STUDY ON LIGHT AND ROOT ZONE ENVIRONMENTS FOR GROWTH AND CAMPTOTHECIN ACCUMULATION OF *OPHIORRHIZA PUMILA*

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Graduate School of Horticulture

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(千葉大学審査学位論文)

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CONTENTS

LIST OF ABBREVIATIONS

CPT	Camptothecin
d	Days after transplanting
DAT	Days after start of the treatment
DMF	N, N-dimethylformamide
DLI	Daily light integral
E	Transpiration rate
EC	Electrical conductivity
LED	Light emitting diode
LAI	Leaf area index
MS	Murashige and Skoog
NSC	Nutrient solution concentration
NST	Nutrient solution temperature
PFAL	Plant factory with artificial light
PPFD	Photosynthetic photon flux density
P_n	Net photosynthetic rate
RH	Relative humidity
ROS	Reactive oxygen species
SSS	Strictosidine synthase

ABSTRACT

Ophiorrhiza pumila, a wild medicinal plant, is distributed on the floors of humid inland forests in subtropical areas and accumulates camptothecin (CPT), which is used clinically as an anti-tumor agent. To meet the increasing demand for CPT and facilitate its stable production, it is necessary to clarify the characteristics of gas exchange rates of whole plants and establish the suitable light and root-zone environments for growth and CPT accumulation of *O. pumila* in a plant factory with artificial light (PFAL). At first, the concentration distribution of CPT was investigated in each organ and at each developmental stage (Chapter 2). To produce the maximum CPT content from *O. pumila*, stem and root were the essential organs, and the seed-ripening stage was the best timing for harvest. To determine the suitable light conditions for growth, the net photosynthetic rate (P_n) and transpiration rate (E) of whole plants were measured using an open-type assimilation chamber (Chapter 3). These analyses revealed that the light saturation point was at a photosynthetic photon flux density (PPFD) of 300 μmol m^{-2} s^{-1}, and the E tended to decrease with increasing PPFD above 100 μmol m^{-2} s^{-1}. As a result, we found that 100 μmol m^{-2} s^{-1} PPFD and 28 °C were good conditions of PPFD and air temperature for photosynthesis and transpiration. Also, when compared these results to the P_n and E of lettuce, and *O. pumila* exhibited to be a typical shade plant. To investigate the suitable root-zone conditions, the effects of nutrient solution concentration (NSC; 0.125, 0.25, 0.5, and 1.0 times) and temperature (NST; 10, 20, 26, and 36 °C) were examined respectively (Chapter 4). According to these results, the 0.25 times and 20 °C were the suitable NSC

and NST, respectively, for growth and CPT accumulation. My research revealed the suitable environmental conditions for the growth and CPT accumulation from *O. pumila* cultivated in a PFAL, and it may contribute to the efficient CPT production for a clinical anti-tumor agent.

CHAPTER 1

Introduction

1.1 Background

1.1.1 Camptothecin

In developed countries, the number of cancer patients and the use of anticancer drugs are rising as the aged population increases. Camptothecin (CPT) is a plant-based monoterpenoid indole alkaloid used as a raw material in anticancer drugs (Figs. 1-1A and 1-2). It was initially identified in extracts of *Camptotheca acuminata* (Nyssaceae) stem bark as new steroids from thousands of plants were being screened (Hsiang et al., 1985; Wall et al., 1966). This compound possesses anti-tumor properties owing to its inhibitory activity against DNA topoisomerase I; however, it is not used clinically due to its very low water solubility, strong cytotoxicity, and rapid inactivation at physiological pH (Hsiang et al., 1985; Redinbo et al., 1998). Therefore, the semi-synthetic derivatives of CPT, topotecan and irinotecan (Fig. 1-1, B and C), have been employed worldwide as clinical anti-tumor agents against cancers of the lungs, cervix, ovaries, colon, and others. (Houghton et al., 1992; Li et al., 2006; Saito et al., 2001).

The world market for these CPT derivatives reached 2.2 billion USD in 2008 and has been expected to continue to increase in the future (Cui et al., 2015; Lorence and Nessler, 2004). Despite the high demand for CPT for use in anticancer drugs, CPT has been extracted mainly from two arboreous plants, *C. acuminata* and *Nothapodytes foetida*

(Olacaceae), which have slow growth rates and low productivity (Asano et al., 2013; Cui et al., 2015; Lorence and Nessler, 2004).

As arboreous plants require a large cultivation area and high photosynthetic photon flux density (PPFD), open-field cultivation is necessary. However, it is difficult to produce and supply stable raw materials for drugs via open-field cultivation due to the unpredictability associated with seasonal change, variable weather, and pests (Asano et al., 2013; Cui et al., 2015). Therefore, to meet the increasing demand for CPT, an alternative cultivation method must be developed or an alternative medicinal plant species must be found.

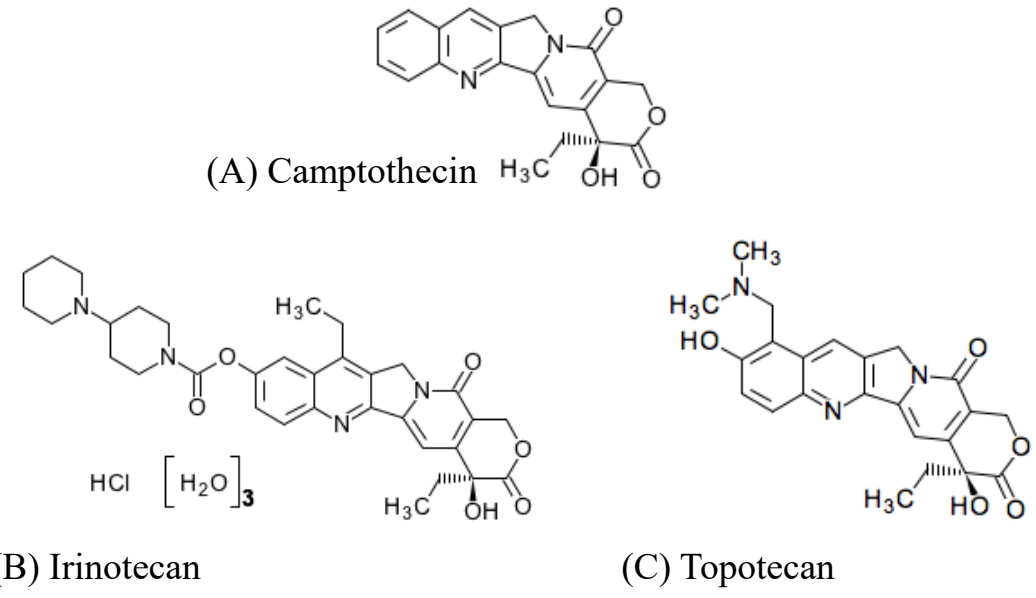

Fig. 1-1 Structural formulas of camptothecin (A), irinotecan (B), and topotecan (C).

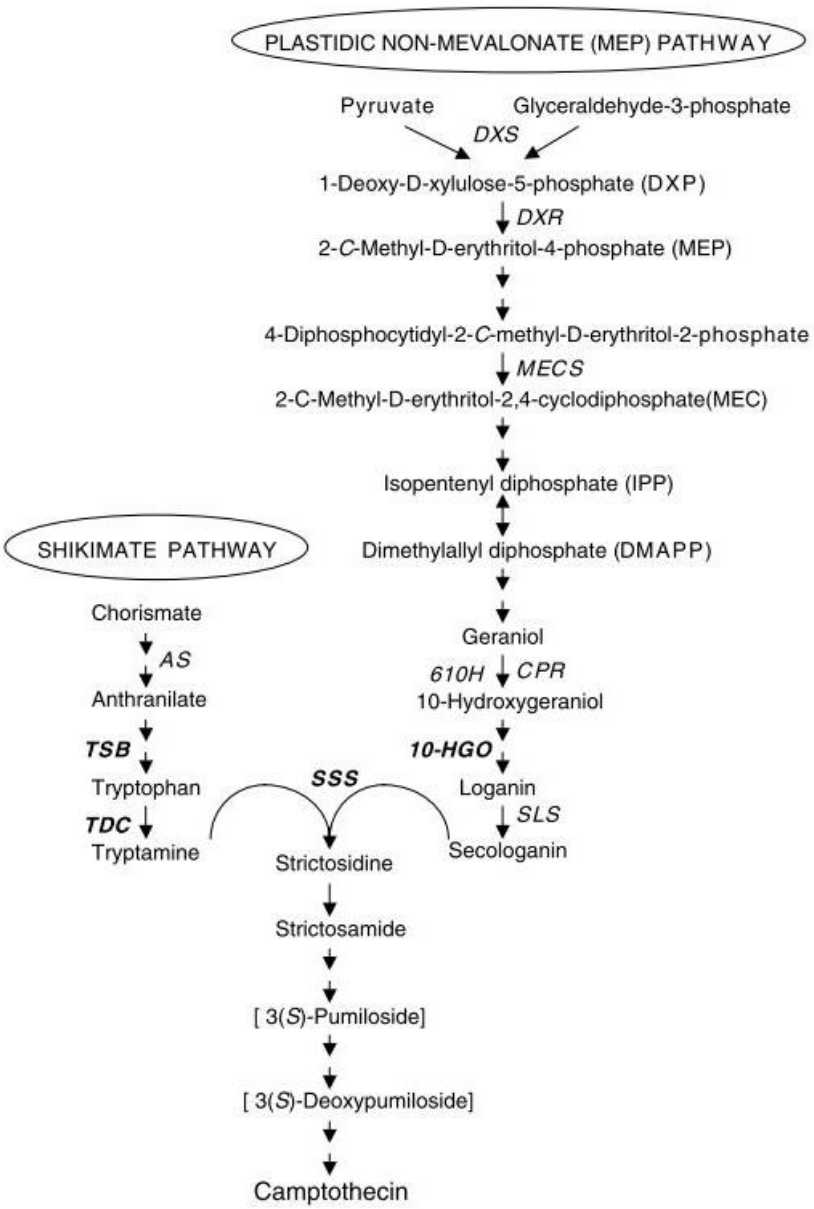

Fig. 1-2 Biosynthetic pathway for terpenoid indole alkaloids in camptothecin-producing plants (Lorence and Nessler, 2004): TSB (β-subunit of tryptophan synthase), TDC (tryptophan decarboxylase), SSS (strictosidine synthase), and 10-HGO (10-hydroxygeraniol oxidoreductase).

1.1.2 Ophiorrhiza pumila

Ophiorrhiza pumila (Rubiaceae) is an herbaceous perennial plant that produces CPT and related alkaloids in all its organs and is viewed as a valuable alternative to arboreous CPT sources (Asano et al., 2013; Cui et al., 2015) (Fig. 1-3). It grows in mountainous, moist, shady habitats in subtropical forests in Ryukyus in southern Japan, Taiwan, southern China, northern Vietnam, and the Philippines (Nakamura et al., 2006). For *O. pumila* with small and unscented flowers, autogamy (self-fertilization in flowering plants) is the main reproductive mechanism (Nakamura et al., 2006).

The genus *Ophiorrhiza* is widely distributed and comprises about 150 species around tropical and subtropical Asia; *O. pumila*, *O. liukiuensis*, and *O. kuroiwai*, which produce CPT and related alkaloids, are known to be distributed in Japan (Aimi et al., 1989; Aimi et al., 1990; Kitajima et al., 2005). The members of Prof. Yamazaki's group at the Graduate School of Pharmaceutical Sciences at Chiba University established hairy root cultures of *Ophiorrhiza* species as a method for CPT production (Asano et al., 2009; Saito et al., 2001; Sudo et al., 2002; Kitajima et al., 2002; Yamazaki et al., 2003). They reported that the hairy root of *O. pumila* produced the highest concentration of CPT (μg g^{-1} DW) among the aforementioned species (Asano et al., 2009). Therefore, *O. pumila* may represent a promising plant material for efficient CPT production. Additionally, because there is no information on the CPT distribution of each organ and the classification of developmental stages in the life cycle of *O. pumila*, it is necessary to investigate and define them.

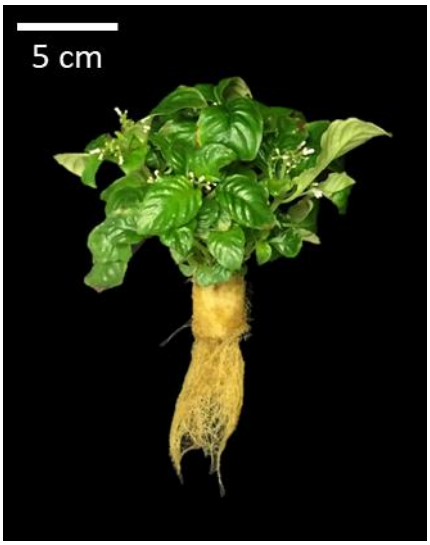

Fig. 1-3 *Ophiorrhiza pumila* cultivated in a plant factory with artificial light.

Saito et al. (2001) reported the CPT production of a hairy root culture of *O. pumila* transformed by *Agrobacterium rhizogenes*. The hairy root of *O. pumila* produced up to 0.1% of CPT per dry weight of cells for five weeks in liquid culture. Also, they reported that a significant amount of CPT accumulated in the culture medium as well as in the hairy root cells. This CPT excretion into the culture medium may inhibit the growth activity of other plants and avoid the cytotoxicity caused by topoisomerase I (Saito et al., 2001). Yamazaki et al. (2003) reported that the highest CPT content among all organs of *O. pumila* was observed in young leaves, flower buds, and roots, which are the most important organs for the propagation and growth of the plant and in withstanding attacks by insects or fungi. They suggested that in *O. pumila*, the root and stem may be the main organs of CPT biosynthesis because SSS (strictosidine synthase) enzymatic activity and

mRNA expression were most active in these organs. The SSS enzyme is one of the key enzymes in the biosynthesis of monoterpenoid indole alkaloids (Fig. 1-2).

The secondary metabolites produced via hairy root or cell suspension cultures can often yield similar metabolite concentrations to those within the whole plant. However, the prohibitive expense of large-scale cultures of hairy root and cell suspension limits this approach. Also, it is required highly skilled labor without aseptic processing and high-cost facilities, including its machines or reagents. Even when secondary metabolite productivity levels were improved, the increased metabolite concentrations were often relatively unstable, and after several subcultures, much lower productivity was observed than that in the initial culture (Verpoorte et al., 2002). It is necessary to find a suitable cultivation system for a high yield of CPT production of *O. pumila*.

1.1.3 Plant factory with artificial light

To obtain stable plant production, a greenhouse or plant factory with artificial light (PFAL) is a more suitable cultivation system than the open-fields where plant environmental control is difficult to achieve. In particular, in a PFAL, plants can be cultivated under controlled environments including air temperature, humidity, gas concentration, and light. This allows the derivation of safe raw materials of a sufficient quality from medicinal plants year-round. Additionally, plant productivity per unit area in a PFAL can be high if multi-layer cultivation shelves are used (Goto, 2012). Such multi-layer cultivation shelves in a PFAL are suitable for plants less than 30 cm in height, such as leafy vegetables and seedlings and provides high plant productivity per unit area (Kozai and Niu, 2016). *O. pumila* is a compact plant with a 5~20 cm height and may have low

PPFD requirements, allowing it to be cultivated in relatively small spaces, such as a PFAL. Since *O. pumila* is a compact herbaceous perennial plant, PFALs are considered optimal cultivation systems for *O. pumila*. Further, plant production in PFALs has already become commercialized owing to the cost savings and yield and quality stability it allows; therefore, it can be applied to *O. pumila* cultivation. If the dry matter of *O. pumila* can increase through environmental control in a PFAL, it has the potential to allowing CPT production beyond that of 0.1% per dry weight within hairy root cultures. Therefore, if *O. pumila* is cultivated in a PFAL, it is expected that high CPT yields can be consistently produced in all plant organs year-round. However, it is necessary to investigate and establish which cultivation environments are most suitable for *O. pumila* growth and CPT production because there is little information on it.

1.1.3.1 Light condition and air temperature

Light and air temperature are important environmental factors that affect gas exchange rates, including net photosynthetic rate (P_n) and transpiration rate (E), along with plant growth and yields (Aleric and Kirkman, 2005; Berry and Björkman, 1980; Fukuzawa et al., 2012; Miller et al., 2001). Using artificial light can induce desired reactions in plants (growth, development, flowering, and others) by controlling light quality, light period, and light intensity (as PPFD). Specifically, an increase of PPFD up to the light saturation point directly increases P_n, yielding an almost linear response of plant growth in a controlled environment (Kang et al., 2013; Ma et al., 2010; weiguuo et al., 2012). Generally, a large daily light integral (DLI; the product of PPFD and light period) usually increases dry matter accumulation in plants. Also, a longer light period

accelerates the flowering of long-day plants. Therefore, control of these light-related environmental factors can be expected to improve plant growth in a PFAL.

Air temperature directly affects the gas exchange rates in all plants, including P_n in a light period and average daily respiration rate. Therefore, the growth rate of plants may be predicted by the sum of P_n and the daily respiration rate under controlled air temperature conditions. To understand the basic characteristics of wild *O. pumila* grown under a humid environment, the response of E under different air temperatures, which affect relative humidity (RH) and the vapor pressure deficit, can provide valuable information. Since no information exists about the gas exchange rates of wild *O. pumila*, it is necessary to investigate the P_n and E values under various PPFDs and air temperatures to establish the optimal cultivation environment. Based on the collected data of gas exchange rates, a practical cultivation experiment in a PFAL is needed to confirm and refine the optimal environmental conditions for long-term cultivation.

1.1.3.2 Root-zone condition

Hydroponic systems that provide sufficient water and nutrition to plants are essential tools for stable cultivation in a PFAL. Optimization of the nutrient solution concentration (NSC) for plant growth is the first step in establishing cultivation methods. NSC significantly affects the absorption of water and nutrients by plant roots. In particular, an excessively low or high NSC often causes a nutritional deficit or unbalanced absorption of mineral nutrients, respectively. The optimal NSC may change depending on the nutritional conditions in a plants' place of origin, plant variety, and growth stage. Therefore, it is necessary to establish the optimal NSC for *O. pumila* growth in a PFAL

and thereafter establish the optimal NSC for CPT accumulation in *O. pumila*.

