, it could be assumed that genetic damages by the treatment with 2,4-D and MNNG attributed to the variation of elongating responses between primary roots and lateral roots which underwent the successive budding of lateral roots, as seen in Figs. 4.2 and 4.3.

RAPD analysis has been developed as one of the techniques to distinguish between species and individuals based on DNA fingerprinting generated by polymerase chain reaction (PCR) (Williams et al., 1990, Labra et al., 2001), and recently it has been applied for assessing genotoxicity of the chemicals in the environments (Savva and Castellani, 1996; Conte et al., 1998; Atienzar et al., 2000). To examine the genotypical changes in primary and lateral roos exposed to 2,4-D and MNNG, RAPD analysis was performed using the roots obtained from cultures with 0.3 μmol/dm^3 of 2,4-D and

cultures with 10 μmol/dm^3 of MNNG at which quite significant variations were observed between primary and lateral roots, as well as the cultures without addition of chemical. **Figure 4.4** shows agarose gel electrophoresis displaying RAPD PCR products with respect to the control cultures free from chemicals for 35 days (lane 1 and 2, primer: A01), cultures for 21 days with addition of 2,4-D (lane 3 and 4, primer: A02) and cultures for 35 days with addition of MNNG (lane 5 and 6, primer: A03). It was found that the pattern of DNA fingerprinting was similar each other for primary and lateral roots in the cases of the control cultures without chemicals. While with addition of 2,4-D or MNNG, it appeared that DNA fingerprinting was different between the primary and lateral roots. According to the results of RAPD analysis, the difference in elongating responses between lateral and primary roots was considered to be attributed to the genetic variation caused by the exposure of the test chemicals to the roots, indicating that the elongating responses can be an index for evaluating the genotoxicity of the chemicals by considering the difference in the primary and lateral root tips.

Fig. 4.4   Agarose gel electrophoresis showing RAPD PCR products. Lane M: phage λ DNA cleaved with *Hind* III, lane 1: primary roots from control culture without chemical exposure, lane 2: lateral roots from control culture without chemical exposure, lane 3: primary roots from culture with 2,4-D (0.3 μmol/dm^3), lane 4: lateral roots from culture with 2,4-D (0.3 μmol/dm^3), lane 5: primary roots from culture with MNNG (10 μmol/dm^3) and lane 6: lateral roots from culture with MNNG (10 μmol/dm^3).

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4.4 Summary

To investigate the influence of chemicals on lateral root budding as the hairy roots responses, the cultures with addition of 2,4-D and MNNG were conducted using both the primary and lateral roots of HT hairy roots of pak-bung, and followings could be concluded.

(1) When compared at a given exposure period and chemical concentration, the lateral roots were found to exhibit the different elongating responses from the primary roots. With addition of 2,4-D, low elongation rate was obtained in the case of lateral roots over the examined 2,4-D concentrations.

(2) In the cultures treated with MNNG, the lateral roots elongated even at the concentration where no elongation was observed in the case of the primary roots. These variation in elongating responses against the additions of test chemicals were significant for increased orders of the lateral roots.

(3) The RAPD analysis revealed that the difference in elongating responses between lateral and primary roots in the presence of 2,4-D or MNNG was attributed to the genetic variation caused by the exposure of the roots to the test chemicals.

Chapter 5

Assessment of Herbicidal Toxicity
Based on Responses of Local Chl Pigmentation of PT Roots

5.1 Introduction

Biological responses of living organisms profoundly reflect the conditions of their surroundings, and have been thereby applicable as bioassay for assessing synergistic toxicities of coexisting pollutants in the terrestrial and aqueous surroundings while complementing the physical and chemical analyses (Fawell and Horth, 1998; Buikema Jr. *et al.*, 1982; Shoji *et al.*, 2000). Though a specific response of a single organism is sometimes used for target chemicals exerting phenotypic or genotypic stimulus on cells, a bioassay system with combinations of plural response indices from test organisms is effective to extend the kinds of detectable chemicals and to cope with the diversity of the pollutants such as heavy metals, herbicides and even unidentified compounds.

The hairy roots display unique growth manner of elongating at root apical meristems and the resultant distribution of cell maturity along the longitudinal direction of the roots as mentioned in Part I, which can induce the positional change in biological responses of the hairy roots to external stimuli. With respect to herbicidal stimuli, the PT hairy root line of pak-bung was found to display the sensitive elongating response against the test chemicals as compared with the heterotrophic root line, as described in Chapter 3. In this chapter, the responses of the local Chl pigmentation in the PT roots against herbicides with different modes of action were examined by non-destructive measurement assisted with an image analysis. Furthermore, both the responses of root elongation and local Chl pigmentation of the roots were applied to the assessment of toxicities of individual herbicides as well as field water as a model pollutant.

5.2 Experimental

5.2.1 Plant materials

The PT hairy root line of pak-bung was used throughout experiments. The hairy roots were maintained by means of regular subcultures in sugar-free MS liquid medium under the same conditions as described in Chapter 1.

5.2.2 Culture conditions and test chemicals

To examine the responses of the root elongation and Chl pigmentation to test herbicides, the cultures were carried out at 25 °C using sterile Petri dishes (90 mm in diameter) containing sugar-free MS medium solidified with 2 kg/m^3 Gelrite® (San-ei Gen F. F. I. Inc., Osaka, Japan). The Petri dishes containing the inoculated roots were located under a 5.0 % carbon dioxide atmosphere in an incubator (Model CFH-305, Tomy Seiko Co., Ltd., Tokyo, Japan) and illuminated with a bank of tubular fluorescent lumps (FL 20SSW, Matsushita Electric Industrial Co., Ltd., Osaka, Japan). The incident light intensity of I at surface of the solid medium was adjusted to 11 or 22 W/m^2.

DCMU, paraquat and 2,4-D were used as test herbicides with distinct herbicidal mechanisms, as mentioned in Chapter 3. These herbicides were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Each medium was supplemented with various quantities of the herbicides, when necessary, according to the procedures instructed in the literatures (Mugnier, 1988; Horn *et al.*, 1983). Field water sample was obtained from a puddle in a Toyonaka district, Osaka, in March 2002, and stored at 4 °C after rough filtration until the use. The water sample was filter-sterilized by passing through 0.2 μm membrane (Millex®-LG, Millipore Co., Bedford, MA, USA) and the MS solid medium containing the indicated ratio of the water sample (R_W) was prepared for the experiments.

5.2.3 Measurement of root elongation rate and analysis of Chl content in hairy roots

The R_G value was recorded by manual measurement according to the method described elsewhere described in Chapter 1. The R_G value was determined during

72-168 h in the course of hairy root culture conducted at $I = 11$ W/m^2, and each data was a mean value of the measurements taken for about fifteen root tips under the respective conditions. The elongating response of hairy roots to the herbicides was estimated by the α value given by Eq. (3.1) in Chapter 3.

The C_{Chl} was measured spectrophotometrically using the roots cut into the segments with length of 5×10^{-3} or 10×10^{-3} m, as described elsewhere (Porra *et al.*, 1989), and recorded as a DW basis of the roots determined gravimetrically by drying the roots at 80 °C for 72 h.

