Breeding Studies on Stable Production of Essential Oil Compounds in *Atractylodes lancea* De Candolle

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Breeding Studies on Stable Production of Essential Oil Compounds in *Atractylodes lancea* De Candolle

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Abbreviation

ABA	: abscisic acid
ANOVA	: analysis of variance
BAP	: 6-benzylaminopurine
GC/MS	: gas chromatography/mass spectrometry
G × E	: genotype–environment interaction
G × L	: genotype–cultivation location interaction
G × Y	: genotype–cultivation year interaction
h_B	: Broad-sense heritability
IAA	: indole-3-acetic acid
JA	: jasmonic acid
SA	: salicylic acid
TCM	: traditional Chinese medicine

Chapter 1

General Introduction

1.1. General Introduction

1.1.1 About Kampo medicines and crude drugs

Kampo medicines are Japanese traditional medicine that have been brought to Japan from China in the fifth century and over time developed into a unique form of medicine influenced by Japanese climate and culture (Yakubo et al., 2014). While Kampo is based on the traditional Chinese medicine (TCM), the diagnostic process and treatment differ (Yu et al., 2006). In addition, some of the materials used in Kampo medicine differ from TCM, owing to the fact that not all materials were readily available in Japan (Yakubo et al., 2014). Materials used in Kampo medicines typically include plant seeds, leaves, rhizomes, and roots that may be dried or steamed, which are used as crude drugs. Fossilized animal bone, insects, and minerals may also be used. Kampo medicines are generally made through blending and extracting two or more of these crude drug preparations (Sato et al., 2008).

During the Meiji era (1868–1912 CE), Kampo medicines were excluded from official medical education, and its usage gradually decreased. However, pharmacognosy and Kampo medicine remained at low levels of use and was preserved by the Japan Society for Oriental Medicine, which was established in 1950. Furthermore, in 1967, the Ministry of Health, Labour and Welfare authorized Kampo medicinal formulations for prescriptions under the national insurance coverage. Currently, there are 148 approved Kampo formulations for prescription under the national insurance coverage (Watanabe et al., 2011; Motoo et al., 2011).

In recent years, Kampo medicine has experienced resurgence in popularity with physicians in Japan, and Kampo formulations have experienced an increase in prescriptions by more than 70% (Motoo et al., 2011; Moschik EC et al., 2014). In recent pharmacological studies, drug efficacy of Kampo has been evaluated using modern experimental methods, and the knowledge gained has been

used to inform clinical practices. Clinical research and the identification of the medicinal action mechanism of Kampo have progressed, especially on Daikenchuto (大建中湯), Rikkunshito (六君子湯), and Yokukansan (抑肝散) (Yoshikawa et al., 2015; Takeda et al., 2013; Mizukami et al., 2009). Kampo medicines are expected to play a significant role as complementary and alternative medicines to the use of western, modern medicine, and the market for Kampo formulations expands every year (Nishimura et al., 2009; E. C. Moschik et al., 2012).

As demand of Kampo medicines has expanded, so has the need for the raw materials used to produce crude drug preparations (Yamamoto et al., 2019). A stable supply of crude drugs is essential to meet the expanding Kampo market needs; however, it has become difficult to maintain a steady supply of raw materials due to climate change, destruction of natural environments, and overexploitation of wild resources. Furthermore, 80% of crude drug preparations used in Japan are dependent on imports from China. Moreover, there has been a rapid rise in price of crude drugs due to increasing labor costs and an increase in commodity price due to both scarcity and Chinese economic growth. Therefore, to produce the raw materials for Kampo medicines in a stable manner, domestication and cultivation of medicinal plants in Japan are required (Yamamoto et al., 2019; Koike et al., 2012). In recent years, Ministry of Agriculture, Forestry and Fisheries in Japan supports to expand domestic cultivation of medicinal plants, and a lot of research institutes and companies in Japan promote to develop cultivation techniques of medicinal plants (Kawashima, 2018).

1.1.2 About *Atractylodes lancea*, the botanical origin of the crude drug "*So-jutsu* (蒼朮)"

Currently, there are 293 species that comprise the crude drugs distributed within Japan. *So-jutsu* was the seventh most commonly used in 2018 with about 800–900 tons consumed in Japan. Its availability is entirely dependent on imports from China, and it is the most commonly imported

Kampo medicine crude drug from China. Based on usage statistics, it is one of the most important crude drugs (Yamamoto et al., 2019).