Root-zone temperature is also an established influencing factor in the nutrient solution absorption rate by roots, which affects plant growth. In particular, the optimal root-zone temperature for growth tends to be lower than that of the above-ground part of plants (Lambers, 2008). Therefore, to promote plant growth, it is necessary to determine the optimal root-zone temperature under the optimal air temperature. For instance, when nutrient solution temperature (NST) increases, the respiration rate of roots increases and often inhibits plant growth due to respiration exhaustion (Du and Tachibana, 1994). Low NST also inhibits plant growth because it suppresses water and nutrient absorption by roots (Yan et al., 2012). Moreover, NSTs of 5 to 15 °C are known to induce oxidative stress via water absorption inhibition and often increase the secondary plant metabolites, e.g., low root-zone temperature increased ascorbic acid concentrations and sugar content in spinach (Chadirin et al., 2011) and bioactive compounds in red perilla (Ogawa et al., 2018) and canola (Son et al., 2020). Furthermore, because the root is one of the important organs in *O. pumila*, the control of the root-zone conditions may significantly impact CPT accumulation. Therefore, in this study, to improve both the growth and CTP accumulation of *O. pumila*, the suitable NST under the suitable air temperature for growth and CPT accumulation is needed to investigate.

1.2 Previous research in a plant factory with artificial light

Many researchers have reported the effects of environmental conditions (light conditions, air temperature, root-zone conditions, and others) on the growth and accumulation of secondary metabolites in horticultural and medicinal plants within

PFALs.

1.2.1 Light condition and air temperature

Kang et al. (2013) reported that the growth and total anthocyanin content of lettuce were improved under an increased PPFD of 290 μmol m^{-2} s^{-1} with a shortened photoperiod of 6/2 h (light/dark). In kale and spinach, the growth increased with increasing PPFD (125 to 620 μmol m^{-2} s^{-1}), and carotenoid concentrations were maximized at a PPFD of 335 μmol m^{-2} s^{-1} for kale and 200 μmol m^{-2} s^{-1} for spinach. In Japanese mint, a PPFD of 200 μmol m^{-2} s^{-1} with a 16 h light period was found optimal for growth and *l*-menthol production (Malayeri et al., 2010); further, increasing the DLI resulted in a higher dry weight, and the prolonged light period increased the number of lateral branches and unfolded leaves by promoting photosynthesis. In contrast, Ma et al. (2010) suggested that a low PPFD of 30 to 50 μmol m^{-2} s^{-1} promoted the growth and total flavonoid production of *Anoectochilus formosanus*, a typical medicinal shade plant.

Miller et al. (2001) measured the whole-plant CO_2 exchange responses of *Angelonia angustifolia* to air temperature and reported that 20 °C was optimal air temperature for their photosynthesis. Timlin et al. (2006) reported that the optimal air temperature for canopy photosynthesis was 24 °C early in the growth period and that decreased to 20 °C as the plant aged for potato (*Solanum tuberosum* L.). The fruit of strawberry growth at 30/22 °C (day/night) had the highest antioxidant activity (Wang et al., 2001).

1.2.2. Root-zone condition

Lu et al. (2017) reported that in red and green perilla plants, an electrical

conductivity (EC) of 1.0 dS m^{-1} and a PPFD of 300 μmol m^{-2} s^{-1} increased the acclimation of rosmarinic acid. Kang and van Iersel (2004) found that the growth and flower quality of salvia (*Salvia splendens*) peaked under a 1.0 times dose of Hoagland solution (EC 2.0 dS m^{-1}); 0.125, 0.25, and 2.0 times (ECs of 0.4, 0.7, and 3.7 dS m^{-1}, respectively). Park et al. (2016) investigated the effects of a newly developed nutrient solution on the growth of *Crepidiastrum denticulatum* and determined that an EC level of 2.0 or 2.5 dS m^{-1} in the nutrient solution was suitable for the accumulation of bioactive compounds.

Sakamoto et al. (2016) reported that 10 and 20 °C of root-zone temperatures significantly increased the total biomass (especially, that of fruits) of strawberries compared to 30 °C. Spinach plants were treated with different root-zone temperatures of 5 °C and 20 °C for two weeks before harvest (Chadirin et al., 2011); low-temperature stress (first week at 5 °C and second week at 20 °C) increased the content of healthful substances for humans such as sugars, ascorbic acid, and Fe. Based on the above findings, this thesis aimed to investigate the effects of various environmental conditions on growth and CPT accumulation in *O. pumila*, an under-researched medicinal plant.

1.3 Study objective

To meet the increasing demand for CPT and facilitate its stable production, it is necessary to clarify the characteristics of gas exchange rates of whole plants and establish the suitable light and root-zone environments for *O. pumila* cultivation in a PFAL. Therefore, in this study, a series of experiments were planned and conducted with the following goals:

1. To identify the distribution of CPT concentrations in each organ and at each

developmental stage;

2. To determine the suitable light conditions (PPFD and light period) and air temperature for growth and CPT accumulation based on the measurement of P_n and E of whole plants under various PPFDs and air temperatures under long-term cultivation;

3. To establish the suitable root-zone environments (NSC and NST) for growth and CPT accumulation.

CHAPTER 2

Variations camptothecin distribution among different organs and developmental stages of *Ophiorrhiza pumila*

2.1 Introduction

Translocation or synthesis of defensive compounds like alkaloids may be related to specific stages in plant reproduction, such as the onset of flowering or the development of fruits (Kaplan et al., 2008; Konchar et al., 2011). In addition, it is well known that young leaves often have much higher concentrations of the secondary metabolites than older leaves, and were considered more valuable to get them (Dam et al., 1996; Kaplan et al., 2008; Ohnmeiss et al., 2000). Furthermore, by acquiring more information on the factors affecting the concentration of target compounds might provide insights into more effective cultivation of little-researched wild plants (Konchar et al., 2011). Therefore, it is necessary to define and investigate the developmental stages and CPT distribution of *O. pumila*.

Yamazaki et al. (2003) reported the CPT concentrations in each organ of a six-month-old *O. pumila*, including the hairy root; however, concentrations in the seed pod were not investigated. Further, these researchers did not describe the environmental conditions under which the specimen were grown or the growth parameters, including the plant size and developmental stage. Therefore, it is necessary to investigate which organs should be targeted to produce high concentrations (mg g^{-1} DW) of CPT, along with the

conditions that lead to high overall CPT content (mg/plant), in *O. pumila*. To establish the optimal harvest-time of *O. pumila* for CPT production, the plant's development stages must be defined. Furthermore, the CPT distribution in each organ and at each developmental stage of *O. pumila* should be standardized.

In this chapter, to compare the CPT concentrations at each developmental stage, seed propagation was employed. Seed propagation is generally an easy propagation method and is widely used for the commercial propagation of many plant species, including medicinal plants (Khanna et al., 2013; Laghmouchi et al., 2017). Therefore, to define the developmental stage of *O. pumila*, it is important to consider plants germinated from seeds at the same time. This chapter aims to determine the target organ and the optimal harvest-time for maximum CPT production after an investigation of the CTP distribution and the definition of the developmental stages in *O. pumila*.

2.2 Materials and methods

2.2.1 Plant material and cultivation environmental conditions

O. pumila seeds were collected from plants grown in a PFAL at Chiba University. The seeds were surface-sterilized with 10% sodium hypochlorite solution for 30 minutes and rinsed five times with sterile distilled water. Sterilized seeds were sown in sterile Petri-dishes containing half-strength Murashige and Skoog (MS) medium. The medium contained 20 g L^{-1} of sucrose, and its pH was adjusted to 5.8 before adding agar. One month after the seeds were sown, the seedlings were transplanted to a plant box containing half-strength MS medium.

Three weeks later, the fresh weight of whole plants averaged 0.1 g. The plants

were transplanted to a urethane sponge as a substrate for hydroponics and watered with Otsuka-A nutrient solution (OAT Agrio Co. Ltd., Japan), which is widely used for leafy and fruit vegetable cultivation in Japan. The EC of the standard concentration of Otsuka-A was 2.7 dS m^{-1} (including the EC of tap water); this concentration is referred to as the 1 times solution in this study. During this period, the 0.125 times solution was used, and the EC of this concentration was 0.6 dS m^{-1}. The seedlings were covered with plastic wrap to maintain a high RH. To perform humidity acclimation, the RH near the seedlings was decreased gradually from 100% to 80 or 70% by increasing the number of holes in the plastic wrap for two weeks.

After the humidity acclimation, the fresh weight of whole plants averaged 0.3 g (n = 15), and the plants were transported to a culture panel, which was floated in a plastic container (18.6 L volume) with 9 L of 0.25 times Otsuka-A nutrient solution. The EC and pH of the solution were 0.9 dS m^{-1} and 6.5, respectively, and the solution was replaced every two weeks. The *O. pumila* plants were grown for 10 weeks after transplanting under the following growth conditions: 16 h light period, PPFD of 100 ± 5 μmol m^{-2} s^{-1} under white LED lamps (Light emitting diode; LDL40S-N/19/25, Panasonic Co., Ltd., Japan), air temperature of 28 °C, RH of 80%, and CO_2 concentration of 1000 μmol mol^{-1}.

2.2.2 Classification of organs and developmental stages

To investigate CPT concentrations in all organs, including reproductive organs, three mature plants were randomly selected 56 to 65 days after transplanted to a hydroponic container. Plant organs were separated into root, young leaf, mature leaf, main stem, lateral stem, flower, ovary, and seed pod (Fig. 2-1). The first leaves from the growth

point were classified as a young leaf, and the leaf lengths were within 2 cm. The other leaves were classified as mature leaf. The main stem was deriving from the seed and other stems were classified as lateral stems. The flower included flower buds, and the ovary containing the developing seeds were what remained after the petals fell. The seed pod was the matured ovaries with seeds.

Plant CPT concentrations were not sampled before 21 d (days after transplanting) owing to insufficient plant weight for CPT analysis. The vegetative stage lasted from 0 to 42 d, the flowering stage from 42 to 56 d, and the seed-ripening stage from 56 to 70 d (Fig. 2-2).

2.2.3 Growth parameters

After measuring the fresh weight of each organ, all organs were lyophilized for 24 h using a freeze dryer (FDU-1110, Eyela, Tokyo Rikakikai, Japan) followed by the measurement of dry weight. The fresh and dry weights of leaf, stem, root, and reproductive organ (including ovary and seed pod), plant heights, total leaf areas, and numbers of seed pods were measured every week from 21 d. Leaf area was calculated from photographs of the leaves using free imaging software (LIA 32 ver. 0.378) after all leaves were cut from the stems.

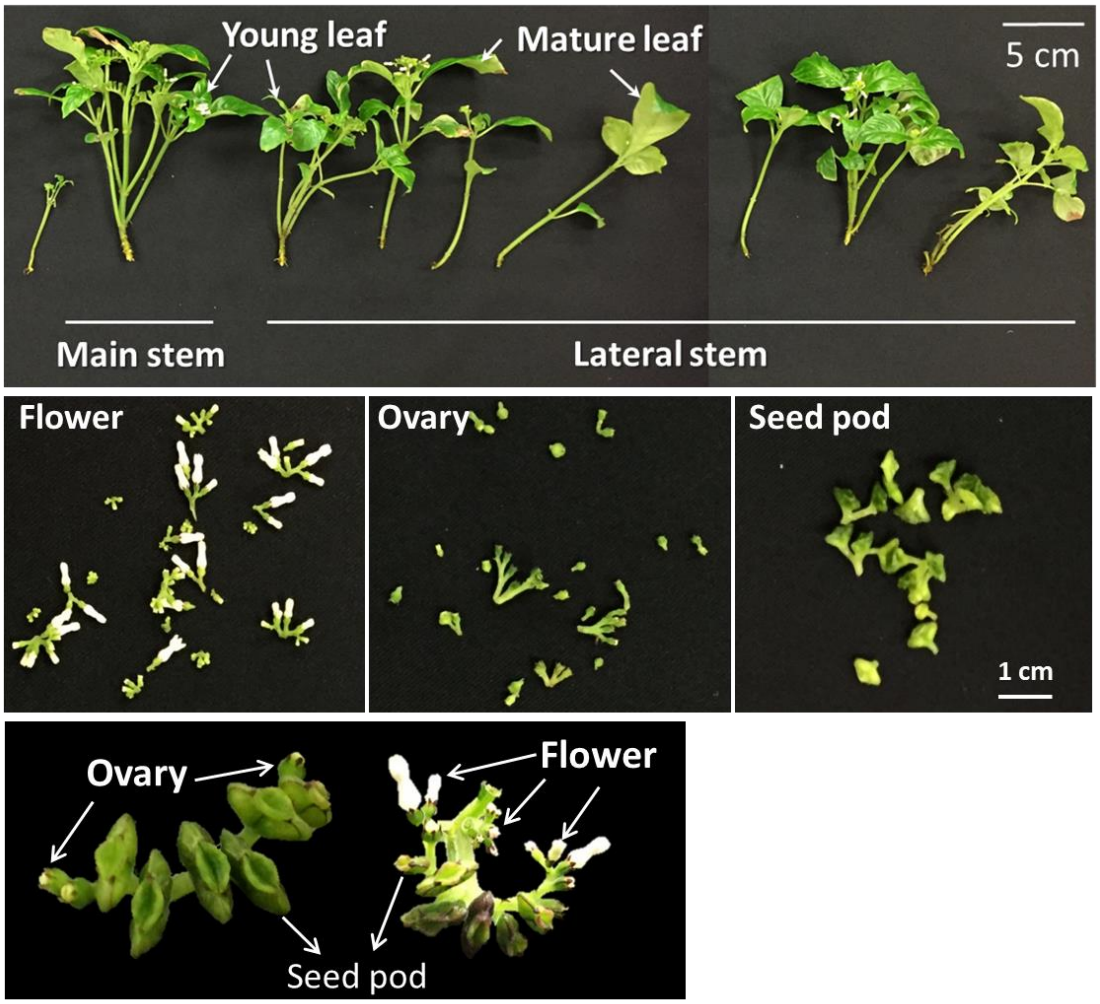

Fig. 2-1 Classification of the plant organs of *Ophiorrhiza pumila*. The first leaves from the growth point were classified as a young leaf, and the leaf size was within 2 cm. The other leaves except for the young leaf were a mature leaf. The main stem was the most central stem, and the lateral stem was the other stems except for the main stem. The flower was included flowers and flower buds, and the ovary was the part after the fall of flower. The seed pod was the organ in which the ovary had matured and contained seeds.

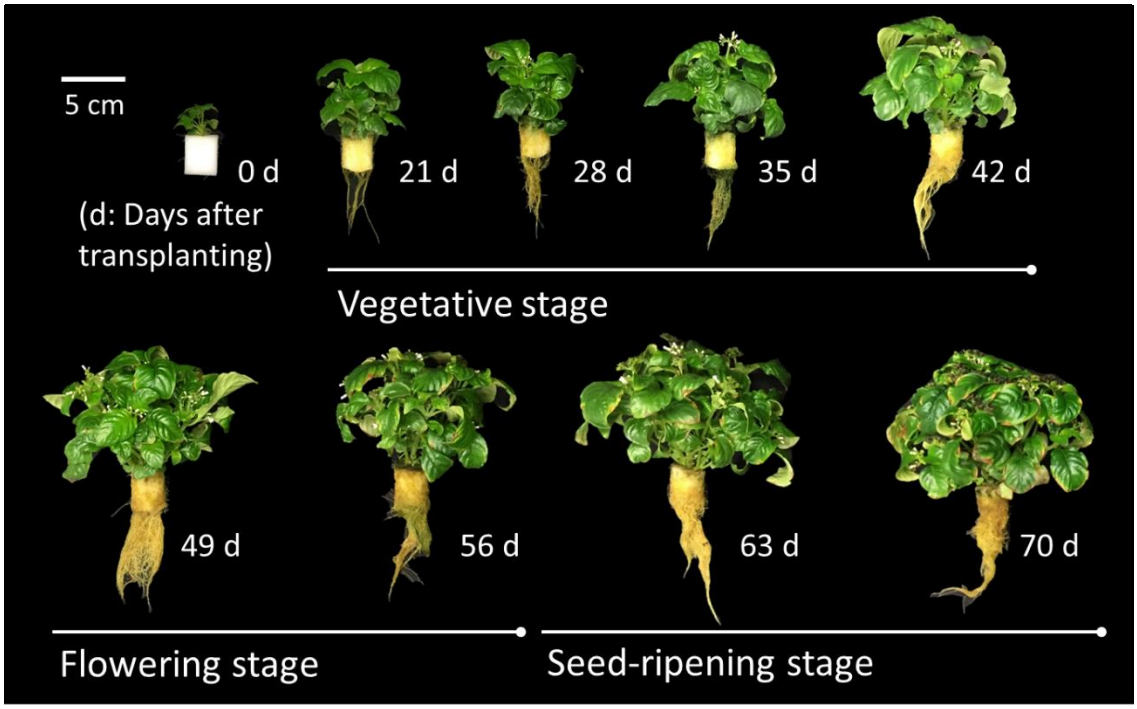

Fig. 2-2 *Ophiorrhiza pumila* grown under a 16 h light period, photosynthetic photon flux density of 100 ± 5 μmol m^{-2} s^{-1} under white light emitting diode lamps, air temperature of 28 °C, RH of 80%, and CO_2 concentration of 1000 μmol mol^{-1}.

2.2.4. Camptothecin analysis

CPT concentrations were analyzed according to the method of Asano et al. (2013) with some modifications. The lyophilized samples of each organ were placed in a 50 mL tube (ST-5010PCR, Yasui Kikai Corporation, Japan) with a stainless steel bead (MC-2235R, Yasui Kikai Corporation, Japan) and shaken by hand 30 times to crush the sample. The powdered sample of each organ (50 mg dry weight) was mixed with 1 mL of methanol (≥ 99.9%) in a 2 mL tube and extracted using an ultrasonic washer (ASU-2, As

One Corporation, Japan; the output of 40 W) for 15 minutes. The extracted sample was stored overnight at 4 °C. The next day, the extracted sample was separated using a centrifuge (MX-305, Tomy Seiko Co., Ltd., Japan) at 10,000 *g* for 10 minutes. The supernatant was filtered through a syringe filter (13HP020AN, Advantec, Japan) and analyzed via high performance liquid chromatography (HPLC; 10AD, Shimadzu Corporation, Japan).