5.3 Image analysis for evaluating local Chl pigmentation in PT hairy roots

5.3.1 Microscopic observation

The Petri dish with the PT hairy roots inoculated was placed on a stage of an optical microscope (Model BX50-34 with a ten-fold objective, Olympus Optical Co.,
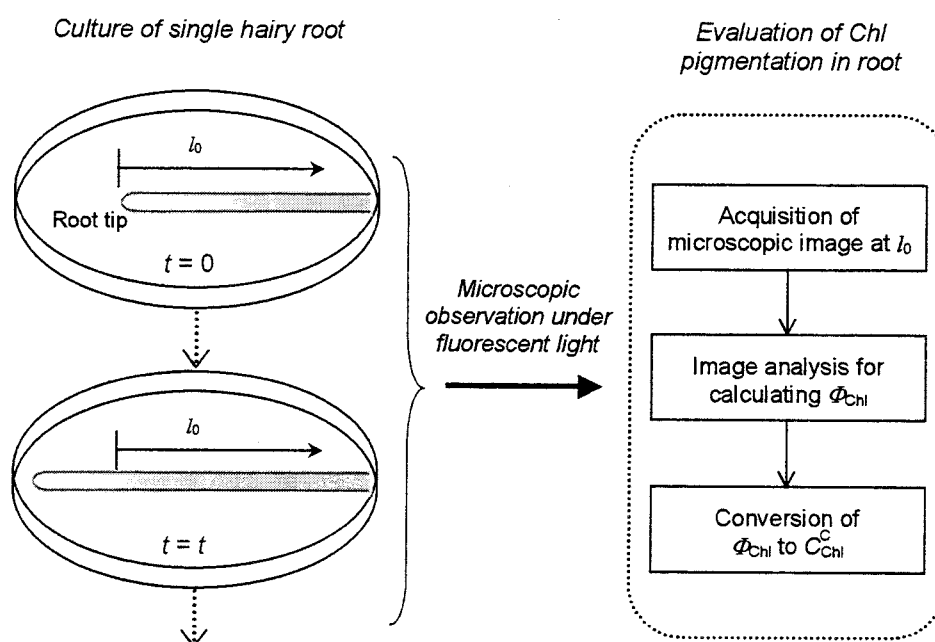

Fig. 5.1 Outline for observation during culture of PT hairy roots on solid medium.

Ltd., Tokyo, Japan) equipped with a BX-FLA fluorescent light device (Olympus Optical Co., Ltd., Tokyo, Japan), and observation was carried out under fluorescent light (excitation and emission wave lengths: 520-550 and above 580 nm, respectively) with the magnification of 10× objective. The dish was marked for the guide of longitudinal position from tip of the roots determined at initial time of the culture (l_0), and each position on the roots was traced for the observation throughout the course of the culture, as illustrated in **Fig. 5.1**. The microscopic observation was performed by fixing a central line of the image at the position of l_0 on the guide. The fluorescent images were acquired using a CCD camera (HCC-600, Flovel Co., Ltd., Tokyo, Japan) attached to the microscope by a connector (U-TV1×, Olympus Optical Co., Ltd., Tokyo, Japan), and inputted into a Pentium based computer with 96 MB RAM *via* an image-acquiring board, as the digitalized data of 480 × 640 pixels to be converted to red, green and blue (RGB) or hue, saturation and intensity (HSI) color coordinates. Each pixel had 256 classified levels of respective color coordinates ranging 0 to 255.

5.3.2 Procedure of color image analysis

Figure 5.2 shows typical fluorescent images of the PT roots under the observing conditions employed in the present study. The hairy roots cultivated on the herbicide-free medium appeared to be reddish owing to the fluorescence from Chl (Fig.
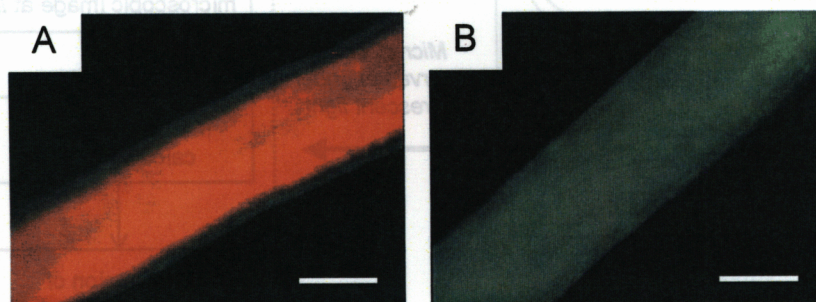
Fig. 5.2 Fluorescent images of PT hairy roots. (A) Culture without herbicide (control) and (B) culture with herbicide (10 μmol/dm^3 paraquat). The hairy roots were cultivated at $I = 22$ W/m^2 on the solid media. The bar shows 0.5 mm.
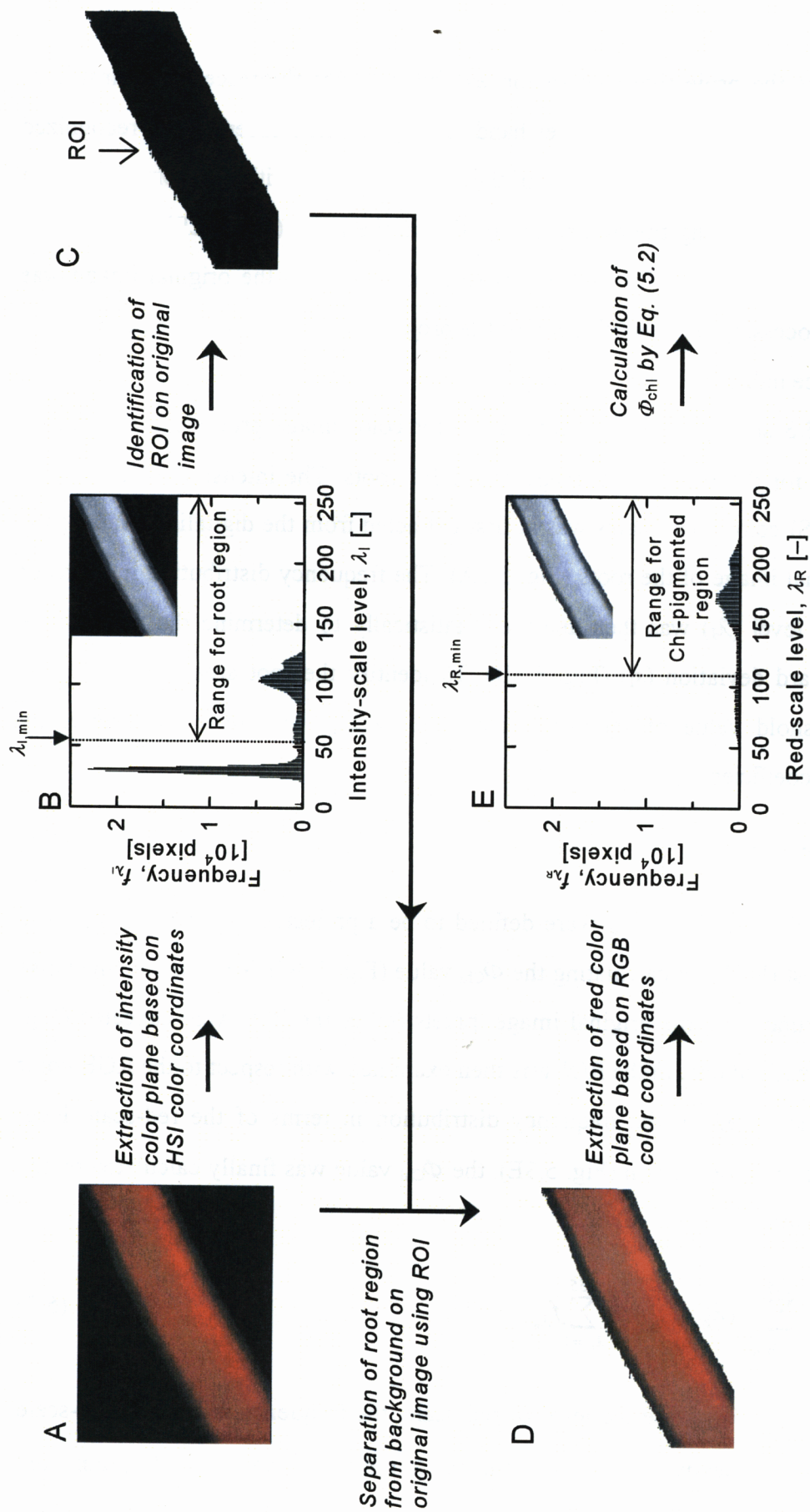
Fig. 5.3 Diagram of image processing procedure to evaluate Chl fluorescent index. (A) Original color image acquired, (B) frequency distribution of intensity scale level on original image, (C) binary image of root region separated from background, (D) color image of root region identified as ROI, (D) color image of root region separated from background, and (E) frequency distribution of red scale level on image of root region.