Recently, there has been a rapid increase in the demand for *So-jutsu.* However, wild resources of *So-jutsu* are faced with the risk of depletion, and this scarcity has led to an increase in its prices every year. Since ~1990, it has been cultivated in China, but there is high variability among individual plants and cultivation is not stable (Chen et al., 2017). Therefore, domestication of *So-jutsu* and determining cultivation practices suited to Japan will promote a steady supply and domestic production for this Kampo raw material.

In the Japanese Pharmacopoeia 17th edition (JP17), *Atractylodes lancea*, *A. chinensis*, and their hybrid species are specified as the botanical origin of *So-jutsu* (2016). *Atractylodes lancea* is a perennial plant that belongs to *Asteraceae*, and it grows naturally in the Hubei, Anhui, Jiangsu, and Henan provinces in China, while *A. chinensis* grows naturally in the Hebei and Shandong provinces (Takeda et al., 1995a; Takeda et al., 1995b). The chromosome number of the diploids *A. lancea* and *A. chinensis* is 2n = 2x = 24 (Hiraoka, 1998; Ge et al., 1987; Duan et al., 2015). *Atractylodes lancea* is highly variable with regard to shoot morphology and medicinal components, and it has a large market distribution compared to *A. chinensis* (Takeda et al., 1997).

The medicinal parts of *A. lancea* are rhizomes, and since ancient times, high-quality *So-jutsu* has ideally contained a large volume of essential oils that form white, cotton-like crystals on the epidermis of the dried rhizomes (Takeda et al., 1995a). Several Kampo formulations require the rhizome, such as Rikkunshito, Yokukansan, and Goreisan (五苓散), and formulations containing the rhizome are mainly used to treat digestive disorders and body fluid imbalances (Kubo et al., 1983; Wang et al., 2008). Given its importance in Kampo formulations and high-quality products produced from *A. lancea*, it has been a topic of research interest. In recent studies, genome

information analyses, such as transcriptomics analysis using next-generation sequencers (NGS), have been promoted in *A. lancea* to assemble basic breeding information that will facilitate genetic improvement and the development of molecular markers (Huang et al., 2016; Ahmed et al., 2016; Chen et al., 2017).

In the 17th edition of the Japanese pharmacopoeia, the quality standard for the essential oil content of *A. lancea* rhizomes prescribed is required to be >0.7 mL in 50 g of dried *Atractylodes* rhizome, making essential oil an important quality standard for *A. lancea* production (Japanese Pharmacopoeia 17th edition, 2016). Major essential oil compounds in *A. lancea* are β-eudesmol, hinesol, atractylon, and atractylodin. Their detailed biosynthesis pathways are not yet elucidated. However, β-eudesmol, hinesol, and atractylon are likely sesquiterpenoids biosynthesized from farnesyl diphosphate in the mevalonate pathway, while atractylodin is a polyacetylene compound biosynthesized from unsaturated fatty acids (Takeda et al., 1996; Minto and Blacklock, 2008; Degenhardt et al., 2009).

The active compounds found in *A. lancea* rhizomes also occur in other cultivated crops. β-eudesmol occurs in plants including *Humulus lupulus* (beer hops), roots of *Chamomilla recutita*, and leaf buds of *Populus nigra*, while hinesol is contained in *Cistus creticus*, *Guatteriopsis blepharophylla*, and *Piper lhotzkyanum* (Irwin, 1989; Szoke et al., 2004; Jerkovic et al., 2003; Paolini et al., 2009; Costa et al., 2008; Moreira et al., 1998). Atractylon is a major component in the *A. lancea* related species of *A. japonica* and *A. macrochephara* (Hatano et al., 1990). In contrast, atractylodin is a highly specific compound not commonly found in other crops besides *A. lancea* and *A. chinensis* (Yosioka et al., 1960). These compounds have been reported to have pharmacological activities. β-Eudesmol and hinesol have been reported to have anti-ulcer activity, while atractylodin has been reported to ameliorate delayed gastric emptying. Overall, these

compounds are pharmacologically active in the gastrointestinal system (Nogami et al., 1986; Nakai et al., 2003).