A TSK gel ODS-100V column (Tosoh, Japan; 4.6 × 250 mm, 5 mm) was used with a methanol:water (7:3, v/v) solvent system. The flow rate of the mobile phase in the column was 1.0 mL min^{-1} for 25 minutes, and the injection volume was 10 μL per each sample. The column temperature was set at 40 °C. The chromatogram was monitored at 254 nm with an ultraviolet-visible photodiode array detector (SPD-M10A, Shimadzu Corporation, Japan). Camptothecin (Sigma-Aldrich, USA) was used as a standard material.

2.2.5. Statistical analysis

Data were statistically evaluated via one-way analysis of variance (ANOVA) with the SPSS program for Windows (Version 24.0; SPSS Inc., Chicago, US). To investigate significant differences among treatments, the means of the measurement parameters were compared using Tukey-Kramer's test at $P < 0.05$.

2.3 Results and discussion

2.3.1 Camptothecin concentration in each organ

The dry weights of each organ ranked as follows: mature leaf (31% of total dry

weight), lateral stem (18%), young leaf (15%), main stem (13%), seed pod (11%), root (7%), flower (3%), and ovary (2%) (Fig. 2-3).

Among the organs, the CPT concentration was the highest in flower; the root and ovary had the second-highest concentrations (Fig. 2-4A). The CPT concentrations of seed pod were lower than those of the flower and ovary. Yamazaki et al. (2003) also reported that the flower bud of six-month-old *O. pumila* plants had the highest CPT concentrations. Among all organs, highest CPT concentrations in flower and root, which are crucial organs for plant reproduction and growth, are presumably beneficial as they can help prevent attacks by insects and fungi (Yamazaki et al., 2003). In this study, the fact that root and reproductive organs displayed the highest CPT concentrations in *O. pumila* suggest that these are important organs for CPT production.

In addition, Yamazaki et al. (2003) reported that the youngest leaf, which is that nearest to the growth point, also had high CPT concentrations as similar to flower and root. However, the young leaf in the present study had significantly lowest CPT concentration among all organs, as this study included somewhat older leaves than did Yamazaki's research. If only the very youngest leaves are considered, their CPT concentrations are expected to be higher than those found in this study. The CPT concentration of the lateral stem was higher than that of the main stem because the lateral stem contained more newly generated stems than the main stem. It is thought that promoting the growth of lateral stem with the highest dry weight can promote high CPT yields. Notably, in this study, the CPT concentrations in all organs were higher than the 0.1% CPT per dry weight reported by Saito et al. (2001). This finding suggests that the *O. pumila* cultivation method presented in this thesis could be implemented to produce

high CPT yields.

2.3.2 Camptothecin content in each organ

The CPT content of the lateral stem was significantly higher than those of the other organs, and there was no significant difference in the CPT content among the other organs (Fig. 2-4B). The CPT contents of the root, main stem, and seed pod were numerically higher than those of the young leaf, mature leaf, flower and ovary. Despite the highest concentration of CPT in flower among all organs, the content was lower than those of the stem and root because the dry weight of the flower accounted for only 3% that of the whole plants. In the present study, it was concluded that the stems, including the main stem and lateral stem, were essential organs for CPT production because the stem accounted for 45% of the total CPT content of the plants. Further, the root had the second-highest CPT content, suggesting their significant role in CPT production. Additionally, we found that seed pod with many seeds could yield high CPT content because of their high dry weight and CPT concentrations.

2.3.3 Developmental stages

Plant heights (Fig. 2-2) peaked at approximately 9.5 cm on 49 d during the flowering stage (Fig. 2-5A). The maximum plant height of *O. pumila* was 10 cm throughout all developmental stages. The total leaf area increased continuously until ~70 d, the seed-ripening stage, after which was tended to decrease (Fig. 2-5B). The number of seed pods increased sharply from 56 d, the flowering stage (Fig. 2-5C); thereafter, the leaf weight, leaf area, and plant height did not increase. At 56 d, the flowering stage, each

plant contained about 14 seed pods, but the seeds inside were immature. At 70 d, the seed-ripening stage, some of the seed pods opened, and the seeds within the opened seed pods were able to germinate. At this stage, one plant had over 100 seed pods, and one seed pod could contain almost 600 matured seeds. Since many tiny seeds can be harvested from a single plant, seed propagation is an efficient method of *O. pumila* propagation.

The fresh and dry weights of the whole plants gradually increased through 70 d, the seed-ripening stage (Fig. 2-6). The fresh and dry weights of the stem and root gradually began to increase rapidly from 42 d (vegetative stage) and possibly continued to increase after 70 d (seed-ripening stage). Flowers appeared from about 28 d (vegetative stage), and the seed pods began to form after the petals fell on or after 42 d. From 63 d, the seed-ripening stage, most of the flowers were transformed into seed pods, and new flowers continued to appear from the lateral stems. At 70 d, the seed-ripening stage, the dry weights of each organ ranked as follows: leaf (48.2%), reproductive organ (25.0%), stem (20.5%), and root (6.3%).

2.3.4 Camptothecin concentration at each developmental stage

Among all organs, the root had the highest CPT concentrations; at 63 d, the seed-ripening stage, the CPT concentration in root was significantly higher (35%) than that before 56 d, during the vegetative and flowering stages (Fig. 2-7A). The CPT concentrations of the stem gradually decreased until 49 d (the flowering stage), after that, they increased continuously (Fig. 2-7B). However, no significant differences were found in the CPT concentrations of leaf which had the lowest CPT concentrations among all organs, at different developmental stages (Fig. 2-7C).

After 49 d, the flowering stage, the growth and generation of lateral stems with higher CPT concentrations than those of the main stem increased; thus, the CPT concentration of the whole stems likely gradually increased from 56 d, the end of the flowering stage. The CPT concentrations of the reproductive organ, including the flowers and seed pods, continuously decreased after flowering (Fig. 2-7D). Flower had significantly higher CPT concentrations compared to those of root (Fig. 2-3), and Yamazaki et al. (2003) also reported that flower bud had significantly higher CPT concentrations than those of all other organs. On the other hand, after flowering, since the number of seed pods with higher dry weights and lower CPT concentrations significantly increased, the CPT concentrations of the reproductive organ were significantly lower than those of the root. The differences in CPT concentrations identified in each organ between this study and that of Yamazaki et al. (2003) may be attributable to differences in the developmental stages of analyzed plants and organ classification. In this study, CPT concentrations were analyzed in various developmental stages and organs were further subdivided than in previous research; thus, the present study might offer more useful data for understanding the distribution of CPT concentrations in *O. pumila*.

2.3.5 Camptothecin content at each developmental stage

The CPT contents at 63 and 70 d, during the seed-ripening stage, were significantly higher (two-fold) than those at 49 and 56 d during the flowering stage (Fig. 2-8). This can be explained as follows: 1) CPT concentrations increased in the roots and stems, and 2) the dry weight of the whole plant, particularly that of the reproductive organ. The CPT content continuously increased as dry weight increases because the CPT

concentrations of all organs were maintained or increased with developmental stages.

In this study, the CPT content did not reach a maximum within 70 d (seed-ripening stage) experimental period. Therefore, the optimal harvest time for *O. pumila* for maximum CPT yields could not be determined based on CPT content alone. However, the optimal developmental stage for harvesting was determined based on the following two considerations. First, the dry weights and CPT concentrations of the roots and stems, which are important CPT-producing organs, reached saturation at 63 d during the seed-ripening stage. Second, the CPT content in the reproductive organs, which accounted for 25% of the total dry weight of the plants, sharply increased leading up to 63 d (the seed-ripening stage); after that, several old leaves fell and generated mold. Since this plant *O. pumila* is not a standardized variety, differences in growth rate are anticipated during cultivation. However, it is possible to identify plant developmental stages via morphological changes to compare future plantings to that described in this study. Therefore, the seed-ripening stage (around 63 d) is optimal for harvesting to realize maximum CPT yields from *O. pumila*.

Finally, in this planting, the maximum CPT content of whole plants was approximately 0.9 mg/plant at 63 to 70 d, during the seed-ripening stage. In the following chapter, the suitable environmental conditions such as air temperature, PPFD, light period, NSC, and NST are investigated to further improve CPT yields.

2.4. Conclusion

In this chapter, I reported that the roots and reproductive organs (flower, ovary, and seed pod) had the highest CPT concentrations among all organs. On the other hand,

the stem, including the main and lateral stems, were essential organs for CPT production, as they accounted for 45% of the total CPT content of the whole plant. Also, the root had the second-highest CPT content may have a significant impact on CPT production. It is concluded that the seed-ripening stage (around 63 d) is the best harvest period to obtain maximum CPT content in *O. pumila*.

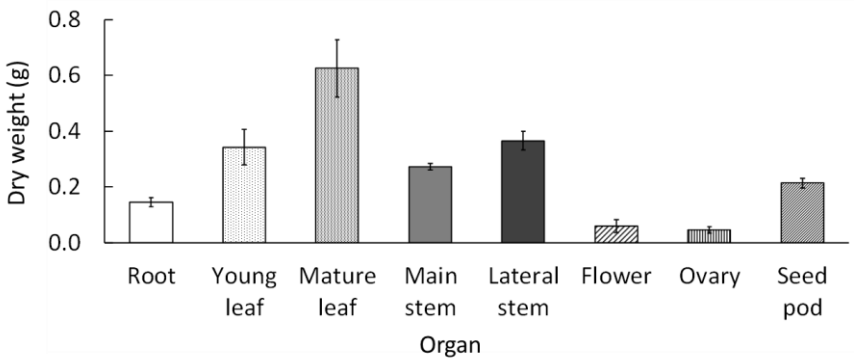

Fig. 2-3 Dry weight of each *Ophiorrhiza pumila* organ at 60 days after transplanting. Vertical bars indicate standard error (n = 3).

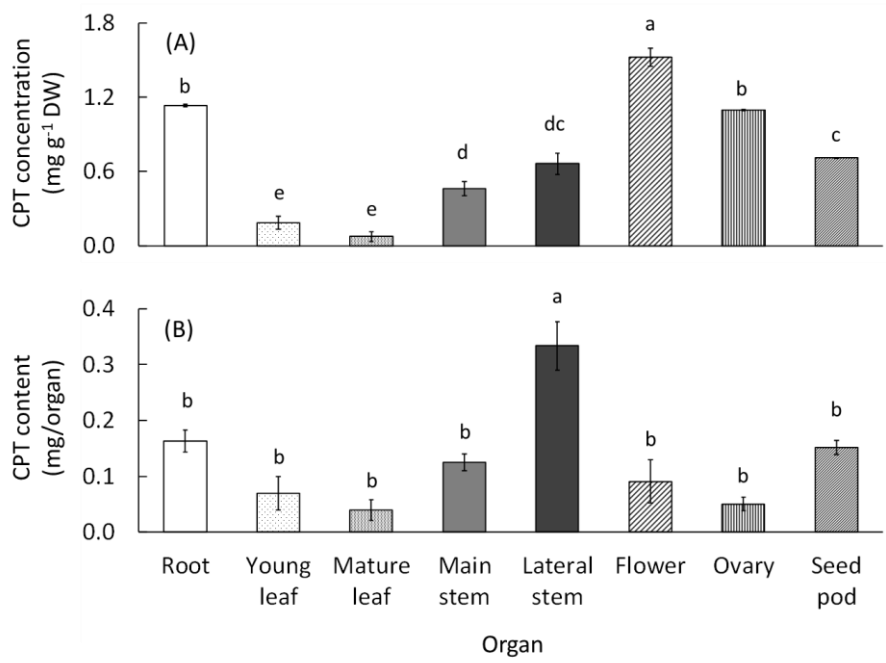

Fig. 2-4 Camptothecin concentration (A) and content (B) in each *Ophiorrhiza pumila* organ at 60 days after transplanting. Vertical bars indicate standard error (n = 3). Different letters indicate significant differences among the organs at $P < 0.05$ by Tukey-Kramer's test.

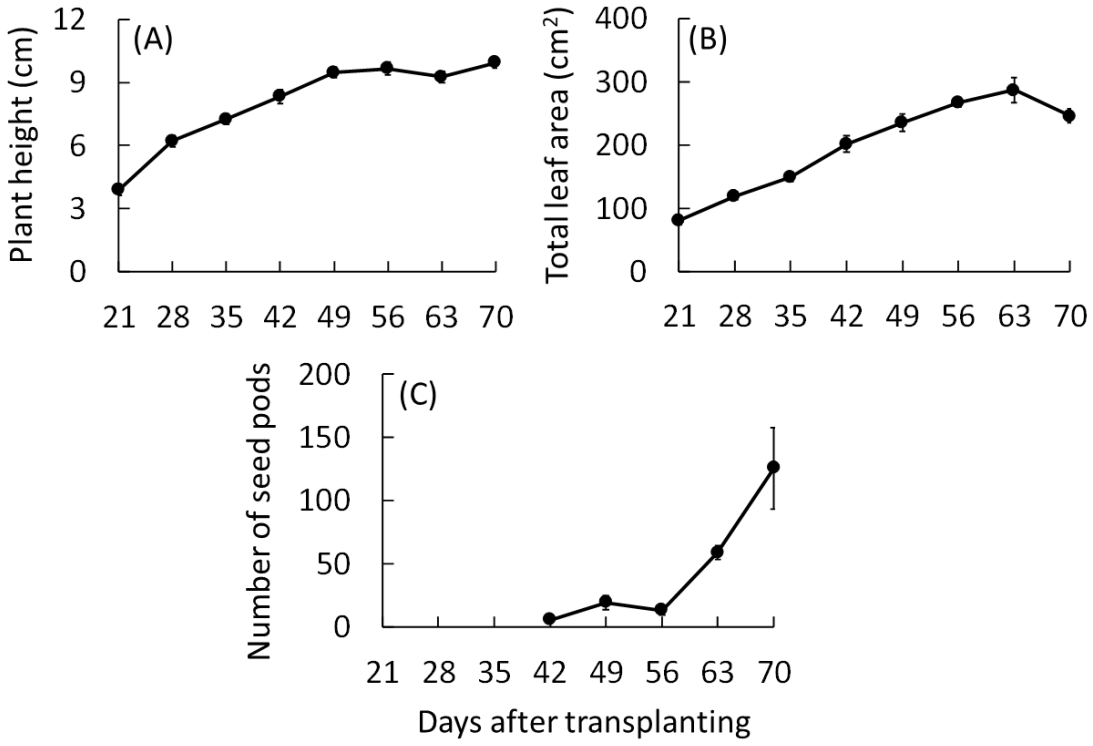

Fig. 2-5 Plant height (A), total leaf area (B), and number of seed pods (C) of whole *Ophiorrhiza pumila* plants 21–70 days after transplanting. The cultivation environments of *O. pumila* plants were as described in Fig. 2-2. Vertical bars indicate standard error (n = 5–8).

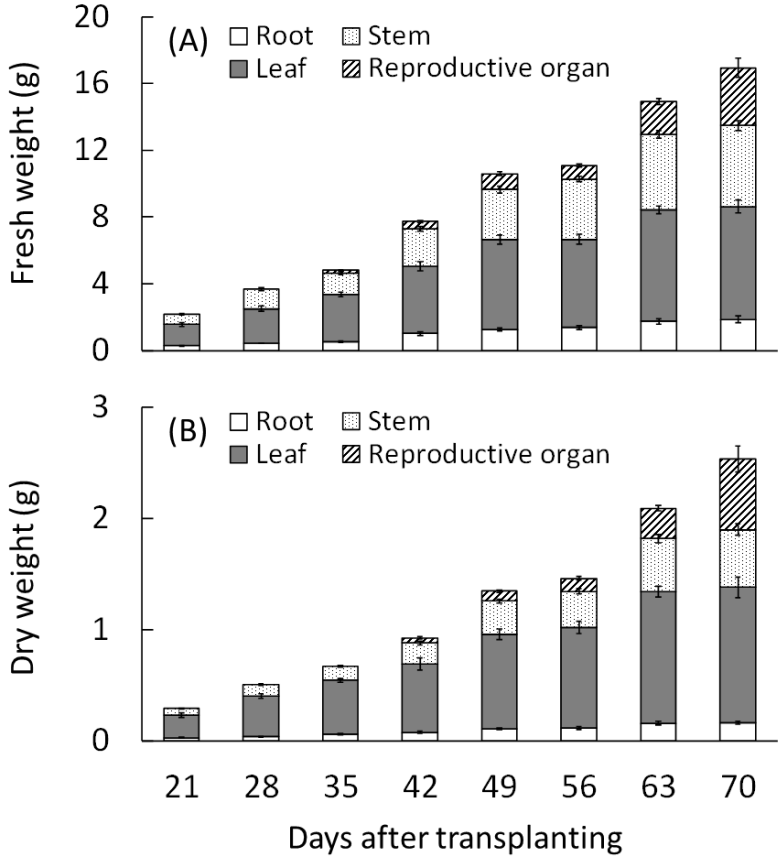

Fig. 2-6 Fresh (A) and dry weights (B) of each *Ophiorrhiza pumila* organ over 21–70 days after transplanting. The cultivation environments of *O. pumila* plants were as described in Fig. 2-2. Vertical bars indicate standard error (n = 5–8).