5.2A), and thus the projective root region exhibited bright fluorescence against dark background on the image. On the other hand, only green fluorescence was recognized on the projective root region in the case of the roots cultivated with addition of paraquat (10 μmol/dm^3), suggesting the diminution of Chl in the roots (Fig. 5.2B). To quantify the fluorescent intensity of Chl pigment as seen in Fig. 5.2A, the original image was subjected to processing for identification of the projective root region and calculating of Chl fluorescence index (Φ_{Chl}).

Figure 5.3 shows a series of procedures for color image processing to evaluate the Φ_{Chl} value for the fluorescent image of the PT roots. The intensity color plane in terms of the HSI color coordinates was at first extracted from the digitalized data of the acquired original image of the roots (Fig. 5.3A). The frequency distribution in terms of intensity-scale level (λ_I) was then analyzed statistically to determine the mean value (m_I) and standard deviation (s_I) (Fig. 5.3B). To identify the root area on the original image, a threshold value of intensity-scale level ($\lambda_{I,min}$) was given as follows by employing the coefficient δ_I.

$$\lambda_{I,min} = m_I + \delta_I \cdot s_I \tag{5.1}$$

Hence, the pixels with $\lambda_I \geq \lambda_{I,min}$ were defined to be a projected root region, that is, a region of interest (ROI) for calculating the Φ_{Chl} value (Fig. 5.3C). To eliminate the noise on the background from the original image, pixels out of the ROI were canceled (Fig. 5.3D). The digitalized data in the ROI was then examined with respect to the RGB color coordinates, and based on the frequency distribution in terms of the red-scale level inside the projective root region (Fig. 5.3E), the Φ_{Chl} value was finally calculated by the following equation.

$$\Phi_{Chl} = \sum_{\lambda_R = \lambda_{R,min}}^{255} (\lambda_R \cdot f_{\lambda_R}) \Bigg/ \sum_{\lambda_R = 0}^{255} f_{\lambda_R} \tag{5.2}$$

where λ_R, f_{λ_R} and $\lambda_{R,min}$ are the level of red scale, the frequency at a given red-scale level of λ_R in the projective root region, and the minimum λ_R value of Chl pigmented

region in the projective root region, respectively.

In the present study, the δ_I value in Eq. (5.1) was determined as -0.29 from comparison between the pixel number inside the ROI (N_P) determined by the image analysis and the N_P value by the manual detection of root edge on the corresponding image. The data of both N_P values using $\delta_I = -0.29$ presented sufficient correlation with a correlation coefficient of 0.950 in terms of 45 root specimens prepared from various l_0 values (see **Fig 5.4**). Moreover, the $\lambda_{R,min}$ value in Eq. (5.2) correlated with m_G and s_G as well as the coefficient δ_G, given as follows.

$$\lambda_{R,min} = m_G + \delta_G \cdot s_G \tag{5.3}$$

Here, the values of m_G and s_G were the mean value and standard deviation from the frequency distribution in terms of green-scale level inside the ROI, and were used as parameters to define the red-scale range corresponding to the Chl pigmented region. The coefficient δ_G in Eq. (5.3) was empirically determined as 0.40, where the Φ_{Chl} value
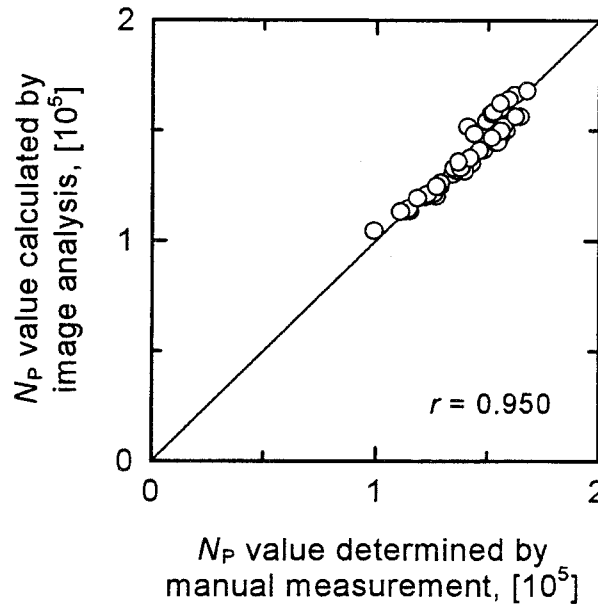
Fig. 5.4 Correlation between the number of pixels in projective root region determined by image analysis and those determined by manual measurement.

calculated for the hairy roots without Chl pigment, *i.e.* HT hairy root line of pak-bung, was assumed to be zero.

The image analysis was performed by employing a computer installed with a LabVIEW™ software including an add-on software for image processing, IMAQ Vision (National Instruments Co., Austin, TX, USA).

5.4 Results and Discussion

5.4.1 Determination of Chl content in roots by image analysis

In the PT hairy roots cultivated under light irradiation, the change in positional Chl content was observed along root length from the tip due to cell maturation arising from their linear growth manner, as described in the Chapter 1. To obtain a calibration

Key	O	◎	o	◉	◐	◑	◒	◓	●
l_0 [10^{-3} m]	2.5	7.5	12.5	17.5	22.5	27.5	35	45	55

Chl content, C_{Chl} [g-Chl/kg-DW]

Chl fluorescent index, Φ_{Chl} [–]

Fig. 5.5 Relationship between Chl content and Chl fluorescent index obtained from various longitudinal positions of PT hairy roots. The hairy roots were cultivated at $I = 11$ W/m^2 on the solid medium without herbicide.