So-jutsu has been mainly used to treat digestive disorders since ancient times, and the medicinal activities of *A. lancea* and the pharmacological activities of the essential oil compounds in *A. lancea* rhizomes are consistent with the prescribed use (Wang et al., 2008). Modern pharmacological research has also reported that these compounds have antitumor activities, pharmacological activities on the nervous system, and anti-inflammatory activity (Koonrungsesomboon et al., 2010; Jun et al., 2018). Clinical uses and research studies on the pharmacological activities of *So-jutsu* require consistent active ingredient concentrations within the *A. lancea* essential oil to maintain consistent dosage in prescriptions containing the crude drug (Chen et al., 2017). However, the crude drugs distributed in the market are highly variable in their contents of essential oil compounds. Cultivation of less variable lines of *A. lancea* will likely improve the consistent and stable production of essential oil compounds (Nishikawa et al., 1975).

In addition, not only is *A. lancea* essential oil content considered an important quality criteria but also the content ratio between β-eudesmol and hinesol. In classical Japanese medicinal literature, white, cotton-like crystals on the *A. lancea* dried rhizome epidermis were an indicator of high quality (Takeda et al., 1998a; Takeda et al., 1998b), and the main components of the crystal are β-eudesmol and hinesol. Furthermore, the content ratio between β-eudesmol and hinesol is an important trait to the crystal deposit (Yosioka et al., 1959; Takeda et al., 1995a). β-Eudesmol and hinesol have similar chemical make-ups in which they share the same molecular weight and are both likely produced from a common germacrane-type ten-membered ring intermediate by the mevalonate pathway (Namba and Tuda, 1990). Therefore, these compounds likely have similar pharmaceutical activities in the treatment of gastric ulcer and other digestive disorders (Koonrungsesomboon et al., 2014).

Despite the chemical similarities between β-eudesmol and hinesol, their mode of action might be different. In the treatment of gastric ulcer, β-eudesmol inhibits Na^+/K^+ ATPase, whereas hinesol inhibits both H^+/K^+ ATPase in gastric membrane vesicles and HCl secretion in the stomach (Satoh et al., 1992; Satoh et al., 2000). In addition, hinesol can inhibit cell growth and induce apoptosis in human leukemia HL-60 cells, suggesting that hinesol may also be useful as an anticancer drug, whereas β-eudesmol shows weak pharmacological activity (Masuda et al., 2015). Thus, the β-eudesmol/hinesol content ratio in *A. lancea* is understandably an important quality criterion. Takeda et al. suggested that the β-eudesmol/hinesol content ratio is mainly influenced by genetic background in *A. lancea*; however, extensive genetic analyses have yet to be conducted (1996). Therefore, a detailed analysis on the heritability of the β-eudesmol/hinesol content ratio in *A. lancea* is also required to better understand and influence the quality of *A. lancea* for its use in crude drug preparations.

The characteristics of *A. lancea* rhizomes are closely related to their natural habitat, and the essential oil content of *A. lancea* varies based on the geographical factors. However, it remains unclear whether the geographical distribution of variation is based more on genetic or environmental factors (Zhen et al., 2012; Takeda et al., 1995a). Generally, accumulation of secondary metabolites, namely, sesquiterpenoids and polyacetylene, is induced by environmental factors, such as biotic and abiotic stresses via phytohormone signaling (Sudha et al., 2002; Bennett and Wallsgrove., 1994; Ramakrishna and Ravishankar, 2011). Several studies have concluded that the essential oil content of *A. lancea* varies in large part due to environmental factors. For instance, soil acidity stimulates β-eudesmol accumulation in *A. lancea* through activation of plant hormones, such as abscisic acid (ABA) and indole-3-acetic acid (IAA) (Yuan et al., 2009). Moreover, it has also been reported that accumulation of β-eudesmol, hinesol, and atractylon is induced by jasmonic acid (JA), salicylic acid

(SA), and ABA activated through fungal or bacterial inoculation (Ren and Dai, 2012). Yuan et al. (2016) also showed that ethylene is an upstream signal of JA and SA, and it plays important roles in the accumulation of essential oil compounds. Thus, the accumulation of essential oil compounds in *A. lancea* may be induced in part by the interaction of various plant hormones triggered by environmental changes.