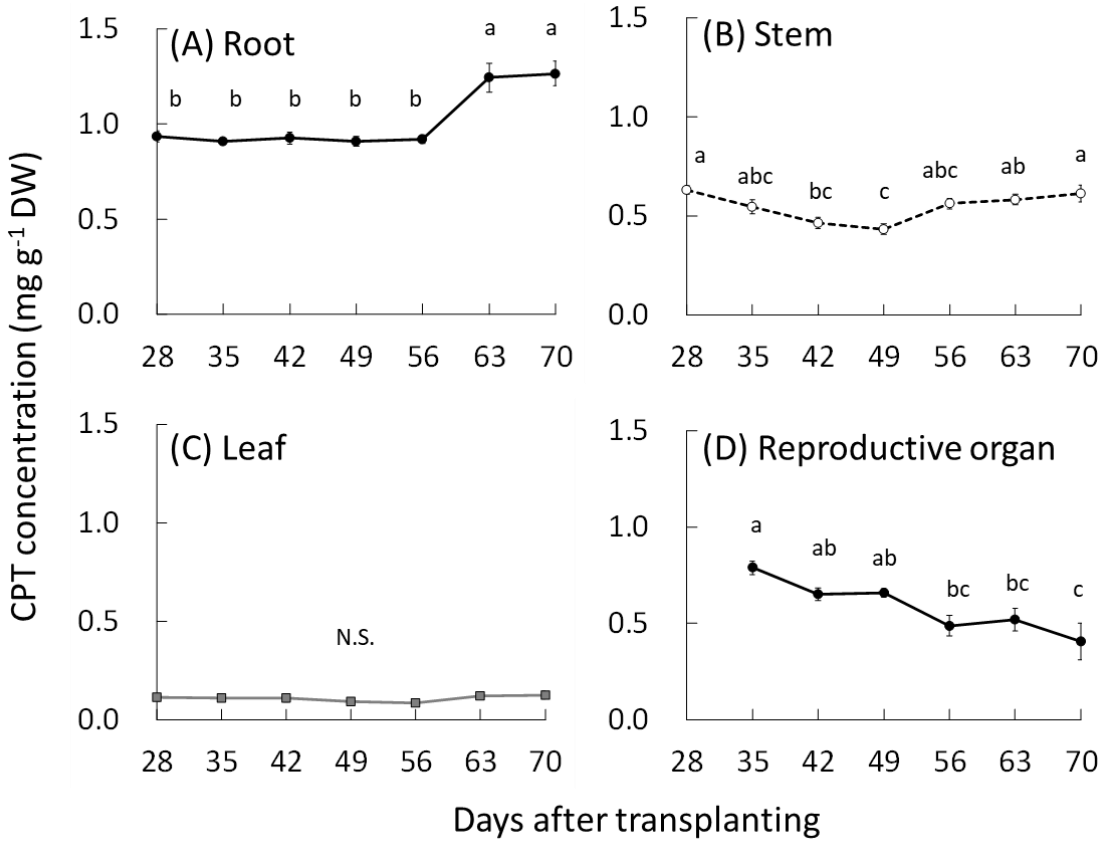

Fig. 2-7 Camptothecin (CPT) concentrations in root (A), stem (B), leaf (C), and reproductive organ (D) of *Ophiorrhiza pumila* from 42 to 70 days after transplanting. The cultivation environments of *O. pumila* were as described in Fig. 2-2. Different letters indicate significant differences in CPT concentrations in each organ at different developmental stages at $P < 0.05$ by Tukey-Kramer's test. (NS: non-significant difference). Vertical bars indicate standard error (n = 5–6).

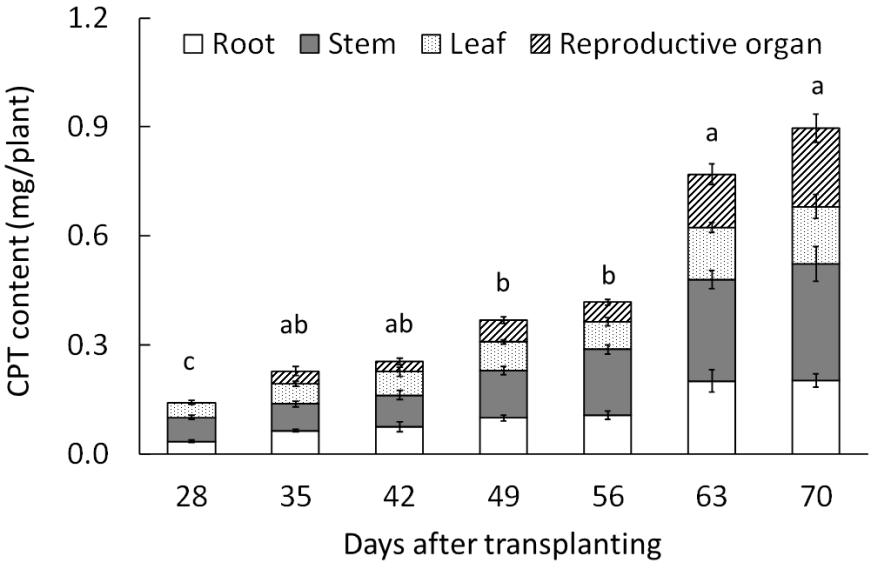

Fig. 2-8 Camptothecin (CPT) content in each *Ophiorrhiza pumila* organ from 42 to 70 days after transplanting. The cultivation environments of *O. pumila* plants were as described in Fig. 2-2. Different letters indicate significant differences in the CPT content of whole plants at different developmental stages at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 5–6).

CHAPTER 3

Photosynthetic photon flux density and light period suitable for growth and camptothecin accumulation of *Ophiorrhiza pumila*

3.1 Introduction

Light and air temperature are important environmental factors that affect P_n and E of plants. Also, the optimal range of light and air temperature conditions can improve the growth and yield of plants. The responses of CO_2 gas and water vapor exchange rates to PPFD or air temperature can provide valuable information to understand the basic characteristics of wild plants unknown such as *O. pumila*. Gas exchange rates have been measured usually on a single-leaf because the measurement of a single-leaf is relatively more accessible than that of a whole-plant. A variety of commercially available measurement instruments are used to measure the P_n and E of a single-leaf. However, the single-leaf measurement of gas exchange rates does not include the respiratory CO_2 efflux from roots, stems, or flowers, and does not take into account the canopy structure, leaf shading, and total leaf area. Therefore, the gas exchange rate of a single-leaf has an insufficient correlation with that of the whole-plant (van Iersel and Bugbee, 2000; van Iersel, 2003). Especially, in the case of *O. pumila,* which has many small leaves and short internodes in high density, whole-plant measurement of gas exchange rates seems to give better indications of the influence of the environmental conditions on growth than single-leaf measurement. Therefore, the P_n and E of *O. pumila* were measured on a whole-plant.

The gas exchange measurement systems for whole plants can be classified into

three types: closed, semi-closed, and open types (Bugbee, 1992; Mitchell, 1992; Takahashi et al., 2008). In the case of the closed type, during a light period, the CO_2 concentration inside the chamber decreases by photosynthesis, while the water vapor concentration increases. The closed type can be used for short-term measurement (within a few minutes), because the CO_2 and water vapor concentrations change rapidly due to photosynthesis and transpiration after the closure of the chamber. Using the semi-closed type, the P_n can be measured by the total amount of CO_2 added or removed to maintain the CO_2 concentration set point inside the chamber. The semi-closed type can be used for the longer-term measurement of P_n and E relative to the closed type. Alternatively, the gas exchange rate measurements of the open type are determined by the difference in the CO_2 or water vapor concentration between the inlet and outlet air of the chamber for plants during a period of light. The open-type can be used for the long-term measurement of P_n and E continuously under stable environmental conditions compared to the closed and semi-closed types because the air continuous to flow stably through the chamber. Moreover, the gas leakage of the open-type assimilation chamber does not affect the measurement of the gas exchange rate compared to the other types, resulting in a higher accuracy of the measurement. Therefore, to measure the gas exchange rates of whole *O. pumila* plants, an open-type assimilation chamber, which allows daily accurate measurements was developed.

To assess the accuracy of the values measured by the developed chamber, the P_n and E of a whole lettuce plant were measured, because several studies of the P_n and E of lettuce, a popular horticultural plant, have been reported under controlled environments (Goto et al., 2014; Kim et al., 2012; Wheeler et al., 1994). Then, the comparison of P_n

and *E* between *O. pumila* and lettuce plant measured by the developed chamber will provide the valuable information to evaluate the performance of photosynthesis and transpiration in *O. pumila*, unknown wild plant. For the next step, based on the collected data of gas exchange rates of *O. pumila*, the practical cultivation experiment in a PFAL is needed to confirm and refine the environmental condition for long-term cultivation (more than a month). PPFD and light period affect morphogenesis such as flowering, branching, lateral shoot emerging, and secondary metabolites accumulation (Ma et al., 2015; Putterill et al., 2004). Therefore, it is needed to investigate the effects of light conditions not only on the gas exchange properties but also growth and CPT content of *O. pumila* grown in a PFAL.

To understand the basic characteristics of the P_n and *E* of *O. pumila*, P_n and *E* of *O. pumila* whole plants were measured under different PPFDs and air temperatures using the open-type assimilation chamber and compared to them of lettuce whole plants (Experiment 1-1 and 1-2; Exp. 1-1 and 1-2). Then, to understand the long-term response of P_n to PPFD, the P_n at 100 and 300 μmol m^{-2} s^{-1} of PPFDs were measured for three days using the open-type assimilation chamber in experiment 1-3 (Exp. 1-3). Finally, to evaluate and conclude the light conditions for practical cultivation, the results of Shimano (2018) was referred, which investigated the effects of PPFD (experiment 2-1; Exp. 2-1) and light period (experiment 2-2; Exp. 2-2) on growth and CPT yield of *O. pumila*.

3.2 Materials and methods

3.2.1 Experiment 1: Assimilation chamber experiment for characteristics of P_n and *E*

3.2.1.1 Plant material and cultivation environmental conditions

O. pumila seedlings propagated by tissue culture were planted in urethane sponges after cutting. Ten seedlings of 1.0 - 2.0 g total fresh weight each were transplanted to a hydroponic container (with internal dimensions of W37 × D25 × H11 cm, and a volume of 11 L) with a nutrient solution (Otsuka-A nutrient solution) in a controlled environment room. The concentration of the nutrient solution was 0.5 times the strength of the Otsuka-A, and the volume of solution was 4 L. To supply sufficient air to roots and to circulate the nutrient solution in each container, aeration was performed using air pumps. EC and pH of the solution were 0.9 dS m^{-1} and 6.5, respectively, and the nutrient solution was replaced every week. To maintain a high RH, a transparent plastic cover (with outer dimension of W40 × D28 × H13 cm) was put on the hydroponic container and it could be fully contained all leaves of the plants on the hydroponic container. Also, the air inside and outside of the cover was ventilated through the small gap between the container and cover. *O. pumila* was grown under the following environmental conditions: light period of 12 h, PPFD of 100 μmol m^{-2} s^{-1} with white LED lamps, air temperature of 28 °C, RH of 90%, and CO_2 concentration of 1000 μmol mol^{-1}. All environmental conditions were measured inside the transparent plastic container and PPFD was measured near the growth point of the plants.

P_n and E were measured at 42 to 49 days after transplanting (Fig. 3-1). One *O. pumila* plant had a total leaf area of 250 cm^2 with a LAI (Leaf area index; total leaf area divided by projected leaf area) of 2.5. Total leaf area was calculated using the same method as mentioned in section 2.2.3. Most of the leaves were young and mature and there were no senescence leaves.

Lettuce was grown under controlled environments as follows: light period of 16 h, PPFD of 200 μmol m^{-2} s^{-1} with white LED lamps, air temperature of 25/20 °C (light/dark periods), RH of 70%, and CO_2 concentration of 1000 μmol mol^{-1}. When the P_n and E were measured, the total leaf area of lettuce (30 days after transplanting) was 325 cm^2 with a LAI of 2.1.

Fig. 3-1 *Ophiorrhiza pumila* after 42 days of the transplanting grown under light period of 12 h, photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}, air temperature of 28 °C, relative humidity of 90%, and CO_2 concentration of 1000 μmol mol^{-1}.

3.2.1.2 Structure of open-type assimilation chamber

A buffer tank for controlling the air temperature, RH, and CO_2 concentration was fabricated using a plastic container (with a volume of 212 L) (Fig. 3-2). The assimilation chamber was also constructed using a plastic container (with a volume of 35.7 L), and acrylic plates were used as covers for the buffer tank and the chamber. The buffer tank, assimilation chamber, and associated parts were located in a controlled environment room.

The chamber was surround by an aluminum cover (thickness 0.8 cm) to maintain the air temperature and was equipped with eight white LED lamps as a light source.

The airflow rate from the buffer tank to the chamber through an air pump was controlled at 3.0 L min^{-1} by a mass flow meter (CMS0050BSRN 200000, Azbil Corporation, Japan) and a precision needle valve. As mentioned above, *O. pumila* seedlings were cultivated under the cover to maintain a high RH. Also, in Exp. 2, the plants were cultivated in the same humidity condition for 35 days. Air current speed inside the chamber was maintained about 0.03 m s^{-1} near the plants, similar to the above cultivation conditions.

3.2.1.3 Control of environmental conditions for the open-type assimilation chamber

The air temperatures inside the buffer tank and assimilation chamber were controlled within ± 0.3 °C with T-type thermocouples and ribbon heaters (100 W) (Fig. 3-2) The RH inside the buffer tank and chamber was maintained within ± 0.3% with a humidity sensor (HMM100, Vaisala, Finland) and a humidifier (ultrasonic atomization unit; HM-303N-SP, Honda Electronics Co., Ltd., Japan). The CO_2 concentration inside the buffer tank was controlled within ± 30 μmol mol^{-1} with a CO_2 sensor (CO_2 Engine K30 EQC, Senseair, Sweden) and by adding pure CO_2 from a CO_2 cylinder. A portable fan (5.0 W) was installed to circulate the air inside the buffer tank.

To measure the P_n, the moisture from the gas samples at the inlet and outlet of the chamber was removed using an electronic dehumidifier (DH-209C-1-R, KELK Ltd., Japan). The CO_2 concentrations of the dehumidified gas samples were measured by two infrared CO_2 analyzers (ZFP9, Fuji Electric Co., Ltd., Japan), respectively. To measure

the *E*, two measurement boxes made from plastic bottles were attached to the entrance and exit of the chamber. Inside the measurement boxes, two T-type thermocouples and two humidity modules were installed to measure the air temperature and RH, respectively. All sensors were connected to a data logger and all measurements were recorded every minute.

3.2.1.4 Measurements of CO_2 gas and water vapor exchange rates

After the environmental conditions (air temperature, RH, CO_2 concentration, and PPFD) inside the chamber had been stabilized for at least 1 hours at each set point, three *O. pumila* plants or four plants of lettuce were placed inside the chamber to acclimate them to the environments. After the acclimation, measurements of the P_n and E were conducted for 30 minutes.

The P_n [μmol CO_2 m^{-2} s^{-1}] was calculated from the difference in CO_2 concentration between the inlet and outlet of the chamber and total leaf area of all plants.

$$P_n = F(C_{in} - C_{out})/A_{leaf} \quad (1)$$

where F is airflow rate through the chamber [mol s^{-1}], C_{in} and C_{out} are CO_2 concentrations at the inlet and outlet of the chamber [μmol mol^{-1}], respectively, and A_{leaf} is the total leaf area of all plants [m^2].

The E [mmol H_2O m^{-2} s^{-1}] was calculated from the mass balance of water vapor between the inlet and outlet of the chamber and total leaf area of all plants.

$$E = F'(W_{out} - W_{in})/A_{leaf} \quad (2)$$

where F' is airflow rate through the chamber [m^3 s^{-1}], W_{in} and W_{out} are absolute humidity at the inlet and outlet of the chamber [mmol m^{-3}], respectively, and A_{leaf} is the same as

above.

3.2.1.5 Environmental conditions for measurements of CO_2 gas and water vapor exchange rates

In Exp. 1-1, for the P_n and E measurements of *O. pumila*, the conditions of the buffer tank were set to PPFDs of 0, 50, 100, 150, 250, 300, and 500 μmol m^{-2} s^{-1}, air temperature of 28 °C, RH of 70%, and CO_2 concentration of 1000 μmol mol^{-1} (Fig. 3-3A). For the lettuce, the conditions were set to PPFDs of 0, 100, 200, and 350 μmol m^{-2} s^{-1}, air temperature of 28 °C, RH of 55%, and CO_2 concentration of 1000 μmol mol^{-1} (Fig. 3-3B). When the RH was controlled at 70% and 55% in the entrance of the chamber, the RH around the plants could be maintained at about 90% and 70%, respectively, which are similar to those of cultivation environments. The P_n and E were calculated per unit leaf area, and the total leaf area of the three *O. pumila* plants and four lettuce plants were 872 and 1300 cm^2, respectively.

In Exp. 1-2 for light response curve, the measurements of the P_n and E of *O. pumila* were measured under different PPFDs (0, 50, 100, 150, 250, 300, and 500 μmol m^{-2} s^{-1}) and air temperatures (25, 28, 31, and 34 °C) (Fig. 3-4). The P_n and E were calculated per unit leaf area, and the total leaf area of the three plants was 872 cm^2.

In Exp. 1-3, the P_n measurements were started, following 1 h of acclimation, and it was conducted at PPFD of 100 (Fig. 3-5A) and 300 μmol m^{-2} s^{-1} (Fig. 3-5B) for three days. The air temperature inside the chamber was set at 28 °C, and the light period was 16 h at each PPFD. Total leaf area of three plants for these experiments was about 901 cm^2.

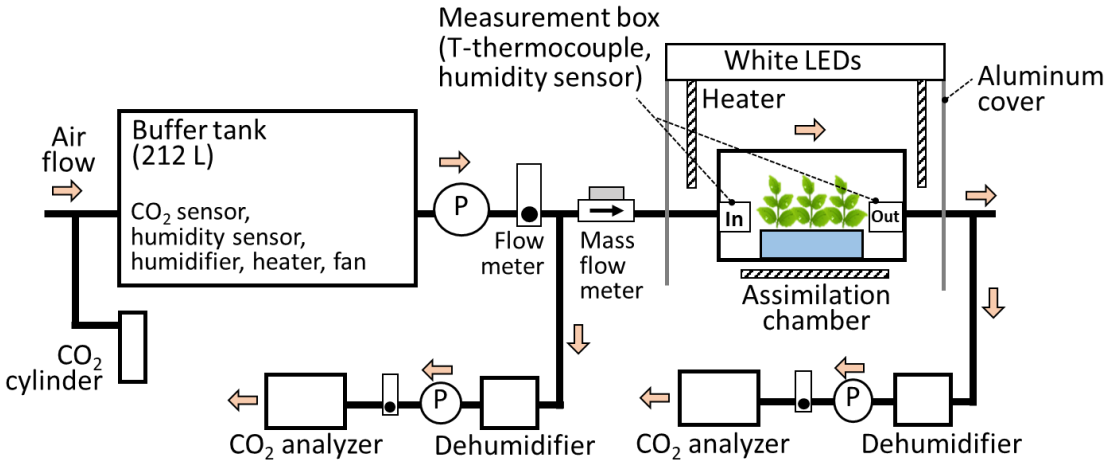

Fig. 3-2 Diagram of an open-type assimilation chamber. LEDs; light emitting diodes. P; air pump.

3.2.2 Experiment 2: Cultivation experiment for long-term responses

This experiment was conducted by Shimano (2018) and his results and discussion were rewritten for the purpose of this paper. The plant material was prepared by the same method as experiment 1. *O. pumila* was grown under the following environmental conditions: air temperature of 28 °C, RH of 90%, and CO_2 concentration of 1000 μmol mol^{-1}.