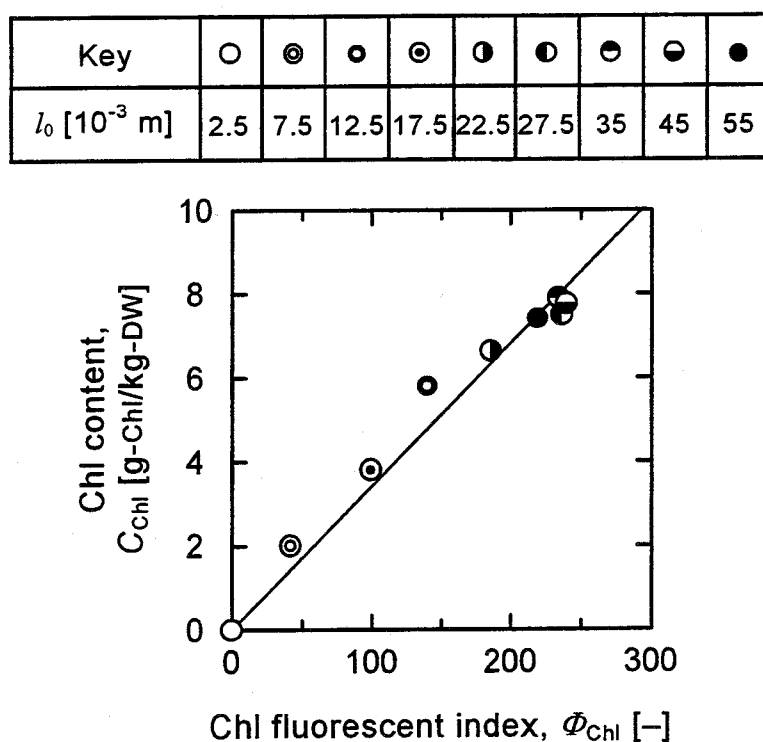line for estimating the Chl level by the image analyzing method, the Φ_{Chl} value was calculated using the images acquired from various longitudinal positions of the PT roots on the usual MS plate. To determine the experimental value of C_{Chl} at the corresponding positions, Chl pigment was simultaneously extracted from the root segments. As seen in **Fig. 5.5**, the C_{Chl} and Φ_{Chl} values examined in the range of $l_0 = 2.5 \times 10^{-3} - 55 \times 10^{-3}$ m showed a good linearity with a correlation coefficient of 0.983. It was therefore judged that the Φ_{Chl} values obtained from the image analysis could be a reliable measure to estimate Chl content in a single segment of the photoautotrophic roots. In the subsequent experiments, the Chl pigmentation was evaluated in terms of the local Chl content (C_{Chl}^C) calculated by $C_{Chl}^C = 3.4 \times 10^{-2} \ \Phi_{Chl}$, which was obtained from the line drawn in Fig. 5.5.

Key	t [h]
○	0
◎	24
●	48

Chl content calculated by image analysis, C_{Chl}^C [g-Chl/kg-DW]

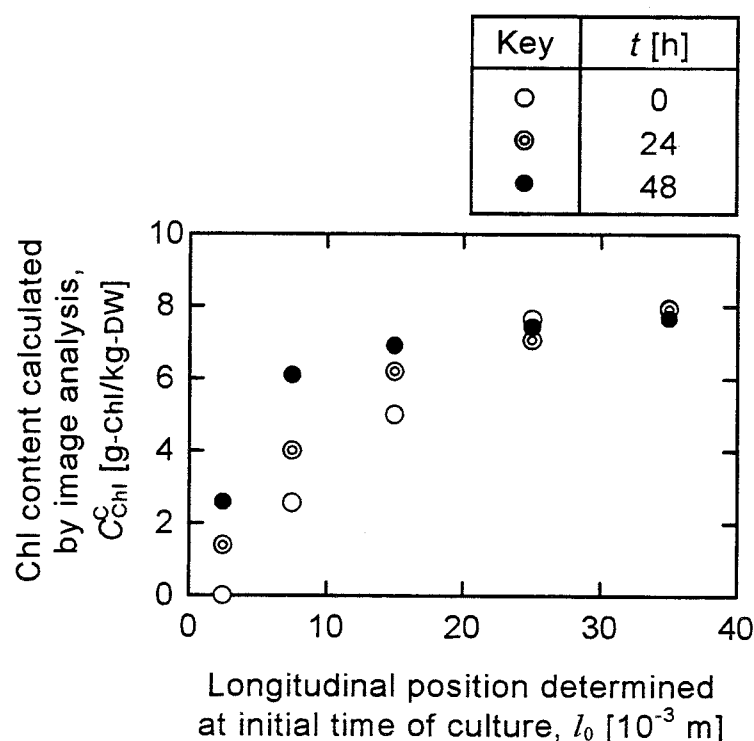Longitudinal position determined at initial time of culture, l_0 [10^{-3} m]

Fig. 5.6 Plots of C_{Chl}^C value against l_0 value in PT hairy roots. The hairy roots were cultivated at $I = 22$ W/m^2 on the solid medium without herbicide.

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Figure 5.6 shows the relation between the I_0 and C_{Chl}^C values in the culture of PT roots on the herbicide-free medium at $I = 22$ W/m^2. As for the longitudinal distribution of the pigment, the C_{Chl}^C value increased with an increasing I_0 value, reaching the saturated level of approximately 8.0 g-Chl/kg-DW. During the culture of t = 0-48 h, the C_{Chl}^C value at the positions of $I_0 = 2.5 \times 10^{-3}$-15×10^{-3} m increased with elapsed time, while no significant change in the C_{Chl}^C value was observed at $I_0 =$ 25×10^{-3} and 35×10^{-3} m and in particular the C_{Chl}^C value at $I_0 = 35 \times 10^{-3}$ m was kept around the saturated level. According to these results, the examinations on the C_{Chl}^C value by the image analysis were conducted at the fixed I_0 values of 2.5×10^{-3} and 35×10^{-3} m, which were regarded as the Chl accumulating and Chl saturating positions, respectively.

5.4.2 Changes of local Chl content in response to herbicides

To examine the changes in local Chl content in the presence of herbicides, the cultures of PT roots were conducted at $I = 22$ W/m^2 on the MS solid medium containing DCMU, paraquat or 2,4-D at each concentration of the EC$_{50}$. As described in foregoing chapter, the EC$_{50}$ values were 0.40, 0.37 and 0.45 μmol/dm^3 for DCMU, paraquat and 2,4-D, respectively, as determined to be concentrations at which the α values based on root elongation rate fall to 50 % of the control. **Figure 5.7A** shows time profiles of the C_{Chl}^C value at the position of $I_0 = 2.5 \times 10^{-3}$ m, where the C_{Chl}^C value was apparently zero at initial time due to the non-pigmented region containing the meristematic root cells. In the control culture without herbicides, the C_{Chl}^C value increased linearly with elapsed time during the culture of $t = 96$ h. Similar courses of the C_{Chl}^C value were observed in the cultures with paraquat and 2,4-D. To the contrary, the addition of DCMU severely suppressed the Chl accumulation throughout the examined culture time. Based on the obtained time profiles of the C_{Chl}^C value at $I_0 = 2.5 \times 10^{-3}$ m, the Chl accumulation rate, $R_A = dC_{Chl}^C/dt$, was determined from the slope of the data shown in Fig. 5.7A. The responses of Chl accumulation to the herbicides were then evaluated by the index, β, given as follows.