In contrast, Takeda et al. (1995) showed that *A. lancea* could be classified into three classes of essential oil contents with regard to β-eudesmol and hinesol compound content depending on their natural habitat and suggested that the contents might largely be influenced by genetic background. The first type has low contents for both β-eudesmol and hinesol, the second type is high in hinesol content but low in β-eudesmol content, and the third type is high in both (Takeda et al., 1995a; Takeda et al., 1996). However, a detailed genetic analysis has not yet been performed to determine whether these three types of *A. lancea* are due to either genetic or environmental factors or a combination of both.

In crops, genetic analysis, the estimation of heritability, and the evaluation of genotype-environment (G × E) interaction are generally studied to establish basic information to assist in breeding (Ukai, 2002). For some medicinal plants, genetic analysis has been performed. In *Artemisia annua*, the heritability of artemisinin content, which is one of the sesquiterpenoids with pharmacological activities against malaria, has been analyzed (Ferreira et al., 1995). However, there are few genetic studies investigating secondary metabolites in the other medicinal plants, including *A. lancea*, and basic genetic studies are not powerful enough to deduce their genetic basis (Ahmed et al., 2016; Huang et al., 2016; Chen et al., 2017). Therefore, evaluation of heritability on the contents of essential oil compounds in *A. lancea* are required to establish basic knowledge, which will be useful in breeding and reducing the variation in quality in *A. lancea*, is needed.

In addition, propagating *A. lancea* seedlings is difficult. There are both female and bisexual *A. lancea* plants with most *A. lancea* flowers being female, and seed harvests are unstable (Hiraoka, 1993). Although *A. lancea* can be propagated by division of rhizomes, the propagation is inefficient, and only a few rhizomes are produced for transplantation every few years. To propagate *A. lancea* seedlings more efficiently, *in vitro* propagation of *A. lancea* has been investigated (Hiraoka et al., 1984). Previous studies have examined micropropagation on solid media, such as agar media, but not on liquid media (Shoyama et al., 1987). Generally, it has been shown that the propagation rate is higher on liquid media than that of solid media (Takayama, 1986). To take advantage of breeding tools that are developed, it is also essential to understand horticultural traits and produce *A. lancea* efficiently and in quantities that permit commercial cultivation.

1.2. The objectives of this study

In this study, I evaluated the heritability of essential oil content and the β-eudesmol and hinesol contents and their ratio to one another using 25 clonally established lines. The 25 clonal lines of *A. lancea* were vegetatively propagated by division of rhizomes. Broad-sense heritability (h_B) of the traits was estimated using calculation models for vegetatively propagated crops such as pasture plants. In addition, G × E interaction analysis on the contents of essential oil compounds and β-eudesmol/hinesol content and ratios were performed using six clonal lines of *A. lancea* cultivated in different years and different locations. Through these experiments, the effects of selective breeding on the essential oil content and make-up of *A. lancea* were evaluated. Furthermore, in this study, *in vitro* shoot propagation in liquid culture was investigated to develop more efficient methods of micropropagation in *A. lancea*.

This study aims (1) to evaluate the genetic and environmental factors influencing the contents

of essential oil compounds in *A. lancea*, (2) to evaluate the heritability of β-eudesmol/hinesol content ratio in *A. lancea*, and (3) to develop efficient methods of *in vitro* shoot propagation in liquid culture of *A. lancea*.

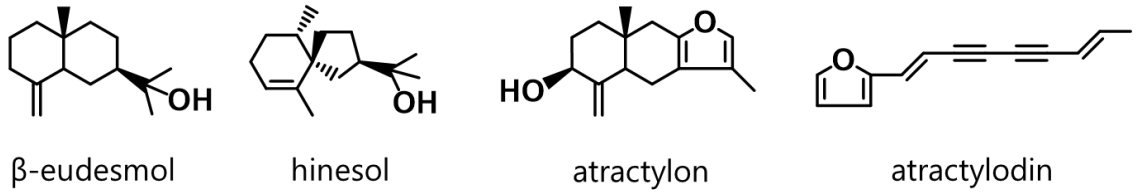

Figure1.1 Chemical structures of essential oil compounds contained in *Atractylodes lancea*.