3.2.2.1 Treatment plots

To investigate the proper PPFD and light period for the growth and CPT content of *O. pumila*, two independent treatments were conducted. In Exp. 2-1, three PPFDs of 50, 100, and 150 μmol m^{-2} s^{-1} were applied to the plants under a light period of 12 h. In Exp. 2-2, three light periods of 8, 12, and 16 h per day were applied to the plants under

PPFD of 100 μmol m^{-2} s^{-1}.

3.2.2.2 Growth parameters

At 35 days after start of the treatment (DAT), the dry weights of leaf, stem, and root, and the leaf area were measured. Total leaf area was calculated using the same method as mentioned in section 2.2.3. The specific leaf weight was calculated using the dry weights and leaf area.

3.2.2.3 Chlorophyll concentration

Chlorophyll was extracted from leaf discs (8.5 mm of diameter) of young leaves with N, N-dimethylformamide (DMF) with reference to a protocol described by Porra et al. (1989). For analysis of the chlorophyll concentration, the leaves were separated into the young and mature leaves. The leaves from the top to the second node were classified as the young leaf, and the leaves under them were classified as the mature leaf. The chlorophyll concentration was determined by measuring the absorbance of leaf extracts at 663.8, 646.8, and 750 nm with an ultraviolet-visible spectrophotometer (Porra et al., 1989) and the concentration was expressed per unit leaf area.

3.2.2.4 Camptothecin analysis

The leaf, stem, and root were frozen in liquid nitrogen and stored at -30 °C in a deep freezer until analysis. They were disrupted using a Multi-beads shocker (MB-601U, Yasui Kikai Corporation, Japan), and MeOH (1 mL) was added per 100 mg (fresh weight) of powdered sample.

The methods of extraction and analysis of the CTP were all the same as mentioned in section 2.2.4.

3.2.2.5 Statistical analysis

Data were statistically evaluated by the same method as mentioned in section 2.2.5.

3.3 Results and discussion

3.3.1 Experiment 1: Assimilation chamber experiment for characteristics of P_n and E

3.3.1.1 Exp. 1-1. Comparison of the P_n and E between O. pumila and lettuce

Net photosynthetic rate (P_n)

Goto et al. (2014) reported that the P_n of whole lettuce plants measured with an open-type assimilation chamber with a buffer tank was approximately 5-6 μmol CO_2 m^{-2} s^{-1} at approximately 400 μmol m^{-2} s^{-1} PPFD when the air temperature was 25 °C and the CO_2 concentration was 1000 μmol mol^{-1}. In this study, the P_n of whole lettuce plants was 6.6 μmol CO_2 m^{-2} s^{-1} at 350 μmol m^{-2} s^{-1} PPFD at an air temperature of 28 °C, and it was similar to the result obtained by Goto et al. (2014). From these results, the chamber developed in this study seems to provide an accurate measurement of P_n.

Fig. 3-3 shows the light response curves of P_n (A) and E (B) of whole plants of *O. pumila* and lettuce at the same air temperature of 28 °C. At a PPFD of 0 μmol m^{-2} s^{-1}, which is the dark period, the respiration rate of *O. pumila* was 0.21 μmol CO_2 m^{-2} s^{-1}, and that of lettuce was 1.01 μmol CO_2 m^{-2} s^{-1} (Fig. 3-3A). The respiration rate per unit leaf

area of shade plants is generally lower than that of sun plants (Lambers et al., 2008). The higher respiration rate of sun plants is due to the greater demand for respiratory energy in leaf cells and chloroplasts compared with that of shade plants (Frak et al., 2001; Lambers et al., 2008).

The light saturation point of *O. pumila* was a PPFD of 300 μmol m^{-2} s^{-1} in this study. The light saturation point of lettuce was expected to be PPFD of 500 μmol m^{-2} s^{-1} (Goto et al., 2014), which is above the range of the measured PPFD (≥350 μmol m^{-2} s^{-1}). When comparing the light saturation point of the whole plant among the experiments, it is important to consider the LAI and the ratio of respiration parts of the plant. In both results, the plants did not include old leaves and had similar low LAI (≈ 2.5). Therefore, the light saturation point of *O. pumila* was considered to be lower than that of leaf lettuce. Additionally, Björkman (1981) reported that the light saturation point of a single leaf of palm lily (*Cordyline rubra*) which is a shade plant, was lower than that of the sun plant, California brittlebush (*Encelia californica*). From these results, *O. pumila* which had the low light saturation point, exhibited typical shade plant photosynthetic properties.

Transpiration rate (E)

As the PPFD increases, the *E* of lettuce showed a tendency to increase in this study (Fig. 3-3B). Conversely, that of *O. pumila* remained constant or decreased slightly. *O. pumila*, which has a lower light saturation point compared to lettuce, was expected to be sensitive to light intensity. In the case of general leafy vegetables, as PPFD increases, the stomata are opened to allow more carbon dioxide into the leaf for photosynthesis (Agata et al., 1985). Stomatal conductance was shown to have a linear correlation with

the P_n within 500 μmol m^{-2} s^{-1} of PPFD in lettuce (Albornoz et al., 2014) and tobacco plant (Baroli et al., 2007). However, the stomata would close to inhibit water loss by transpiration at a higher PPFD to prevent excessive dehydration and physiology damage (Livingston, 1911). In the case of *O. pumila*, the stomata seemed to start closing to prevent dehydration over 100 μmol m^{-2} s^{-1} of PPFD. It is also thought that at around 100 μmol m^{-2} s^{-1} of PPFD, *O. pumila* absorbed sufficiently the water and nutrients in the nutrient solution by root. Furthermore, there are few reports on the *E* response to the PPFD in shade plants. Thus, the observed *E* response of *O. pumila* to the PPFD was considered to be a characteristic of shade plants. These results for P_n and *E* suggest that *O. pumila* is a typical shade plant. In addition, it is concluded that less than 300 μmol m^{-2} s^{-1} PPFD, which is the light saturation point, are suitable PPFDs for photosynthesis of *O. pumila*.

3.3.1.2 Exp. 1-2. Light response curve

Net photosynthetic rate (P_n)

The P_n and *E* of *O. pumila* were measured using the open-type assimilation chamber under seven PPFDs and four air temperatures (Fig. 3-4). The P_n of the whole plant of *O. pumila* increased with an increase in the PPFD regardless of the air temperature, and the light saturation point was approximately 300 μmol m^{-2} s^{-1} of PPFD (Fig. 3-4A). The light saturation points at all air temperatures were the same as the result for *O. pumila* in Exp. 2-1 (Fig. 3-3A). Also, for *O. pumila*, less than PPFD of 300 μmol m^{-2} s^{-1}, which is the light saturation point, seemed to be suitable for photosynthesis.

In many plant species, the P_n increases as the air temperature increases, and it decreases at a higher air temperature than the optimal air temperature for plant growth and

photosynthesis (Berry and Björkman, 1980). Regardless of the PPFD in this study, the P_n at 28 and 31 °C tended to be higher than those at 25 and 34 °C (Fig. 3-4A). It is considered that air temperatures between 28 and 31 °C are suitable for photosynthesis in *O. pumila*.

Transpiration rate (E)

At all air temperatures, the *E* peaked at PPFD of 100 μmol m^{-2} s^{-1} and after that remained unchanged or decreased with increasing PPFD. Also in this study, the stomata might be closed to prevent dehydration over 100 μmol m^{-2} s^{-1} of PPFD, and thus, over this PPFD seemed to be excessively high PPFD for *O. pumila.* Therefore, 100 μmol m^{-2} s^{-1} of PPFD, which had no negative response in the *E*, seemed to be suitable for transpiration of *O. pumila*.

Between 0 and 300 μmol m^{-2} s^{-1} of PPFD, the *E* of *O. pumila* increased with an increase in air temperature, especially above 31 °C. RH was maintained at 70%, as a result vapor pressure deficit in the air increased at higher temperatures. An acceleration in cuticular and stomatal transpiration occurs at higher air temperatures (Lambers et al., 2008). In this measurement, the wilt of *O. pumila* leaves was observed when they were exposed to 31 and 34 °C of air temperature for several hours. Therefore, it appeared that the air temperatures lower than 31 °C were suitable ones for transpiration of *O. pumila*.

From the results of P_n and *E* of the whole *O. pumila* plant, I determined that an air temperature of 28 °C and PPFD of 100 μmol m^{-2} s^{-1} were suitable conditions for short-term photosynthesis and transpiration.

3.3.1.3 Exp. 1-3. P_n measurement for three days

To understand the P_n response to PPFD more, the P_n was measured at 100 and 300 μmol m^{-2} s^{-1} of PPFDs for three days using the open-type assimilation chamber. The P_n at PPFD of 100 μmol m^{-2} s^{-1} was almost constant in at 2.2 μmol CO_2 m^{-2} s^{-1} for 3 days (Fig. 3-5A). The P_n at PPFD of 300 μmol m^{-2} s^{-1} on Day 1 was 6.6 μmol CO_2 m^{-2} s^{-1} for 6 h after the start of the light period (Fig. 3-5B). However, after that, the P_n gradually decreased and reached 6.1 μmol CO_2 m^{-2} s^{-1} at the end of the day. The P_n at 300 μmol m^{-2} s^{-1} was 5.7 μmol CO_2 m^{-2} s^{-1} on average on Day 3. Additionally, the leaves color at 300 μmol m^{-2} s^{-1} became lighter from Day 2 (data not shown). It is possible that starch accumulation by excessive irradiation under the saturated PPFD caused to some visible discoloration via destroyed chloroplasts (Nebauer et al., 2011). From the result, PPFD of 300 μmol m^{-2} s^{-1} may not be suitable for long-term cultivation. Additionally, in Exp. 1-2, PPFD of 100 μmol m^{-2} s^{-1} was considered a suitable condition for the photosynthesis and transpiration of *O. pumila*. From these results, the treatments of PPFD in Exp. 2-1 were set at 50, 100 and 150 μmol m^{-2} s^{-1} of PPFDs to find a suitable PPFD for long-term cultivation of *O. pumila*.

3.3.2 Experiment 2: Cultivation experiment for long-term responses

3.3.2.1 Exp. 2-1. PPFD

At 35 DAT, the total dry weight at 100 μmol m^{-2} s^{-1} of PPFD was significantly higher than those at 50 and 150 μmol m^{-2} s^{-1} (Table 1). In Exp. 1-2, the P_n at 50 μmol m^{-2} s^{-1} was 1.6 μmol CO_2 m^{-2} s^{-1} and a half of that at 100 μmol m^{-2} s^{-1} (Fig. 3-4A). The PPFD of 50 μmol m^{-2} s^{-1} was too low to accumulate the photo-assimilates through the experiment period. There were no significant differences in total leaf area and chlorophyll

concentration among the treatments, however the leaf rolling was observed at PPFD of 150 μmol m^{-2} s^{-1}. The specific leaf weight at a PPFD of 100 μmol m^{-2} s^{-1} was significantly higher than that at a PPFD of 50 μmol m^{-2} s^{-1}, probably because as PPFD increased, leaf thickness also increased.

In Exp. 1-1 for 30 minutes, the P_n at PPFD of 150 μmol m^{-2} s^{-1} was higher than at 100 μmol m^{-2} s^{-1}, however, in the 35 days of cultivation, most of the growth parameters at 100 μmol m^{-2} s^{-1} were greater than those at 150 μmol m^{-2} s^{-1}. The continuous excessive-irradiation, which is out of the photosynthetic capacity of the plant, may cause the inactivation of photosynthetic apparatus, resulting in growth inhibition (Barth, 2001). In particular, photo-inhibition often occurs on shade plants even at a low PPFD because of the small capacity for the utilization of absorbed light (Öquist et al., 1992; Powles 1984). Therefore, since *O. pumila* is a shade plant, photo-inhibition likely occurred even at 150 μmol m^{-2} s^{-1}. From these results, PPFD of 100 μmol m^{-2} s^{-1} was suitable for *O. pumila* cultivation for a long period.

The CPT content of leaves was the highest among the organs because the leaf had the highest dry weight and is the critical organ for CPT accumulation (Fig. 3-6). At 100 μmol m^{-2} s^{-1} of PPFD, the CPT content of whole plant was significantly higher than at other PPFDs. Therefore, PPFD of 100 μmol m^{-2} s^{-1} was considered suitable not only for growth promotion but also CPT accumulation in *O. pumila*.

3.3.2.2 Exp. 2-2. Light period

At 35 DAT, the total dry weight, total leaf area, and specific leaf weight increased as the light period increased, especially the total dry weight under the light period of 16

h, which was about four times higher than at 8 h (Table 2). Generally, a DLI (the product of PPFD and light period) usually increases dry matter accumulation and accelerates the flowering of long-day plants. In this experiment, the dry weights of leaves and stems for the 16 h light period were four to five times higher than those in the 8 h period, even though the DLI of the 16 h period (5.8 mol m^{-2} d^{-1}) was twice that of the 8 h period (2.9 mol m^{-2} d^{-1}). This was probably because the plants in the 16 h treatment generated many lateral shoots and branches compared to other treatments (Fig. 3-7).

The CPT content significantly increased with an increase in DLI. The content for the 16 h light period was approximately three times higher than for the 8 h period (Fig. 3-8). In particular, in the stem, the CPT content under the 16 h light period was significantly higher (2.7 times) than the 12 h treatment. It was determined that, under the longest light period, 16 h, *O. pumila* had many lateral shoots and branches, which contained high CPT content. In these results, the 16 h light period produced large amounts of stems that were suitable not only for growth promotion but also CPT accumulation of *O. pumila*.

According to the results of Exp. 2-1 and 2-2, it suggests that the combination of PPFD of 100 μmol m^{-2} s^{-1} and light period of 16 h (DLI: 5.8 mol m^{-2} d^{-1}) is suitable light condition for both growth promotion and CPT accumulation of *O. pumila*.

3.4 Conclusion

From the measurements of the open-type assimilation chamber, it was clarified that the light saturation point was PPFD of 300 μmol m^{-2} s^{-1} at all air temperatures, and the air temperature of 28 °C and PPFD of 100 μmol m^{-2} s^{-1} were suitable for

photosynthesis and transpiration of *O. pumila*. When comparing the P_n and E between *O. pumila* and lettuce, the characteristics of photosynthesis and transpiration in *O. pumila* show the typical shade plant. For practical cultivation, PPFD of 100 μmol m^{-2} s^{-1} with the light period of 16 h (DLI: 5.8 mol m^{-2} d^{-1}) was the suitable light condition for both growth promotion and CPT accumulation of *O. pumila* under an air temperature of 28 °C, RH of 90%, and CO_2 concentration of 1000 μmol mol^{-1}.

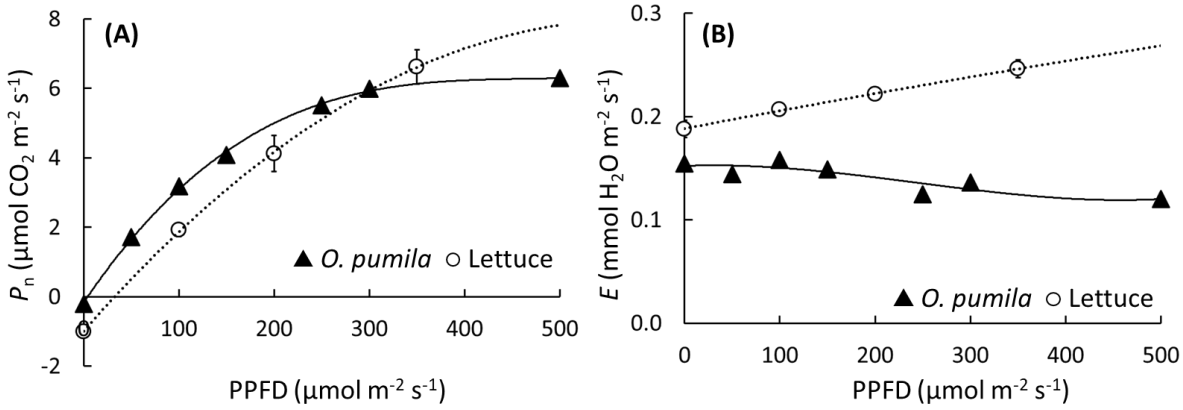

Fig. 3-3 Light response curves of net photosynthetic rate (P_n; A) and transpiration rate (E; B) of *Ophiorrhiza pumila* and lettuce measured using an open-type assimilation chamber. The setting values of the open-type assimilation chamber were air temperature of 28 °C and CO_2 concentration of 1000 μmol mol^{-1}. The relative humidity was set to 55 and 70% for lettuce and *O. pumila*, respectively. The airflow rate was 3.0 L min^{-1}. Symbols represent the mean value, and vertical bars indicate standard error (n = 2 and 3). PPFD; photosynthetic photon flux density.

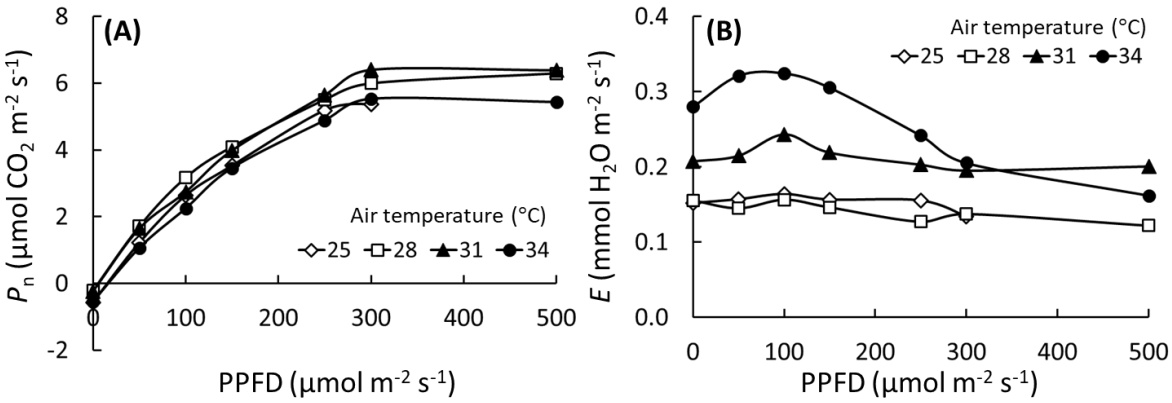

Fig. 3-4 Light response curves of net photosynthetic rate (P_n; A) and transpiration rate (E; B) of *Ophiorrhiza pumila* measured using an open-type assimilation chamber under different PPFDs (0, 50, 100, 150, 250, 300, and 500 μmol m^{-2} s^{-1}) and air temperatures (25, 28, 31, and 34 °C). The setting values of the open-type assimilation chamber were relative humidity of 70%, and CO_2 concentration of 1000 μmol mol^{-1}. The airflow rate was 3.0 L min^{-1}. Symbols represent mean values (n = 2). PPFD; photosynthetic photon flux density.