Key	Fitted line	Herbicide
○	————	Control
△	— — —	DCMU
□	- - - - -	Paraquat
◇	··············	2,4-D
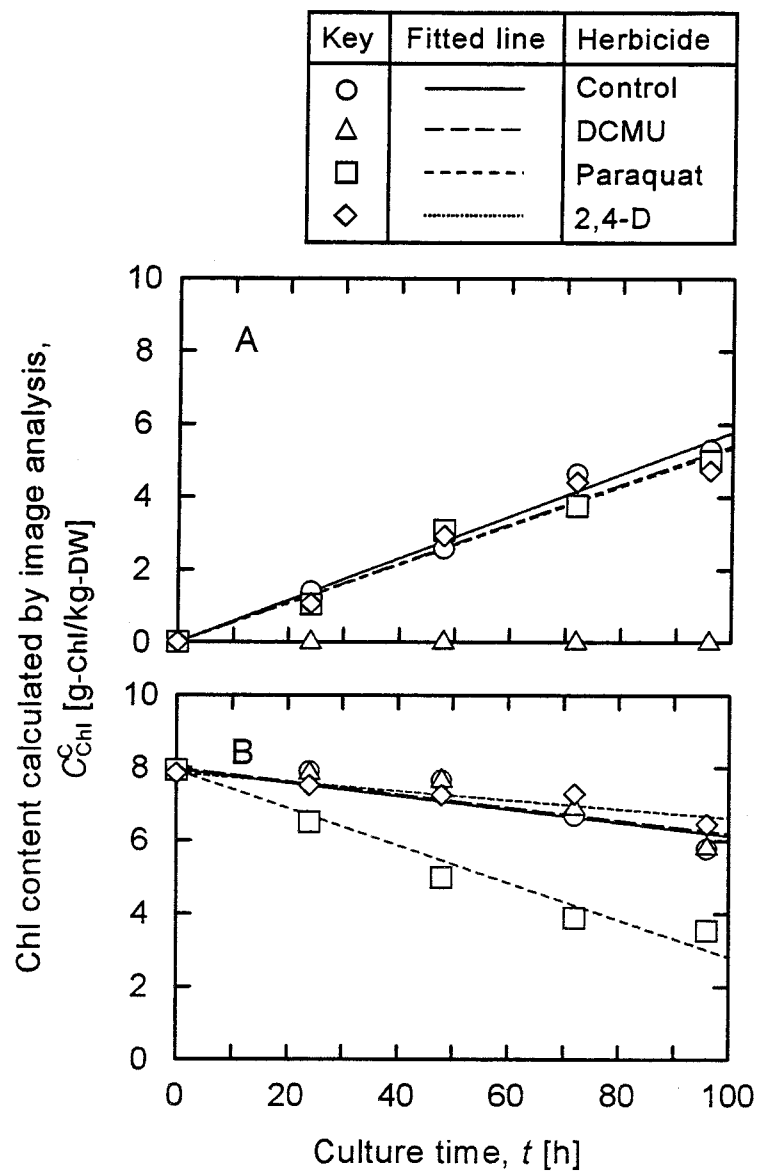
Fig. 5.7 Time courses of C_{Chl}^{C} value in PT hairy roots. (A) $I_0 = 2.5 \times 10^{-3}$ m and (B) 35×10^{-3} m. The hairy roots were cultivated at $I = 22$ W/m^2 on the solid media to which DCMU, paraquat and 2,4-D were added at 0.45, 0.37 and 0.40 μmol/dm^3, respectively.

$$\beta = \frac{R_A \text{ for herbicide-containing culture}}{R_A \text{ for control culture free from herbicide}} \times 100 \qquad (5.4)$$

Under the light condition of $I = 22$ W/m^2, the values of β were determined as 0, 93.6 and 93.8 % for DCMU, paraquat and 2,4-D, respectively, as listed in the upper part of **Fig. 5.8**. Similar tendencies of β values for the respective herbicides were obtained when the hairy roots were cultivated at $I = 11$ W/m^2.

	Chl accumulating index, β [%]		Chl degrading index, γ [%]	
	$I = 11$ W/m^2	$I = 22$ W/m^2	$I = 11$ W/m^2	$I = 22$ W/m^2
DCMU	0	0	91.1	98.4
Paraquat	90.6	93.6	92.9	282
2,4-D	95.8	93.8	105	86.5
Field water	110	105	112	217
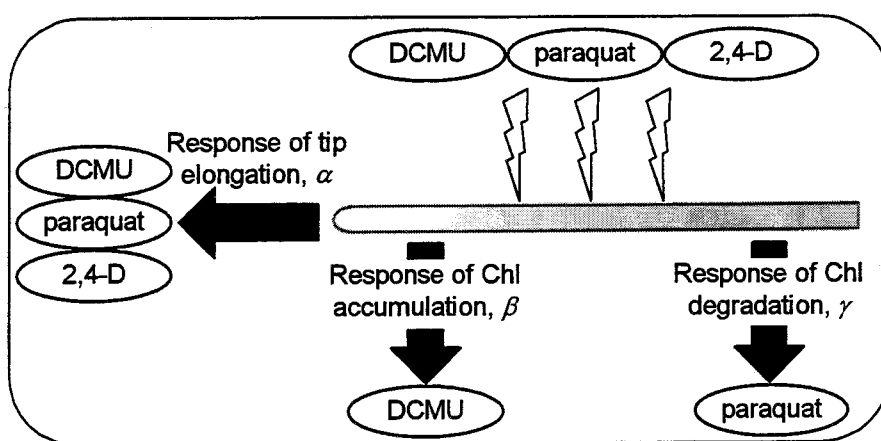
Fig. 5.8 Summary of responses of PT hairy roots against herbicidal stimuli based on tip elongation and Chl pigmentation.

Figure 5.7B shows the time profiles of the C_{Chl}^C value at the position of $l_0 = 35 \times 10^{-3}$ m where the initial C_{Chl}^C value was 7.9 g-Chl/kg-DW in the cultures at $I = 22$ W/m². In the control culture, the C_{Chl}^C value decreased slightly with elapsed time during the course of the culture. The overlapped profiles of the C_{Chl}^C value were found in the cultures containing DCMU and 2,4-D. On the other hand, the addition of paraquat accelerated the drop in the C_{Chl}^C value within the examined culture period. To evaluate the obtained time profiles of C_{Chl}^C at $l_0 = 35 \times 10^{-3}$ m, Chl degradation rate, $R_D = -dC_{Chl}^C/dt$, was determined from the slope of the data in Fig. 5.7B. The degradation of Chl in response to the herbicides was evaluated by the index, γ, given as follows.

$$\gamma = \frac{R_D \text{ for herbicide-containing culture}}{R_D \text{ for control culture free from herbicide}} \times 100 \qquad (5.5)$$

As indicated in Fig. 5.8, the values of γ values were determined as 98.4, 282 and 86.5 % for DCMU, paraquat and 2,4-D, respectively. Here, the γ values obtained in the cases of $I = 11$ W/m² were 91.1, 92.9 and 105 % for DCMU, paraquat and 2,4-D, respectively, and no significant degradation of Chl against paraquat was observed, as listed in the upper part of Fig. 5.8. In this figure, the γ values obtained at $I = 11$ W/m² are also included, and the β value for paraquat was found to be lowered unlike that obtained at $I = 11$ W/m².