Chapter 2

Genetic and environmental factors influencing the contents of essential oil compounds in *Atractylodes lancea*

2.1. Introduction

Atractylodes lancea De Candolle (Compositae) is a medicinal plant that is distributed in East Asia, mainly in central China (Shiba et al., 2006). Dried rhizomes of *A. lancea* are prescribed in Japanese and Chinese herbal medicines as the crude drugs (Society of Japanese Pharmacopoeia, 17th edition, 2016; Chinese Pharmacopoeia, 2015). In Japan, the crude drug is called "*So-Jutsu*," whereas in China, it is called "*Chang-zhu*"(Mizukami et al., 2000). The rhizomes of *A. lancea* have mainly been used for digestive disorders and body fluid imbalance (Kubo et al., 1983; Wang et al., 2008). Major active ingredients in *A. lancea* were essential oil compounds, namely, sesquiterpenoids (β-eudesmol, hinesol, atractylon) and polyacetylene (atractylodin) (Koonrungsesomboon et al., 2014). The sesquiterpenoid compounds have been reported to have anti-ulcer activity, while atractylodin has been reported to ameliorate the delayed gastric emptying (Nogami et al., 1986; Nakai et al., 2003). Based on pharmacological studies, such compounds are considered important bioactive ingredients in *A. lancea* (Kubo et al., 1983). However, the drugs distributed in the market have hugely varied in contents of the major essential oil compounds, posing a challenge for the cultivation of *A. lancea* with constant quality (Nisikawa et al., 1975). Therefore, it is critical to investigate the factors influencing the variation in the contents of essential oil compounds in *A. lancea* to keep pharmacological activities constant in many traditional prescriptions containing the crude drug (Nisikawa et al., 1975).

Generally, phenotypic variation is a product of both genetic and environmental variation (Ukai, 2002). The qualities of *A. lancea* samples are closely associated with their habitat, and the contents of essential oil compounds in *A. lancea* vary considerably based on geographical factors. However, it remains unclear whether the geographical variations are based on genetic or environmental factors (Zhen et al., 2012; Takeda et al., 1995a).

Some studies have reported that the activation of phytohormones such as jasmonic acid and abscisic acid through symbiosis with several endophytes induces the production of the essential oil compounds in *A. lancea* (Ren and Dai, 2012; Wang et al., 2015). In addition, Yuan et al. reported that soil acidity stimulated the accumulation of essential oils in *A. lancea*, particularly β-eudesmol, by influencing the concentrations of phytohormones such as abscisic acid (Yuan et al., 2009). Such results suggest that the contents of essential oil compounds in *A. lancea* could vary depending on environmental factors such as biological and abiotic stress. In contrast, Takeda et al. (1996) suggested that the contents of essential oil compounds are influenced largely by genetic factors based on a comparative study of the essential oil compounds between wild and cultivated *A. lancea*. However, extensive genetic analyses, particularly the estimation of heritability and Genotype-Environment (G × E) interaction analyses associated with the contents of the essential oil compounds in *A. lancea*, have not been performed.

In the present study, to evaluate effects of genetic factors on the contents of essential oil compounds in *A. lancea*, I cultivated twenty-five clonal lines of *A. lancea* in a micro-environment and estimated broad sense heritability for β-eudesmol, hinesol, atractylon, and atractylodin contents. In order to estimate the broad sense heritability, I employed the calculation model for vegetatively propagated crops such as orchardgrass, bromegrass, and tall fescue (Kalton et al., 1952; McDonald et al., 1952; Burton et al., 1953). In addition, to examine G × E interaction and the effects of environmental factors, I cultivated six clones in different years (2016, 2017) and different locations (Hokkaido, Ibaraki), and determined contents of β-eudesmol, hinesol, atractylon, and atractylodin in the clones. Subsequently, I evaluated genetic variances, environmental variances, and G × E interaction variance based on the compound contents.

2.2 Materials and Methods

2.2.1. Plant materials

In the present study, seeds of *A. lancea* obtained in a previous study were used (Takeda et al., 1997). The seeds were originally obtained from the People's Republic of China and were from different genotypes. In other words, the seeds were harvested in natural mating. The plants were identified by the author himself as *A. lancea* due to morphological characteristics. A clonal line of *A. lancea* was propagated from a rhizome of a single plant. Twenty-five clonal lines (line1–line25) with different genotypes were used in the present study.