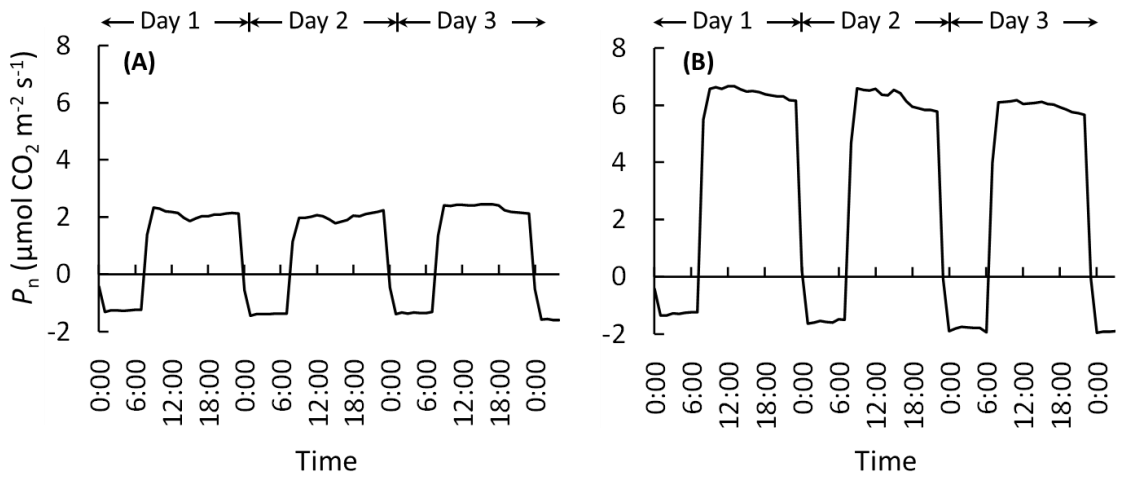

Fig. 3-5 Net photosynthetic rate (P_n) of *Ophiorrhiza pumila* measured using an open-type assimilation chamber at photosynthetic photon flux densities of 100 and 300 μmol m^{-2} s^{-1} (A and B, respectively) for three days. The setting values of the open-type assimilation chamber were light period of 16 h, air temperature of 28 °C, relative humidity of 70%, and CO_2 concentration of 1000 μmol mol^{-1}. The airflow rate was 3.0 L min^{-1}.

Table 3-1 Effects of photosynthetic photon flux density (PPFD) on dry weight, leaf area, specific leaf weight, and chlorophyll concentration of *Ophiorrhiza pumila* at 35 days after the start of treatment (n = 10).

PPFD[z] ($\mu mol\ m^{-2}\ s^{-1}$)	Dry weight (g)				Leaf area (cm^2)	Specific leaf weight ($gDW\ m^{-2}$)	Chlorophyll concentration ($\mu g\ cm^{-2}$)
	Leaf	Stem	Root	Total			
50	0.14 b[y]	0.05 b	0.07 b	0.26 b	73.4	35.8 b	39.8
100	0.25 a	0.11 a	0.10 a	0.49 a	98.8	51.2 a	42.5
150	0.17 b	0.07 b	0.07 b	0.31 b	74.9	41.8 ab	40.1

[z]PPFD: Photosynthetic photon flux density.

[y]Different letters within columns indicate a significant difference among the treatments at $P < 0.05$ by Tukey-Kramer's test.

O. pumila grew under light period of 12 h, air temperature of 28 °C, relative humidity of 90%, and CO_2 concentration of 1000 $\mu mol\ mol^{-1}$.

This experiment was conducted by Shimano (2018), and this table was remade by this thesis.

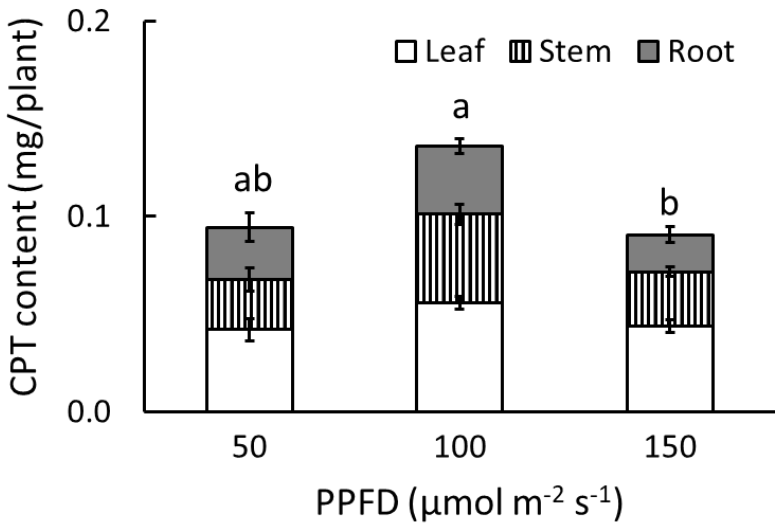

Fig. 3-6 Effects of photosynthetic photon flux density (PPFD) on camptothecin (CPT) content of *Ophiorrhiza pumila* at 35 days after the start of treatment. The treatments were PPFDs of 50, 100, and 150 µmol m^{-2} s^{-1}. This experiment was conducted by Shimano (2018), and it was remade by this thesis. The environmental conditions of cultivation were light period of 12 h, air temperature of 28 °C, relative humidity of 90%, and CO_2 concentration of 1000 µmol mol^{-1}. The CPT content was calculated using the CPT concentration analyzed by HPLC and dry weight of each organ. Different letters indicate significant difference among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 10).

Table 3-2 Effects of light period on dry weight, leaf area, specific leaf weight, and chlorophyll concentration of *Ophiorrhiza pumila* at 35 days after the treatment (n = 10).

Light period (h)	Dry weight (g)				Leaf area (cm^2)	Specific leaf weight ($gDW\ m^{-2}$)	Chlorophyll concentration ($\mu g\ cm^{-2}$)
	Leaf	Stem	Root	Total			
8	0.13 b[z]	0.07 b	0.07 b	0.27 b	58.7 c	49.0 b	24.9
12	0.22 b	0.10 b	0.09 b	0.41 b	113.2 b	36.6 c	24.8
16	0.58 a	0.33 a	0.15 a	1.06 a	163.9 a	66.5 a	27.8

[z]Different letters within columns indicate a significant difference among the treatments at $P < 0.05$ by Tukey-Kramer's test.

O. pumila grew under photosynthetic photon flux density of 100 µmol m^{-2} s^{-1}, air temperature of 28 °C, relative humidity of 90%, and CO_2 concentration of 1000 µmol mol^{-1}.

This experiment was conducted by Shimano (2018), and this table was remade by this thesis.

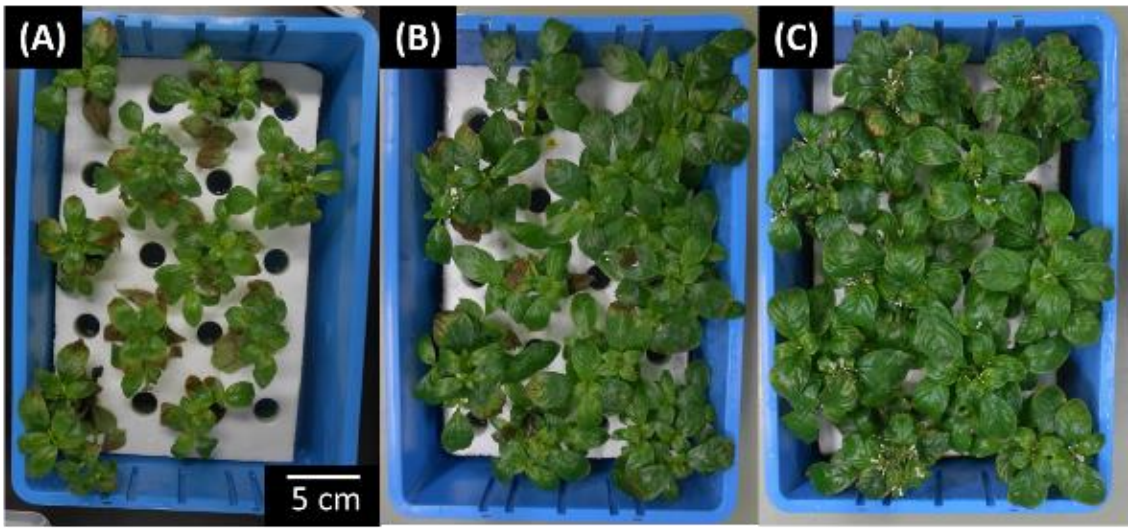

Fig. 3-7 Pictures of *Ophiorrhiza pumila* grown under the light periods of 8, 12, and 16 h (A, B, and C, respectively) in experiment 2-2. This experiment was conducted by Shimano (2018), and it was remade by this thesis. The environmental conditions of cultivation were photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}, air temperature of 28 °C, relative humidity of 90%, and CO_2 concentration of 1000 μmol mol^{-1}.

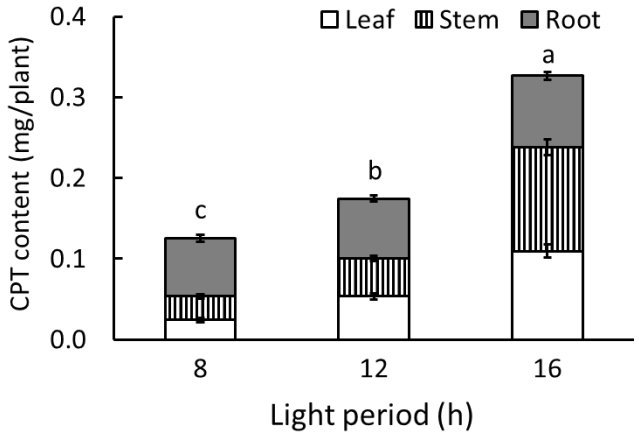

Fig. 3-8 Effects of light period on camptothecin (CPT) content of *Ophiorrhiza pumila* at 35 days after the start of treatment. This experiment was conducted by Shimano (2018), and it was remade by this thesis. The treatments were light periods of 8, 12, and 16 h. The environmental conditions of cultivation were the same as Fig. 3-7. The CPT content was calculated using the CPT concentration analyzed by HPLC and dry weight of each organ. Different letters indicate significant difference among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 6–10).

CHAPTER 4

Effects of concentration and temperature of nutrient solution on growth and camptothecin accumulation of *Ophiorrhiza pumila*

4.1 Introduction

In chapter 3, I established the suitable air temperature and light conditions (PPFD and light period) for growth and CPT accumulation of *O. pumila* in a PFAL. As a next step, the conditions of the root-zone are established to improve the growth and CPT accumulation of *O. pumila*.

Hydroponic systems that provide sufficient water and nutrition to plants are essential tools for stable cultivation in a PFAL. Optimization of the NSC for plant growth is the first step in establishing of cultivation methods. NSC significantly affects the absorption of water and nutrients by the roots. In particular, excessively low or high NSC often causes a nutritional deficit or unbalanced absorption of mineral nutrients by roots, respectively. Many researchers have studied the optimal NSCs for the growth of horticultural crops (Abou-Hadid et al., 1996; Rouphael et al., 2012) and medicinal plants (Park et al., 2016). Because wild *O. pumila* is grown under nutrient-poor conditions, it is hypothesized that the optimal EC of the nutrient solution for growth will be lower than that of horticultural crops. An important second step is establishing the optimal NSC for CPT accumulation of *O. pumila*.

The root-zone temperature is also known to be an effective factor for plant growth. For instance, when the NST increases, the respiration rate of the roots increases, which

often inhibits plant growth due to respiration exhaustion. Low NST also inhibits plant growth because it suppresses water absorption by roots. Moreover, NSTs of 5 °C to 15 °C were found to induce oxidative stress through the suppression of water absorption and increase the ascorbic acid concentration and sugar content in spinach (Chadirin et al., 2011) and the bioactive compounds in red perilla (Ogawa et al., 2018) and canola (Son et al., 2020). Previous studies reported that oxidative stress imposed by, for example, treatment with hydrogen peroxide (H_2O_2), can induce the accumulation of indole alkaloids by root cultures and cell suspensions of the medicinal plant *Uncaria tomentosa* (Huerta-Heredia et al., 2009; Vera-Reyes et al., 2013). Therefore, it is thought that NSTs that induce oxidative stress may increase the CPT content of *O. pumila*. In addition, because the main organs of CPT accumulation in *O. pumila* are the stems and roots (Yamazaki et al., 2003), it is possible that the NST, which influences the root-zone, will affect the CPT accumulation in *O. pumila*.

Therefore, the objectives of this study were to investigate the effects of the NSC (experiment 1; Exp. 1) and the NST (experiment 2; Exp. 2) on the growth and CPT accumulation of *O. pumila* in PFAL.

4.2 Materials and methods

4.2.1 Plant material and cultivation environmental conditions

Before transplanting to a hydroponic container, the cultivation method was the same as mentioned in section 2.2.1.

Three weeks after cutting, the seedlings with four leaves and 50 ~ 100 mg of total fresh weight were transplanted to a container with a nutrient solution and started the NSC

treatment mentioned below. *O. pumila* was cultivated under a transparent plastic cover to maintain the RH at 90%. The environmental conditions of except for the NSC and NST follows: 16 h light period, PPFD of 100 ± 5 μmol·m^{-2}·s^{-1} with white LED lamps, 28 °C of air temperature, RH of 90%, and CO_2 concentration of 1000 μmol mol^{-1}. PPFD was measured near the growth point of plants.

4.2.2 Treatment plots

4.2.2.1 Exp. 1 Nutrient solution concentration (NSC)

The nutrient solution used for this experiment was a commercial product, Otsuka-A nutrient solution. The composition for a standard concentration (one-strength) was 16 mM NO_3^-; 4 mM $H_2PO_4^{3-}$; 4 mM Ca^{2+}; 2 mM Mg^{2+}; 8 mM K^+ and 1.3 mM NH_4^+ and a micronutrient solution. The EC of the standard concentration of Otsuka-A was 2.7 dS m^{-1} (including the EC of tap water) and is called 1.0 NSC in this chapter. The pH of this composition was 5.5–6.5. Since *O. pumila* is a wild plant with a slow growth rate compared to horticultural crops, the NSC treatments were set at lower concentrations than that of the 1.0 NSC. Four treatments were set in total at 0.125, 0.25, 0.5, and 1.0 NSCs and the experiment continued for 63 days. The ECs of these treatments were 0.6, 0.9, 1.5, and 2.7 dS m^{-1}, respectively.

Twenty seedlings for each treatment were transplanted to a container filled with 4 L of the nutrient solution. When the volume of nutrient solution in the container decreased to approximately 80% (3 L), further nutrient solution at the original concentration was supplied to bring each container back up to 4 L. The additional nutrient solution in all treatments except for 1.0 NSC was done at 32 and 56 DAT, and that of 1.0

NSC was done at 56 DAT. The pH of all nutrient solutions was adjusted to maintain 6.5 using a portable pH meter (Cyberscan pH 310, Eutech Instruments, Singapore), every 3 to 4 days. The EC of each nutrient solution was measured using a portable conductivity meter (Cyberscan CON 400, Eutech Instruments, Singapore) at the same time as the pH adjustment. In this experiment, NST was approximately 26 °C without any control under the air temperature of 28 °C. To supply sufficient air to roots and to circulate the nutrient solution in each container, aeration was performed using air pumps and air stones.

4.2.2.2 Exp. 2 Nutrient solution temperature (NST)

The seedlings were cultivated hydroponically for 120 days under the same environmental conditions as Exp. 1. Based on the results of Exp. 1, the NSC in the containers was 0.25 NSC of Otsuka-A. Ten plants with an average 14.2 g of total fresh weight were transplanted to a container filled with 10 L solution of 0.25 NSC, and the NST treatments mentioned below were started at 0 DAT (Fig. 4-6).

The NST treatments were set at 10, 20, 26, and 36 °C (as T10, T20, T26, and T36, respectively), which were lower and higher temperatures around the ambient NST (26°C). The NSTs, except for T26, were maintained and controlled using a handy cooler (TRL-107NHF, Tomas Kagaku Co., Ltd., Japan) and a thermal controller (TC-107, Tomas Kagaku Co., Ltd., Japan). Additionally, to keep the NST uniform and to supply air to the roots, aeration was performed using air pumps and air stones. The NST in the containers was measured by two thermocouples in each treatment and recorded by a data logger at 15-minute intervals.

4.2.3 Growth parameters

All of the growth parameters mentioned below were measured at 63 DAT in Exp. 1 and at 0 and 35 DAT in Exp. 2. In both experiments, each organ of *O. pumila* plants with 4-6 stems, in which each stem had 4-6 nodes, was classified as follows to measure the fresh and dry weights and analyze CPT (Figs. 4-2 and 4-6). The leaves from the top to the second nodes were classified as young leaves, and the leaves below that were classified as mature leaves. In Exp. 1, all stems and flower buds were classified as a stem, because the flower buds were too small to be analyzed for CPT. On 0 and 35 DAT, in Exp. 2, all reproductive organs, including flowers, ovaries, and seed pods, were classified as "Others", because the mature plants bore many flowers and seeds. Because the numbers of flowers and ovaries varied among the plants in each treatment, the fresh and dry weight of the others were measured, but their CPT was not analyzed.

In Exp. 1, for all treatments, the different organs from sampled plants were stored in a freezer at -30 °C to analyze the concentration of chlorophyll and CPT. At the same time, the different organs from other plants were dried at 50 °C for 72 hours in a convection oven (MOV-112F drying chamber, Panasonic Cor., Japan) to measure the dry weight. In Exp. 2, each organ was lyophilized for 24 hours using a freeze-dryer (FDU-1110, EYELA, Japan). After measurement of the dry weight of a lyophilized organ, CPT was analyzed using the same organ because the number of plants was limited.