Though the primary effect of DCMU is to block the photosynthetic electron transport (PET) system involved in chloroplast of plants (Fuerst and Norman, 1991; Wakabayashi and Sato, 1992), this herbicide is known to destroy the Chl molecule when administered in large doses (Sandmann and Böger, 1982; Barry, 1990). As for the Chl responses against the sub-lethal concentration of PET inhibitors, François and Robinson (François and Robinson, 1990) reported that atrazine inhibited Chl accumulation especially in the exponential growth phase rather than the stationary phase in algal cultures. This is a similar phenomenon to the finding that DCMU suppressed the accumulation of the Chl in the active growing-cells near the apical meristems in the hairy roots. Paraquat interferes with the redox reactions involved in photosynthesis and

respiration, forming active oxygen radicals harmful to cells (Fuerst and Norman, 1991).

The higher value of γ observed at $I = 22$ W/m^2 with addition of paraquat may be attributed to the frequent formation of radicals due to functionally maturated Chl pigment as a site of photosynthesis at the position of $l_0 = 35 \times 10^{-3}$ m as compared with the tip position of the roots. With respect to 2,4-D, both the values of β and γ for the respective longitudinal positions of $l_0 = 2.5 \times 10^{-3}$ and 35×10^{-3} m were around 100 %, indicating that this chemical is less phytotoxic against Chl synthesis and degradation. The primary herbicidal function of 2,4-D can be disruption of phytohormone without specific effect on Chl pigmentation (Mugnier, 1988), which supports the results obtained in the present study. As illustrated in the lower part of Fig. 5.8, it is therefore expected that the combined assay based on the Chl accumulating and degrading responses of the PT roots contributes to the detection of the herbicidal chemicals and the assessment of their toxicities according to their distinct mechanisms.

5.4.3 Application of root responses to evaluating toxicity of field water

To extend the availability of the responses of photoautotrophic roots for
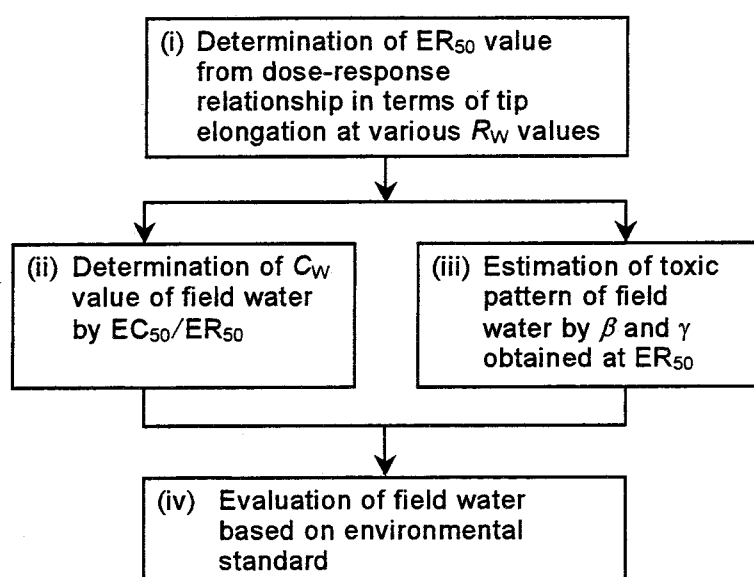
Fig. 5.9 Procedure for evaluating toxicity of field water based on responses of PT hairy roots.

evaluating environmental pollution in terms of herbicidal toxicity, model water obtained from field was subjected to the test according to the procedure as indicated in **Fig. 5.9**.

(i) From a dose-response relationship obtained from the root elongating responses to the field water, median effective ratio (ER_{50}) defined as the R_W value at which the α value falls to 50 % of the control is determined. (ii) The concentration of chemicals equivalent to field water toxicity (C_W) is estimated by EC_{50}/ER_{50}. (iii) On the MS plate prepared at $R_W = ER_{50}$, the values of β and γ are determined from the changes in C_{Chl}^C at the indicated I_0 values in the same manner as mentioned in the foregoing section. (iv) The C_W value for the test water evaluated through the procedures, (i)-(iii), is compared with an environmental standard.

In the dose-response relationship obtained for the field water at $I = 11$ W/m^2 (**Fig. 5.10**), a substantial decrease in the α value was observed at $R_W = 0.5$. The ER_{50} value

Fig. 5.10 Dose-response relationship of tip elongation of PT hairy roots on medium containing field water. The hairy roots were cultivated at $I = 11$ W/m^2 on the solid media to which field water was added at various ratios. The vertical bars show standard deviation. The line was drawn by matching Eqs. (3.2) and (5.6) to the data.

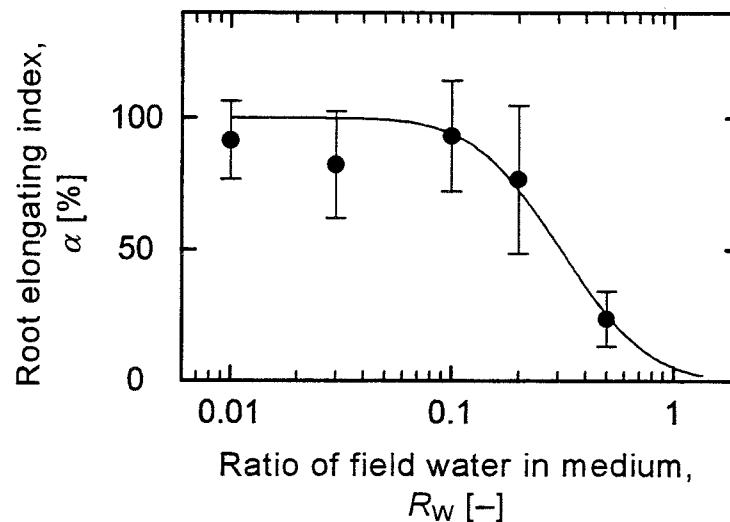was determined from the experimental data, by expressing the change in the α value with R_W on the basis of the normal distribution probability function, as described by Eq. (3.2) in Chapter 3. Here, the z value in Eq. (3.2) is correlated with the R_W and ER_{50} values, as well as the coefficient σ_W, using the following equation.

$$z = \frac{\log(R_W / ER_{50})}{\sigma_W} \tag{5.6}$$

By matching Eqs. (3.2) and (5.6) to the experimental data using the nonlinear least-squares method, as shown by the curve in Fig. 5.10, the ER_{50} values were determined as 0.31. By comparison of the obtained values of ER_{50} for the field water and EC_{50} based on elongating responses for the known test chemicals, the C_W value was determined to be 1.45, 1.19 and 1.29 $\mu mol/dm^3$ for DCMU, paraquat and 2,4-D, respectively. Moreover, the values of β and γ were determined as 110 and 112 % at $I =$ 11 W/m^2, and 105 and 217 % at $I = 22$ W/m^2, respectively, from tracing the Chl changes in the roots on the plate containing the field water at ER_{50} values, as listed in Fig. 5.8. Judging from the both indices in terms of the accumulation and degradation of Chl in the roots, the field water tested was considered to display "paraquat-like" toxicity against the roots. With respect to paraquat, the environmental standard is evaluated as 0.39 $\mu mol/dm^3$ in terms of 10-day Human Health Advisory (HA_{10d}), which is defined as an expected level of chemical at which no adverse effect on human health is induced through its intake for up to 10 days (Zavaleta et $al.$, 1993). Thus, it is known that the C_W value of the field water tested in the present study can be 3 times high as the HA_{10d} value.