2.2.2. Cultivation of *A. lancea*

The 25 clonal lines were cultivated in an experimental field located in Ami-machi, Inashiki-gun, Ibaraki prefecture (35°.99'N, 140°.20'E), Japan, in 2017. Rhizomes of the 25 clonal lines were divided into about 50 g. The rhizomes were planted in November 25, 2016 and harvested on November 23, 2017 (Figure 2.2). The cultivation was performed with 5–20 biological replicates for each clonal line. The replicates for each clonal line were as follows; line1–line17 (n = 20), line18–line24 (n = 10), line25 (n = 5).

To assess the effects of cultivation year on the contents of essential oil compounds in *A. lancea*, six clones (line1–line6) were also grown in the fields located in Ibaraki prefecture in 2016. The plants were cultivated from November 25, 2015 to November 23, 2016 in Ibaraki prefecture. The cultivation was performed with 20 biological replicates for each clonal line. The six clones were also cultivated in an experimental field located in Kyowa-town, Hokkaido prefecture (43°.01'N, 140°.53'E), Japan, in 2017, to evaluate the effects of cultivation location on the contents of essential oil compounds in *A. lancea*. Rhizomes of the plants were planted on October 21, 2016 and harvested

on October 19, 2017. The plants were cultivated with 8–20 biological replicates for each clonal line. The replicates for each clonal lines were as follows; line1 (n = 20), line2 (n = 12), line3 (n = 8), line4 (n = 20), line5 (n = 18), line6 (n = 20).

2.2.3. GC-MS analysis of essential oil compounds

The harvested rhizomes of *A. lancea* were dried in a convection oven at 50℃ for 7 days and their dry weights were determined. The dried rhizomes were pulverized using a vibrating mill (Cosmic Mechanical Technology, TI-200). The contents of four essential oil compounds, namely, β-eudesmol, hinesol, atractylon, and atractylodin, were quantified by gas chromatography-mass spectrometry (GC-MS) analysis as follows. Powdered samples of *A. lancea* rhizomes (0.5 g) were extracted with *n*-hexane (25 mL) for 15 min using a recipro shaker (Taitec, model SR-1) and centrifuged at 1660 × g for 10 min. The supernatant was separated, and the residue was re-extracted with n-hexane (20 mL) in a similar manner as above. The supernatant was combined, and phenanthrene (1.5 mg in 1 mL *n*-hexane) was added as an internal standard. A solution with a total volume of 50 mL was made by adding of *n*-hexane. An aliquot (1 μL) of the solution was injected into GC-MS. GC-MS analysis was carried out using an Agilent 7890 gas chromatograph equipped with a 5975 mass spectrum detector (MSD) (Agilent Technologies). Chromatography was performed with a DB-WAX GC column (polyethylene glycol, 30 m×250 μm i.d., 0.25 μm film; Agilent J&W Scientific). The column was used with the following temperature program: column held at 160℃ for 2 min after injection, increased by 5℃/min to 200℃, then increased by 8℃/min to 240℃, and held for 5min. Injection temperature was set to 160℃. Helium was used as the carrier gas and the flow rate was 1.0 mL/min.

The quantitative analyses of β-eudesmol, hinesol, atractylon and atractylodin were calculated on the basis of peak-area ratio to the I.S. in TIC chromatogram and regression analyses were performed. Standards of β-eudesmol (14.08 mg) and hinesol (20.16 mg), provided by Tsumura & Co. (Japan), were initially dissolved in each 10 mL *n*-hexane, and then stepwise-diluted with hexane followed by adding the I.S. (1.5 mg, in 1 mL *n*-hexane) to make series of the standard solutions ranging in concentration from 0.005 to 0.7 mg/mL of each compounds. Standard of and atractylon (4.37 mg), provided by Tsumura & Co. (Japan), was initially dissolved in 10 mL *n*-hexane, and then stepwise-diluted with *n*-hexane followed by adding the I.S. (1.5 mg, in 1 mL *n*-hexane) to make series of the standard solutions ranging in concentration from 0.001 to 0.2 mg/mL. Standard of atractyloidin (1.18 mg), provided by Tsumura & Co. (Japan), was initially dissolved in methanol, and then stepwise-diluted with *n*-hexane followed by adding the I.S. (1.5 mg, in 1 mL *n*-hexane) to make series of the standard solutions ranging in concentration from 0.0005 to 0.06 mg/mL of each compounds. The correlation coefficients for the standards of β-eudesmol, hinesol, atractylon and atractylodin were 0.99, 1.00, 0.99 and 0.99, respectively. The contents of the sesquiterpenoids were expressed based on the dry weight of the powdered sample.