Total leaf area was calculated from a photograph of all separated leaves from the stems using free imaging software (LIA 32 ver. 0.378). The projected leaf area in Exp. 1 was calculated every week using the same software from a photograph taken from the top of the plant canopy.

4.2.4 Chlorophyll concentration

In Exp. 1, the chlorophyll concentration was determined by the method of Porra et al. (1989). The young leaves were disrupted using a Multi-beads shocker (MB-601U, Yasui Kikai Corporation, Japan). The powdered sample (approximately 20 mg) was weighed and incubated at room temperature in 1 mL of DMF for two days to extract the chlorophyll. The chlorophyll concentration was determined by measuring the absorbance of leaf extracts at 663.8, 646.8, and 750 nm with a spectrophotometer (V-550), and was expressed per unit leaf area by using the specific leaf area in each treatment.

4.2.5 Camptothecin analysis

Through a preliminary experiment, the ratio of extraction solution volume to sample was optimized depending on the water content in the sample, i.e., frozen (fresh) one in Exp. 1 and lyophilized (dried) one in Exp. 2.

The powdered sample of each organ (100 mg of fresh weight and 50 mg of dry weight) was mixed with 1 mL of methanol (≥99.9%) in a 2 mL tube and extracted with an ultrasonic washer (ASU-2; the output of 40 W) for 15 minutes. The methods of extraction and analysis of the CTP were all the same as mentioned in section 2.2.4.

4.2.6 Statistical analysis

Data were statistically evaluated by the same method as mentioned in section 2.2.5.

4.3 Results

4.3.1 Exp. 1 Nutrient solution concentration (NSC)

Electronic conductivity, growth parameters, and chlorophyll concentration

The EC at all NSCs was maintained at approximately the set value throughout the experiment (Fig. 4-1). The EC at 1.0 NSC increased slightly from 21 DAT and increased more evidently from 45 to 49 DAT, reaching 3.2 dS m^{-1}. At 0.125, 0.25, and 0.5 NSCs, the EC was stable and decreased slightly from 35 DAT. From 0 to 56 DAT, the absorbed amount of the nutrient solution at 1.0 NSC was 1 L (daily absorbed rate was 1 mL per plant) and those at the other three treatments were 2.0 L (daily absorbed rate was 2 mL per plant).

At 63 DAT, the fresh weights of young leaves and stems at 0.25 and 0.5 NSCs were significantly higher than those at 0.125 and 1.0 NSCs (Table 4-1 and Fig. 4-2). Because the fresh weight of stems occupied the largest proportion among the organs in all treatments, the total fresh and dry weights at 0.25 and 0.5 NSCs were also significantly higher than those at the other two NSCs. There was no significant difference in the fresh weights of mature leaves and roots among the treatments.

The projected leaf areas in all treatments except for 1.0 NSC increased rapidly from 35 to 42 DAT, after which those at 0.25 and 0.5 NSCs increased more rapidly than the other two treatments (Fig. 4-3A). The rate of increase of the projected leaf area at 0.125 NSC was lower from 42 DAT onwards as compared to those at 0.25 and 0.5 NSCs. The projected leaf area at 1.0 NSC was the lowest among the treatments throughout the experiment. The rate of increase of the projected leaf area at 1.0 NSC was especially low until 42 DAT; thereafter, the rate was similar to 0.25 and 0.5 NSCs. At 63 DAT, the total

leaf areas at 0.125, 0.25, and 0.5 NSCs were higher than that at 1.0 NSC (Fig. 4-3B).

The chlorophyll concentration at 0.125 NSC was significantly lower than that at the other NSCs, approximately half of that observed with the other three treatments (Fig. 4-4). The chlorophyll concentration was saturated at approximately 65 to 70 μg cm^{-2} at treatments above 0.25 NSC.

Camptothecin

In all treatments, the highest CPT concentration was found in the stems, followed by that in the roots, young leaves, and mature leaves (Table 4-2). In young leaves, the CPT concentrations at 0.25 and 0.5 NSCs were significantly higher than those at 0.125 and 1.0 NSCs. Moreover, in the roots, the CPT concentration at 0.25 NSC was 1.6 times higher than those at the other NSCs. In mature leaves and stems, there was no significant difference in CPT concentration among the treatments.

The CPT content was significantly higher at 0.25 NSC (Fig. 4-5). In all treatments, the stems had the highest CPT content among all the organs.

4.3.2 Exp. 2 Nutrient solution temperature (NST)

Growth parameters

At T36, 50% of the treated plants died by 14 DAT, and 80% of them died by 35 DAT (data not shown). Furthermore, by 35 DAT, the plants showed marked damage, and the root color had changed to brown compared to the other treatments. Therefore, the results of T36 were omitted from this study.

On 35 DAT, total fresh and dry weights and leaf area were significantly higher at

T20 than with the other treatments. The fresh weight of the others, comprising the reproductive organs that include flowers, ovaries, and seed pods, was significant (Table 4-3). As the NST decreased, the dry matter ratio of the shoots and roots tended to increase, and thus, the dry matter of the roots at T10 was significantly higher than that at T26.

The plants at T20 showed vigorous growth and bore many lateral shoots and flowers (Fig. 4-6). On the other hand, most of the mature leaves at T10 seemed to be aged and brown in color by 35 DAT unlike with the other treatments.

Camptothecin

The CPT concentration in the roots was the highest among the organs, regardless of the NST (Table 4-4). The CPT concentrations in the stems and roots were two to three times higher than those in the leaves regardless of the NST. The CPT concentration in the young leaves was not affected by NST treatment, but the concentration in the mature leaves was significantly higher at T10 than for the other NSTs. In the stems and roots, the CPT concentrations at T10 and T20 were significantly higher than those at T26.

The parts that contributed most to the CPT content were the stems and roots in all treatments (Fig. 4-7). The total CPT contents at T10 and T20 were higher than that at T26. The CTP content at T20 was approximately three times higher than that at T26 and this was statistically significant.

4.4 Discussion

Exp. 1 Nutrient solution concentration (NSC)

In this study, we found that the 0.25 and 0.5 NSCs were more suitable for growing

O. pumila than the 0.125 and 1.0 NSCs. It is known that excessively high or low concentrations of a nutrient solution negatively affects plant growth, yield, and quality (Park et al., 2016; Rouphael et al., 2012). Kang and van Iersel (2004) found that the growth and flower quality of salvia (*Salvia splendens*) showed a peak at 1.0 times of Hoagland solution (EC 2.0 dS m^{-1}), while at 0.125, 0.25, and 2.0 times (EC 0.4, 0.7, and 3.7 dS m^{-1}), they were lower. According to our results, the change in the EC, projected leaf area, and absorbed amount of the nutrient solution explained the differences in water uptake rate and growth of *O. pumila* among the treatments. At 1.0 NSC, the EC was stable or slightly increased, while the decrease in the volume of the nutrient solution and the increase rate of projected leaf area was low compared to those of the other treatments until 42 DAT. This suggested that the plant at 1.0 NSC absorbed little of the nutrient solution until 42 DAT because of osmotic stress and that the EC in 1.0 NSC was higher than the absorption concentration of *O. pumila*. Other researchers have also reported that high NSC and high EC caused a reduction in plant growth through osmotic stress (Ding et al., 2018; Fallovo et al., 2009; Rouphael et al., 2012). However, after 42 DAT, the growth inhibition at 1.0 NSC was alleviated and the rate of increase of the projected leaf area at 1.0 NSC was the same as those of the other treatments. Therefore, it is possible that the roots of *O. pumila* acclimated to a high NSC in the latter growth stage and that the optimal NSC may change depending on the nutritional conditions in the place of origin, plant varieties, and growth stage.

At 42 DAT, the 0.125 NSC in this study showed a deficit in the nutrient elements required by *O. pumila*. Because, in our experiment, the volume of the nutrient solution in each treatment was the same, the total amount of mineral nutrition was the lowest at 0.125

NSC. This NSC might have an insufficient supply of minerals such as N and Mg for growth and chlorophyll production in *O. pumila* compared to the 0.25 and 0.5 NSCs. Ding et al. (2018) reported that the chlorophyll content of pakchoi (*Brassica campestris* L. ssp. Chinensis) significantly decreased at lower concentrations of nutrient solution (EC 0 to 0.6 dS m^{-1}). Our results indicated that both the maximum growth and a high chlorophyll concentration (indicating a high photosynthetic ability) were obtained when *O. pumila* was grown in 0.25 and 0.5 NSCs (EC 0.9 and 1.5 dS m^{-1}, respectively).

In our study, the CPT concentrations of the young leaves and roots were higher at 0.25 and 0.5 NSCs, where growth was promoted, than at 0.125 and 1.0 NSCs. It is known that secondary metabolites are synthesized from primary metabolites; therefore, the NSC for growth promotion may also be appropriate for CPT accumulation in *O. pumila*. Although there was no difference in growth between the 0.25 and 0.5 NSCs, the CPT concentration in the roots at 0.25 NSC was significantly higher than that at 0.5 NSC. Roots are one of the main organs for CPT production in *O. pumila* (Yamazaki et al., 2003). The 0.25 NSC treatment might be suitable for not only the nutrient and water uptake but also for CPT biosynthesis or accumulation in the roots of *O. pumila*. Further experiments are needed to elucidate the mechanism of CPT accumulation by NSC.

The CPT content at 0.25 NSC was significantly higher than those at the other NSCs because the total dry weight and CPT concentration of young leaves and roots were the highest at this NSC. Within the range of NSCs and the treatment period in this experiment, 0.25 NSC was the most suitable NSC for plant growth and CPT accumulation in *O. pumila*.

Exp. 2 Nutrient solution temperature (NST)

It is known that a root-zone temperature that is too high for optimal growth will decrease the photosynthetic rate (Xu et al., 2002), increase the respiration rate, and change the absorption of water and nutrients by the roots (Klock et al., 1997). In creeping bentgrass, when the roots were exposed to 35 °C, the respiration rate of the whole plant exceeded the net photosynthetic rate of the canopy, resulting in growth inhibition (Xu and Huang, 2000). In our study, we found that NSTs of 26 °C and 36 °C significantly inhibited growth compared to 20 °C NST, although the habitat of wild *O. pumila* is subtropical (Nakamura et al., 2006). An NST of 36 °C was too high to maintain the growth of *O. pumila* even if the NST treatment period was 14 days. It is possible that the main reason for growth inhibition at T36 is an excessive respiration rate in roots because the root respiration rate at 36 °C may be almost 2.5 times as high as that at 20 °C, according to the theory of temperature coefficients (Q10) (Atkin and Tjoelker, 2003).

At low NSTs, Sakamoto et al. (2016) reported that a 10 °C and a 20 °C of root-zone temperatures increased both total biomass and reproductive organs (including the fruits) of strawberries, compared to 30 °C. For temperate crops, many researchers report photosynthesis inhibition, leaf area reduction, and growth inhibition caused by reducing the water absorption by roots exposed to 12 °C in the case of cucumber (Tachibana, 1987), and 14 °C in the case of rice (Nagasuga et al., 2011). From our results, an NST of 20 °C seemed to be more suitable for vegetative growth and reproductive growth in *O. pumila*, compared to 10 °C. The plant growth and the fresh weight of the others were significantly higher at T20 than those at T10 and T26. In addition, in *O. pumila*, most of the mature leaves at T10 withered, and the dry matter ratios of the shoots and roots were the highest

out of all the NSTs. According to these results, the water absorption of *O. pumila* may have been suppressed at 10 °C NST, which inhibited growth even though there was sufficient water in the root-zone.

In chapter 3, the air temperature of 28 °C used in this experiment was the suitable condition for the photosynthesis, transpiration and the growth of above-ground parts of *O. pumila*. Therefore, these findings showed that the combination of different temperature controls for the root-zone (20 °C) and above-ground parts (28 °C) was more suitable to maximize the whole plant growth in *O. pumila*.

In this study, the CPT concentrations of all organs at T26 were markedly lower than those at T10 and T20. It is suggested that the high respiration rate of the roots at T26 negatively affected not only plant growth but also CPT accumulation. Srivastava et al. (2004) mentioned that the availability of precursors through primary photosynthetic metabolites markedly influenced alkaloid accumulation. This suggests that CPT as an alkaloid accumulation at T26 may also require primary photosynthetic metabolites.

In general, low NST inhibited water absorption and often induced water stress, thus generating reactive oxygen species (ROS). This ROS generation is known to be a trigger of secondary metabolite production in many vegetables (Chadirin et al., 2011) and medicinal plants (Ogawa et al., 2018; Son et al., 2020). Although there are few reports on alkaloid production under low NST, Malik et al. (2013) reported that the expression levels of alkaloid biosynthetic enzymes and target alkaloid accumulation in all organs of *Catharanthus roseus* and the roots of *Nicotiana tabacum* were higher at root-zone temperatures of 12 °C than at 25 °C and 30 °C. In our experiment, at T10, the plants showed a typical water stress phenomenon because of low NST; however, the CPT

concentration at T10 was the same as that at T20. These results indicate that 10 °C NST did not promote CPT accumulation in any of the organs of *O. pumila* compared to 20 °C.

It should be noted that the NST treatment in Exp. 2 started 120 days after transplantation to a hydroponic container, i.e., when the plants were bigger and more mature than in the NSC treatment in Exp. 1. The difference in CPT concentrations of 0.25 NSC and T26, which are the same NSC and NST conditions, is thought to be related to the growth stage (Tables 4-2 and 4-4).

To produce the raw materials for drugs, it is important to understand and increase the total amount of target compounds as well as overall growth. In all the treatments in our experiment, we found that the stems and roots were the main organs contributing to a high CPT content compared to the other organs. The CPT content at T20 was higher than that at T10, although CPT concentrations at T10 and T20 were the same. Thus, it is thought that an NST of 20 °C was suitable for both growth and CPT accumulation in *O. pumila* because of the adequate absorption rate and the respiration rate of the roots.

4.5 Conclusion

In conclusion, these results showed that the suitable NSC and NST were the 0.25 NSC and 20 °C, respectively to improve the growth and CPT accumulation of *O. pumila*. These findings also showed the necessity of appropriate NSC control and the different temperature control for root-zone and above-ground part to maximize the CPT yield from *O. pumila*.

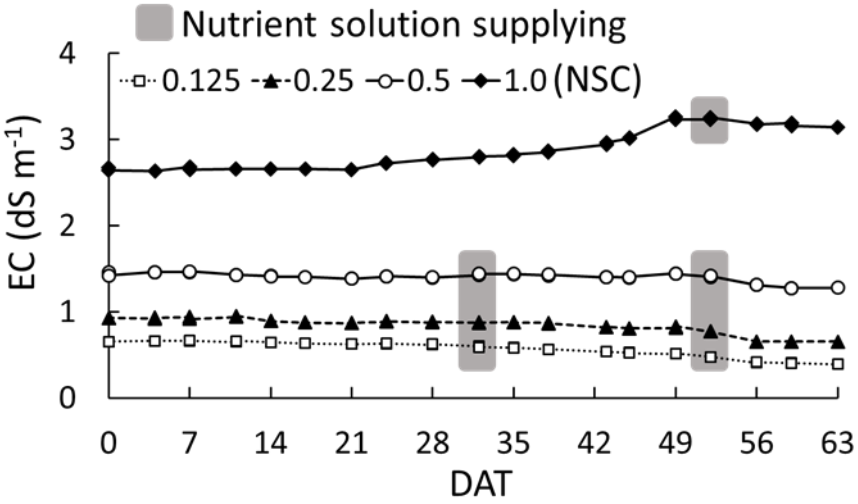

Fig. 4-1 Change in electrical conductivity (EC) in each nutrient solution concentration (NSC) for 63 days after the start of the treatment (DAT) in experiment 1. The treatments were 0.125, 0.25, 0.5, and 1.0 NSC. 1.0 NSC was the standard concentration of Otsuka-A nutrient solution. The grey marked DATs indicate the timing of the supply of the nutrient solution.

Table 4-1 Effects of nutrient solution concentration (NSC) on fresh and dry weights of *Ophiorrhiza pumila* at 63 days after start of the treatment (DAT) in experiment 1 (n = 8).

NSC	Fresh weight (g)					Total dry weight (mg)
	Leaf[y]		Stem	Root	Total	
	Young	Mature				
0.125	0.9 b[x]	1.0	1.7 b	0.7	4.3 b	36.6 b
0.25	1.3 a	1.1	2.9 a	0.7	6.0 a	51.3 a
0.5	1.3 a	1.1	2.9 a	0.8	6.0 a	46.3 a
1.0[z]	0.5 b	0.8	1.2 b	0.7	3.2 b	31.5 b

[z] 1.0 NSC is the standard concentration of Otsuka-A nutrient solution.
[y] Leaf from the top to the second node were classified as a young leaf, and the leaves under that were classified as a mature leaf.
[x] Different letters within columns indicate significant difference among the treatments at $P < 0.05$ by Tukey-Kramer`s test.

Fig. 4-2 Pictures of *Ophiorrhiza pumila* grown in each nutrient solution concentration (NSC) at 63 days after the start of the treatment (DAT) in experiment 1. The treatments were 0.125, 0.25, 0.5, and 1.0 NSC. 1.0 NSC was the standard concentration of Otsuka-A nutrient solution.

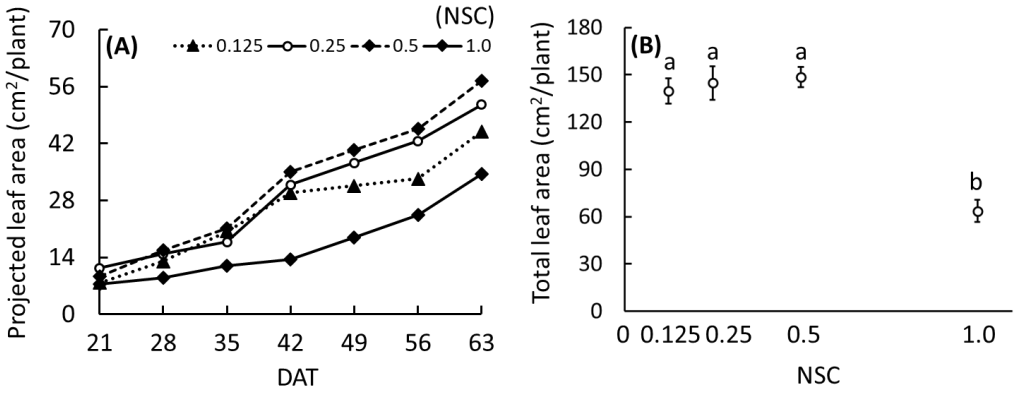

Fig. 4-3 Effects of nutrient solution concentration (NSC) on projected leaf area (A) and total leaf area (B) of *Ophiorrhiza pumila* in experiment 1. The projected leaf area was measured every week after 21 days after the start of the treatment (DAT), and the total leaf area was measured at 63 DAT. The treatments were 0.125, 0.25, 0.5, and 1.0 NSC. Different letters indicate significant differences among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 8).