5.5 Summary

Change of local Chl content in PT hairy roots of pak-bung was evaluated at incident light intensities of $I = 11$ and 22 W/m^2 by non-destructive measurement of the pigment based on a color image analysis, and followings could be concluded.

(1) During the herbicide-free culture of t = 0-48 h, the C_{Chl}^C value at the positions of l_0 = 2.5×10^{-3}-15×10^{-3} m increased with elapsed time, while no significant change in the C_{Chl}^C value was observed at l_0 = 25×10^{-3} and 35×10^{-3} m, where the C_{Chl}^C value at l_0 = 35×10^{-3} m was kept around the saturated level of 8.0 g-Chl/kg-DW.

(2) With addition of DCMU, paraquat and 2,4-D to medium at the median effective concentrations (0.40, 0.37 and 0.45 μmol/dm^3 for DCMU, paraquat and 2,4-D, respectively), the PT roots showed different responses of Chl pigmentation to the test herbicides when measured at longitudinal lengths of l_0 = 2.5×10^{-3} m (Chl accumulating position) and l_0 = 35×10^{-3} m (Chl saturating position) under the light irradiation. Chl accumulating index, β and Chl degrading index, γ were determined from the changes in Chl content at l_0 = 2.5×10^{-3} and 35×10^{-3} m, respectively, during the cultures for 96 h: β = 0 (DCMU), 93.6 (paraquat) and 93.8 % (2,4-D), and γ = 98.4 (DCMU), 282 (paraquat) and 86.5 % (2,4-D) at I = 22 W/m^2.

(3) The bioassay system with the PT hairy roots was applied to evaluating a model sample of field water. The values of β and γ for the field water were determined as 105 and 217 % at I = 22 W/m^2 respectively, from which the field water tested was judged to be a "paraquat-like" toxicant against the roots.

General Conclusion

Responses of root tissues against various chemical stimuli were studied with respect to the morphological and physiological properties unique to plant root system, for the application to the assessment of chemical toxicity in environment. In this work, plant hairy roots were used as the representative of the root tissues, which exhibit stable and infinite propagation keeping their differentiated phenotype as adventitious roots under controlled culture conditions. It is demonstrated that proliferative and physiological features of the hairy roots, such as root elongation, lateral root budding and cell maturity along longitudinal direction, were available as a measure for estimation of chemical stress, and strategies for assessing toxicity of chemicals were proposed from the viewpoint of tissue-specific responses including the foregoing outcrops distinct from death of cells or individuals. The major findings obtained throughout this thesis are concluded as follows.

In Part I including Chapters 1 and 2, as the fundamental study for application of the hairy roots as the tool of the bioassay, both the potentials of root elongation and tip budding were characterized, respectively, considering changes in cell maturity along the direction of single root. In Chapter 1, the elongating potentials of pak-bung hairy roots were characterized in terms of ATP contents in root tip parts or carbon balance of the roots. In the cultures of HT roots with various values of initial ATP content in tip parts conducted in sucrose-free medium, root elongation was found to cease at ATP content below about 2.0×10^{-4} mol/m. In the case of the PT roots cultivated under light irradiation, the occurrence of root elongation depended on the root length from root tips as well as the culture conditions, $i.e.$, the roots with or less than 2.0×10^{-2} m did not elongate. From the time profiles of root elongation and ATP contents, cell yield of elongating roots and maintenance energy on an ATP basis were estimated, which coincided closely with each other for the HT roots and the PT roots under the examined conditions. Furthermore, carbon balance in the PT roots was calculated from CO_2 fixation rate taking account into the longitudinal distribution of Chl content and carbon

consumption rates for both anabolic and catabolic metabolisms. As a result, the minimum carbon requirement for the root elongation was estimated to be almost constant under the examined culture conditions.

In Chapter 2, root budding potential from segmental roots was characterized in terms of oxygen uptake rate of the roots. The positional distributions in potential of lateral root budding and oxygen uptake rate were examined using the segments of madder and horseradish hairy roots obtained at different mean distances from root tips. The root budding rates and oxygen uptake rates of the root segments obtained from the positions near tip parts were relatively higher and gradually decreased with the increases in distance from root tips in both the cases of madder and horseradish hairy roots. Positive relations were observed between the rates of lateral root budding and oxygen uptake of both the hairy roots. The relation indicated that the potential of lateral root budding was suppressed at the oxygen uptake rates of 1.5×10^{-6} and 3.2×10^{-6} mol-O_2/(h·m) for madder and horseradish hairy roots, respectively. These values were approximately comparable to the oxygen uptake rates of these hairy roots required for maintenance metabolism under a lowered sucrose concentration.

In Part II which covers Chapters 3, 4 and 5, the hairy root responses against chemical stimuli were evaluated based on the root elongation at apical meristems, tip budding as lateral roots or cell maturity along the longitudinal direction. In Chapter 3, hairy root responses to chemicals were evaluated by employing root elongation as an index. The HT and PT hairy roots originated from pak-bung plant were found to display different elongating responses to DCMU, paraquat, pyributicarb and 2,4-D depending on the respective necrotic actions of the herbicides. The combination of both types of hairy roots will provide a useful bioassay system for the detection of toxic environmental pollutants as well as for the primary distinction between chemicals with different herbicidal mechanisms. Moreover, an observation system was developed to evaluate the elongating behavior of pak-bung hairy roots through automatic tracing of the root tip points employing computer-aided image analyzing technique. It was concluded that the predictive evaluation of elongating responses by the transient data

acquired with the observation system constructed in the present study could facilitate prompt assessment of herbicidal toxicities with high sensitivity.

In Chapter 4, hairy root responses to chemicals were evaluated taking account of lateral root budding. To investigate the influence of chemicals on lateral root budding as the hairy roots response, elongating properties were analyzed in the presence of 2,4-D and MNNG using both the primary and lateral roots of HT hairy roots of pak-bung. From the data at a given exposure period and chemical concentration, the lateral roots were found to exhibited the different elongating responses as compared with those of the primary roots. With addition of 2,4-D, reduced elongation rates were obtained in the cases of lateral roots over the examined 2,4-D concentrations. In the cultures treated with MNNG, root elongation of lateral roots occurred at the MNNG level where there is no elongation in the case of the primary root tips. These differences of elongating responses against test chemicals were increased with increasing in the lateral root orders. As a result of RAPD analysis, the difference in elongating responses between lateral and primary roots was attributed to the genetic variation caused by the exposure of the roots to the test chemicals.

In Chapter 5, hairy root responses to chemicals were evaluated based on the longitudinal distribution of local Chl pigment in the PT hairy roots of pak-bung. At the longitudinal lengths of $= 2.5 \times 10^{-3}$ and 35×10^{-3} m, which were Chl accumulating and Chl saturating positions, respectively, the roots exhibited the different Chl responses to the herbicides depending on the respective necrotic mechanisms of DCMU, paraquat and 2,4-D, when the herbicides were administered at the EC_{50} values determined from the root elongating potential. The obtained results suggested that the assay based on the root elongating index as well as Chl accumulating and degrading indices contributed to the detection of the chemicals and the classification of their mechanisms. These indices could be determined by tracing the changes of tip position and Chl pigmentation of the roots on the images, employing the non-destructive measurement method together with the computer-aided color image analysis. Therefore, the present study provides a methodology for monitoring and assessing the environmental pollution with sorting of

herbicidal functions. Moreover, the bioassay system with the PT hairy roots was found to be available for evaluating toxicity of field water.