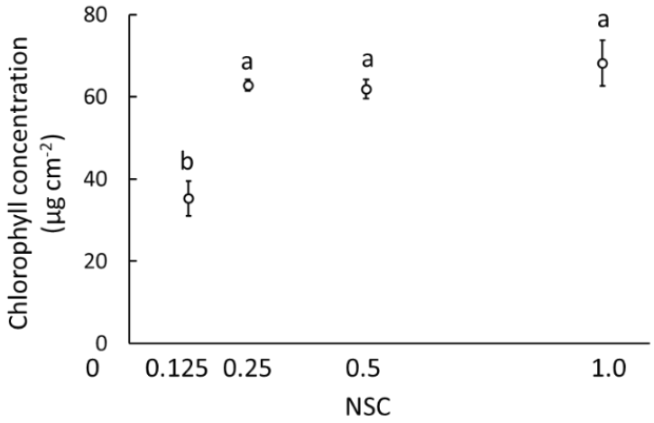

Fig. 4-4 Effects of nutrient solution concentration (NSC) on the chlorophyll concentration in young leaf of *Ophiorrhiza pumila* at 63 days after start of the treatment (DAT) in experiment 1. The treatments were 0.125, 0.25, 0.5, and 1.0 NSCs. Different letters indicate significant differences among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 8).

Table 4-2 Effects of nutrient solution concentration (NSC) on camptothecin concentration of *Ophiorrhiza pumila* organ at 63 days after start of the treatment (DAT) in experiment 1 (n = 8).

NSC	Camptothecin concentration (mg g^{-1} DW)			
	Leaf[y]		Stem	Root
	Young	Mature		
0.125	0.8 b[x]	0.1	2.2	1.0 b
0.25	1.2 a	0.2	2.0	1.7 a
0.5	1.0 a	0.2	2.0	0.9 b
1.0[z]	0.7 b	0.1	1.9	1.0 b

[z] 1.0 NSC is the standard concentration of Otsuka-A nutrient solution.
[y] Leaf from the top to the second node were classified as a young leaf, and the leaves under that were classified as a mature leaf.
[x] Different letters within columns indicate significant difference among the treatments at $P < 0.05$ by Tukey-Kramer`s test.

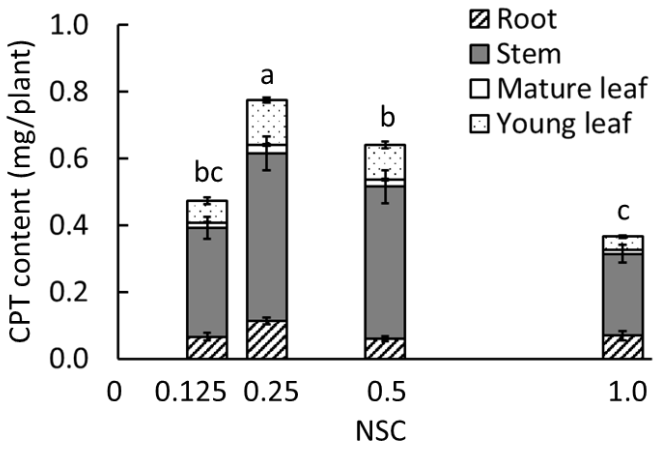

Fig. 4-5 Effects of nutrient solution concentration (NSC) on the camptothecin (CPT) content in the whole plant of *Ophiorrhiza pumila* at 63 days after start of the treatment (DAT) in experiment 1. The treatments were 0.125, 0.25, 0.5, and 1.0 NSCs. The CPT content was calculated using the CPT concentration determined by HPLC and dry weight of each organ. Different letters indicate significant differences among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 8).

Table 4-3 Effects of nutrient solution temperature (NST) on growth of *Ophiorrhiza pumila* at 35 days after start of the treatment (DAT) in experiment 2 (n = 3).

DAT	NST (°C)	Fresh weight (g)						Total dry weight (g)	Dry matter ratio (%)		Leaf area (cm^2)
		Leaf[y]		Stem	Others	Root	Total		Shoot	Root	
		Young	Mature								
0		0.5	2.6	3.8	2.3	2.5	14.2	1.4	10.8	4.3	35.9
35	T10	1.3	3.0	4.5	4.5 b	4.7	18.0 b	2.9 b	19.4	7.2 a	151.7
	T20	1.4	5.3	7.4	9.7 a	6.2	30.1 a	4.6 a	17.7	6.0 ab	254.2
	T26 [z]	1.1	4.1	6.3	3.3 b	4.2	18.9 b	2.6 ab	16.3	5.2 b	202.0

[z] NST in T26 was not controlled.
[y] Leaf from the top to the second node were classified as a young leaf, and the leaves under that were classified as a mature leaf.
[x] Different letters within columns indicate significant difference among the treatments on 35 DAT at $P < 0.05$ by Tukey-Kramer`s test.

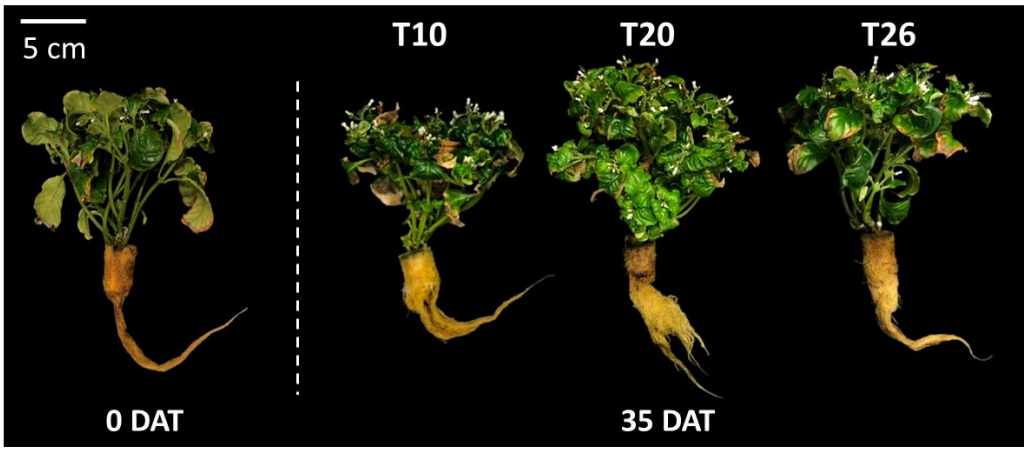

Fig. 4-6 Pictures of *Ophiorrhiza pumila* treated at each nutrient solution temperature (NST) at 0 and 35 days after start of the treatment (DAT) in experiment 2. The NST treatments were set at 10, 20, and 26 °C as T10, T20, and T26, respectively. The NSTs at T10 and T20 were maintained using a handy cooler, and that at T26 was not controlled.

Table 4-4 Effects of nutrient solution temperature (NST) on camptothecin concentration of *Ophiorrhiza pumila* at 35 days after start of the treatment (DAT) in experiment 2 (n = 3).

NST (°C)	Camptothecin concentration (mg g^{-1} DW)			
	Leaf[y]		Stem	Root
	Young	Mature		
T10	0.5	0.3 a[x]	1.3 a	1.8 a
T20	0.5	0.1 b	1.4 a	2.0 a
T26[z]	0.4	0.1 b	0.8 b	1.0 b

[z] NST in T26 was not controlled.
[y] Leaf from the top to the second node were classified as a young leaf, and the leaves under that were classified as a mature leaf.
[x] Different letters within columns indicate significant difference among the treatments at $P < 0.05$ by Tukey-Kramer`s test.

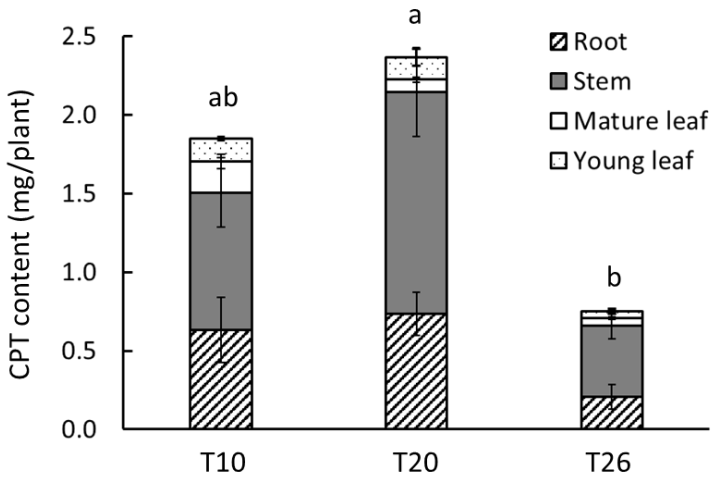

Fig. 4-7 Effects of nutrient solution temperature (NST) on the camptothecin (CPT) content in whole plant of *Ophiorrhiza pumila* at 35 days after start of the treatment (DAT). The NST treatments were set at 10, 20, and 26 °C as T10, T20, and T26, respectively. The NSTs at T10 and T20 were maintained using a handy cooler, and that ay T26 was not controlled. The CPT content was calculated using the CPT concentration determined by HPLC and dry weight of each organ. Different letters indicate significant differences among the treatments at $P < 0.05$ by Tukey-Kramer's test. Vertical bars indicate standard error (n = 3).

CHAPTER 5

Conclusions and future work

5.1 Summary

To meet the increasing demand for CPT and facilitate its stable production, it is necessary to clarify the characteristics of gas exchange rates of whole plants and establish the suitable light and root-zone environments for growth and CPT accumulation of *O. pumila* in a PFAL.

In chapter 2, the concentration distribution of CPT in different plant organs were initially analyzed to identify target organs. The root and reproductive organs (flower, ovary, and seed pod) had the highest CPT concentration among all organs. On the other hand, the stem, including the main stem and lateral stem, was an essential organ to CPT production because the stem accounted for 45% of the total CPT content of the whole plant. Also, it was concluded that the suitable harvested timing for *O. pumila* was the seed-ripening stage (around 63 days after transplanting to a hydroponic container).

In chapter 3, to determine the suitable light conditions for growth, the P_n and E of whole plants were measured using the open-type assimilation chamber. These analyses revealed that the light saturation point was at a PPFD of 300 μmol m^{-2} s^{-1}, and the E tended to decrease with increasing PPFD above 100 μmol m^{-2} s^{-1}. As a result, it suggested that 100 μmol m^{-2} s^{-1} PPFD and 28 °C are good conditions of PPFD and air temperature for photosynthesis and transpiration. Also, when compared these results to the P_n and E of lettuce, and *O. pumila* exhibited to be a typical shade plant.

In chapter 4, to investigate the suitable root-zone conditions, the effects of

nutrient solution concentration (NSC; 0.125, 0.25 0.5, and 1.0 times) and temperature (NST; 10, 20, 26, and 36 °C) were examined respectively. According to these results, the 0.25 times solution and 20 °C was the suitable NSC and NST, respectively, for growth and CPT accumulation.

My research revealed the suitable environmental conditions for the growth and CPT accumulation from *O. pumila* cultivated in a PFAL, and it may contribute to the efficient CPT production for a clinical anti-tumor agent.

5.2 Camptothecin production by *Ophiorrhiza pumila*

In this study, *O. pumila* was suggested as an alternative medicinal plant for CPT production instead of *C. acuminata*, so the CPT productions of the two plants were compared. In the case of *C. acuminata*, alternative or more sustainable methods have been studied because of the problems of storage of plant material and environmental concerns (climate or season). Vincent et al. (1997) suggested the CPT production method that is continue to harvest of young leaves of *C. acuminata* repeatedly, without the destruction of the tree in a greenhouse. The CPT from young leaves of *C. acuminata* was produced a total of 87.5 mg m^{-2} by 6-week interval harvest for 12 weeks. Because *C. acuminata* is a deciduous tree, it was considered that harvest period is 12 weeks a year. Therefore, it is assumed that a total of 87.5 mg m^{-2} is collected in a year.

On the other hand, under the controlled environments: 16 h light period, PPFD of 100 μmol m^{-2} s^{-1} of white LED lamps, air temperature of 28 °C, RH of 80%, and CO_2 concentration of 1000 μmol mol^{-1}, the CPT from *O. pumila* whole plant was 37 mg m^{-2} after 9 weeks of transplanting to a hydroponic container. At this time, the plant density

was 48.1 plant m^{-2}. When it is assumed that a PFAL has 7 floors of multi-layers, the CPT from *O. pumila* might be produced 259 mg m^{-2}. One of advantages of PFAL is possible to cultivate the plants of uniform quality through a year, so *O. pumila* can be harvested 5.8 times in a year. Therefore, in the PFAL, *O. pumila* can produce a total CPT of 1502.3 mg m^{-2} y^{-1}. The annual CPT production of *O. pumila* cultivated in a PFAL (1502.3 mg m^{-2} y^{-1}) is 17 times higher than that of *C. acuminata* cultivated in a greenhouse (87.5 mg m^{-2} y^{-1}) when energy efficiency is not considered.

When considering the production of a target compound in the different cultivation systems, it is better to take into account the energy cost required for plant production. Graamans et al. (2018) compared an annual dry matter production and energy required for lettuce production between a PFAL and a greenhouse. The annual dry matter production of lettuce cultivated in the PFAL was estimated approximately 5 kg m^{-2} y^{-1}, and it was over 2 times higher than that cultivated in the greenhouse because the PFAL can optimize plant environments and result in high productivity. The PFAL requires 3.5 times higher energy input than the greenhouse; to produce the dry matter, the electrical energy uses were 247 kWh kg^{-1} of PFAL and 70 kWh kg^{-1} of greenhouse. The electrical energy uses for PFAL or greenhouse was calculated considering LEDs, heating, sensible cooling, and dehumidification or latent cooling, and for a greenhouse, the total solar radiation was also considered.

With reference to the results in the above study, the CPT production with consideration of energy demand is compared between *O. pumila* cultivation in a PFAL and *C. acuminata* in a greenhouse. The CPT concentrations were 1.3 g kg^{-1} DW in *O. pumila* in a PFAL (reported this study) and 3.0 g kg^{-1} DW in *C. acuminata* in a greenhouse

(Vincent et al., 1997), and they were used to calculate the electrical energy use to produce CPT. To produce the CPT, the electrical energy uses are considered 191.5 kWh g^{-1} CPT for a PFAL and 23.3 kWh g^{-1} CPT for a greenhouse. Even if a PFAL requires higher energy demand than a greenhouse for CPT production, it has many advantages such as high yield per cultivation area, uniformity of environmental conditions, uniform quality throughout the year compared to a greenhouse. In particular, in a PFAL, because *O. pumila* requires a lower PPFD and higher temperature than those for lettuce, the energy demand for *O. pumila* is possible to decrease than that for lettuce. Additionally, when the NST was controlled at 20 °C, the CPT production of *O. pumila* increased three times than that at 26 °C (Chapter 4). Therefore, the annual CPT production of *O. pumila* cultivated in a PFAL is possible to be higher than that of *C. acuminata* because of the above consideration.

Finally, the energy demand is compared between a PFAL and a greenhouse for CPT production by *O. pumila*. To produce the CPT of *O. pumila*, the electrical energy uses are considered 191.5 kWh g^{-1} CPT for a PFAL and 54.3 kWh g^{-1} CPT for a greenhouse. To get the high yield of CPT, the best timing to harvest of *O. pumila* is at the seed-ripening stage (Chapter 2). Because the suitable light period is 16 h to accelerate flowering (Chapter 3), it is required to control the light period using the supplemental lighting, which increases the electrical energy use, in a greenhouse. Therefore, the electrical energy use for a greenhouse may increase than 54.3 kWh g^{-1} CPT.

A PFAL has the following advantages: 1) Production of uniform quality and yield of plant for a year through optimal environmental control regardless of weather and season: 2) 50% reduction of fertilizer consumption by recycling of circulating the nutrient

solution: 3) 50-70% reduction in working hours and labor per unit yield: 4) Low product loss due to physical, chemical, and biological damage to plants (Kozai and Nui, 2016). More than one hundred of PFALs that produce lettuce have been already commercialized in Japan because they have commercial advantages described above even though the energy demands in the PFALs are still high compared to those in the greenhouses. For example, a PFAL with fluorescent lamps, which is operated by Spread Co., Ltd. in Kyoto, produces 23,000 leaf lettuce heads daily (Kozai, 2016). Since the plant size and plant density of *O. pumila* are similar to those of lettuce, it is possible to put CPT production of *O. pumila* in practical use in a PFAL using already developed technologies from the viewpoint of production system. Therefore, it is expected that the annual production of *O. pumila* in a PFAL will be commercialized in the near future.

5.3 Suggestions for future research

This study investigated the characteristics of P_n and E under various air temperatures and PPFDs using the open-type assimilation chamber and determined the suitable environmental condition for growth and CPT accumulation of *O. pumila*. Still, the various light conditions can be controlled to improve the growth and the accumulation of secondary metabolites of *O. pumila*. Especially, increasing DLI usually increases dry matter accumulation and improves plant quality. This study concluded the light conditions with PPFD of 100 μmol m^{-2} s^{-1} and light period of 16 h was suitable for growth and CPT accumulation of *O. pumila*, and the DLI was 5.76 mol m^{-2} d^{-1}. However, there is a possibility that the growth and CPT accumulation of *O. pumila* can further be improved under higher DLI than 5.76 mol m^{-2} d^{-1}.

In chapter 3, it was reported that the P_n of *O. pumila* gradually decreased after 6 hours when *O. pumila* was exposed at PPFD 300 μmol m^{-2} s^{-1} which is the light saturation point. Based on those results, the DLI can increase using a PPFD combination with PPFD 300 μmol m^{-2} s^{-1} for less than 6 h and PPFD 100 μmol m^{-2} s^{-1} for the other hours, within light period of 16 h, resulting to improve the growth and CPT accumulation. Therefore, it can be expected that the combination of low and high PPFDs under a light period of 16 h will further improve the growth and CPT accumulation of *O. pumila*.

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