In this study, responses of plant hairy roots against chemical stimuli have been investigated with respect to the morphological and physiological features of the root system, and the strategies for assessing toxicity of chemicals have been discussed from the viewpoint of both biological and bioengineering points of view. It is considered that most properties of the hairy roots examined in this study are applicable to normal excised roots, and therefore the outcomes obtained in this work will make contributions to design the methodologies for evaluating chemical responses of plant root tissues in the fields of the environmental and agrochemical studies.

Nomenclature

A_{Chl}	=	gross amount of Chl in hairy roots	[g]
$\hat{A}_{ATP}$	=	ATP amount in root tip part	[mol]
C_C	=	carbon content in hairy roots	[g/kg-DW]
C_{Chl}	=	Chl content in hairy roots determined spectrophotometry	[g/kg-DW]
C_{Chl}^{C}	=	Chl content in hairy roots calculated by image analysis	[g/kg-DW]
C_{Chl}^{i}	=	Chl concentration at inside of chloroplast	[g/m³]
C_{Chl}^{o}	=	Chl concentration at outside of chloroplast	[g/m³]
$C_{Chl,S}$	=	saturated value of C_{Chl} in Eq. (1.6)	[g/kg-DW]
C_H	=	herbicide concentration in medium	[μmol/dm³]
C_W	=	chemical concentration equal to toxicity of tested field water	[μmol/dm³]
$\overline{C}_{Chl}$	=	average Chl content in hairy roots	[g/kg-DW]
$\hat{C}_{ATP}$	=	ATP content in root tip part	[mol/m]
$\hat{C}_{ATP,E}$	=	$\hat{C}_{ATP}$ value at $t = t_E$	[mol/m]
$\hat{C}_{ATP,0}$	=	$\hat{C}_{ATP}$ value at initial time of culture	[mol/m]
D	=	root diameter	[m]
EC_{50}	=	median effective concentration based on root elongation rate	[μmol/dm³]
ER_{50}	=	median effective ratio based on root elongation rate	[–]
f_{λ_I}	=	frequency at intensity-scale level of λ_I in projective root region	[–]
f_{λ_R}	=	frequency at red-scale level of λ_R in projective root region	[–]
F_B	=	normal distribution probability function for ΔN_B	[h⁻¹]
I	=	incident light intensity	[W/m²]
j	=	line segment perpendicular to vector, v	[–]
k	=	parameter concerning Chl formation and cell maturation in Eq. (1.6)	[m⁻¹]
k_{Chl}	=	apparent permeation rate constant through envelope of chloroplast	[h⁻¹]
k'	=	kinetic parameter in Eq. (3.4)	[h⁻¹]
l	=	longitudinal position from root tip	[m]
l_0	=	longitudinal position from root tip determined at initial time of culture	[m]

L	=	root length from tip	[m]
L_E	=	root length from tip at $t = t_E$	[m]
L_G	=	length of GPs	[m]
L_0	=	inoculum root length from tip	[m]
$L_{0,min}$	=	minimum L_0 value required for root elongation	[m]
$\hat{L}$	=	length of root tip part	[m]
m_{ATP}	=	maintenance energy on ATP basis	[mol/(h·m)]
m_G	=	mean value in frequency distribution of green-scale level	[−]
m_I	=	mean value in frequency distribution of intensity-scale level	[−]
N_B	=	number of BTs per length of segment	[BTs/m]
$N_{B,max}$	=	maximum number of BTs per length of segment	[BTs/m]
N_P	=	number of pixels in projective root region	[−]
P	=	coordinate of root tip point	[−]
Q	=	oxygen uptake rate of roots per length of segment	[mol/(h·m)]
Q_{min}	=	minimum Q value required for root budding	[mol/(h·m)]
r	=	correlation coefficient	[−]
r_{ANA}	=	carbon consumption rate for anabolism	[mol/h]
r_{CAT}	=	carbon consumption rate for catabolism	[mol/h]
r_{CO_2}	=	CO_2 fixation rate of hairy roots	[mol/h]
R_A	=	Chl accumulation rate	[g/(h·kg-DW)]
R_{CAT}	=	r_{CAT} value based on root mass	[mol/(h·kg-DW)]
R_D	=	Chl degradation rate	[g/(h·kg-DW)]
R_G	=	root elongation rate determined by manual measurement	[m/h]
R_G^C	=	root elongation rate calculated by image analysis	[m/h]
$R_{G,S}^C$	=	saturated value of R_G^C determined from Eq. (3.4)	[m/h]
$R_{G,5}^C$	=	R_G^C value at $t = 5$ h in Eq. (3.4)	[m/h]
R_W	=	ratio of field water in medium	[−]
$\tilde{R}_B$	=	average rate of lateral root budding	[BTs/(h·m)]
$\bar{R}_{CO_2}$	=	average CO_2 fixation rate of hairy roots	[mol/(h·kg-DW)]

s_G	=	standard deviation in frequency distribution of green-scale level	[–]
s_I	=	standard deviation in frequency distribution of intensity-scale level	[–]
t	=	culture time	[h]
t_E	=	time of ceasing in root elongation	[h]
t_F	=	final measurement time	[h]
$\tilde{t}_B$	=	mean time for lateral root budding in Eq. (2.1)	[h]
v	=	vector showing elongating direction	[–]
w	=	water content of hairy roots	[–]
x	=	x coordinate of root tip point calculated by image analysis	[–]
y	=	y coordinate of root tip point calculated by image analysis	[–]
Y_{ATP}	=	cell yield of elongating root on ATP basis	[m/mol]
z	=	variable in Eq. (3.2)	[–]
α	=	root elongating index	[%]
β	=	Chl accumulating index	[%]
γ	=	Chl degrading index	[%]
δ_G	=	coefficient in Eq. (5.3)	[–]
δ_I	=	coefficient in Eq. (5.1)	[–]
$\Delta\hat{C}_{ATP}$	=	change in ATP content in root tip part	[mol/m]
ΔL	=	root elongation length determined by manual measurement	[m]
ΔL^C	=	root elongation length calculated by image analysis	[m]
ΔN_B	=	change in number of BTs per length of segment	[BTs/m]
Δt	=	measurement period	[h]
ε	=	coefficient in Eqs. (A1.4) and (A1.5)	[–]
λ_I	=	level of intensity scale	[–]
$\lambda_{I,min}$	=	minimum λ_I value in projective root region	[–]
λ_R	=	level of red scale	[–]
$\lambda_{R,min}$	=	minimum λ_R value in Chl pigmented region	[–]
$\tilde{\mu}$	=	average of specific elongation rate	[h⁻¹]
ρ	=	density of hairy roots	[kg-FW/m³]

σ_B = coefficient in Eq. (2.1)   [h]

σ_H = coefficient in Eq. (3.3)   [−]

σ_W = coefficient in Eq. (5.6)   [−]

Φ_{Chl} = Chl fluorescent index   [−]

ψ_{ATP} = coefficient in Eq. (1.4)   [mol/(h·g)]

ψ_C = coefficient in Eq. (1.8)   [mol/(h·g)]

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List of Publications

Original papers

1. Elongating Potential of Pak-Bung Hairy Roots under Photoautotrophic Culture Condition

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