2.2.4. Statistical analysis

Statistical analyses, including one-way ANOVA, two-way ANOVA, and correlation analysis (Pearson's correlation coefficient) were performed for β-eudesmol, hinesol, atractylon, and atractylodin contents using R (version 3.5.0). Broad sense heritability (h_B) was estimated from variance components in ANOVA as follows: $h_B = \sigma^2_G / (\sigma^2_G + \sigma^2_E)$, where σ^2_G = genotypic variance, σ^2_E = environmental variance (Ukai, 2002; Burton et al., 1953). Effective number of replicates (r) for the estimation of σ^2_G was calculated using the following formula: $r = (\sum_{i=1}^{a} r_i - \sum_{i=1}^{a} r_i^2 /$

$\sum_{i=1}^{a} r_i) \,/(\mathrm{a} - 1)$, where a = the number of clonal lines (Yoshida, 1998).

2.3. Results

2.3.1. Estimation of broad sense heritability for the contents of essential oil compounds and rhizome weight

To evaluate the heritability of the contents of essential oil compounds in *A. lancea* under a microenvironment, 25 clonal lines were grown in Ibaraki prefecture in 2017 and the contents of the compounds in individual plantlets were determined. Figure 2.3 illustrates the distribution of each compound in different clonal lines. In most clonal lines, the ranges of variation in the contents of β-eudesmol, hinesol, atractylon, and atractylodin were lower than varietal differences, although some lines exhibited relatively high variations among individual plantlets in the same line, for example, line20 and line23 (Figure 2.3). One-way ANOVA and the estimation of broad sense heritability of those compound contents were performed using the above results. Table 1 shows that the differences in varietal compound contents were significant ($P > 0.01$) and the heritability for the contents of β-eudesmol, hinesol, atractylon, and atractylodin were high at 0.84, 0.77, 0.87, and 0.86, respectively (Table 2.1).

The range of variation in rhizome weight was smaller than varietal differences as well as in the contents of essential oil compounds (Figure 2.4). Although the difference in varietal rhizome weight was significant, the heritability of rhizome weight was not high at 0.31 (Table 2.1).

2.3.2. Relation between rhizome yields and the contents of essential oil compounds

Correlation analyses among various compound contents and rhizome yield were performed, since there is potential linkage among different traits. According to the results, there was few correlation between rhizome yields and the contents of essential oil compounds, and the correlation coefficient between rhizome yields and contents of β-eudesmol, hinesol, atractylon and atractylodin

were 0.25, 0.35, -0.01 and 0.15 respectively (Figure 2.5). In addition, it was confirmed that a strain with high essential oil contents and high rhizome yield as in line 5 (Figure 2.5). In contrast, the correlation between β-eudesmol and hinesol contents was relatively high, although the correlations among the other compounds were comparatively low (Table 2.4).

2.3.3 Environmental conditions of cultivation years and locations

Climatic data for each cultivation years and locations were used the data issued by the Japan Meteorological Agency (JMA: Japan Meteorological Agency, 2019). Climatic conditions for the period of cultivation in Ibaraki prefecture were slightly different between 2016 and 2017. Mean temperatures in 2016 and 2017 were 14.9℃ and 15.4℃, and accumulated rainfall in 2016 and 2017 were 1243 mm and 1196 mm, respectively (Table 2.2). Environmental conditions of cultivation locations were more differed than that of cultivation years. Hokkaido is located in north of Japan, and mean temperature in Hokkaido is lower than mean temperature in Ibaraki prefecture. Mean temperatures for the period of cultivation in Hokkaido and Ibaraki prefecture were 15.4℃ and 8.3℃, respectively (Table 2.2). In addition, accumulated rainfall was differed between Hokkaido and Ibaraki prefecture. The accumulated rainfall for the period of cultivation in Hokkaido and Ibaraki prefecture was 1915 mm and 1487 mm, respectively (Table 2.2). Further, there were difference in soil type and soil texture between 2 locations. The soil type and soil texture in Ibaraki prefecture were andosol and loam, and that in Hokkaido were alluvial soil and sandy loam (Table 2.2).

2.3.4. Effects of cultivation year on contents of the essential oil compounds

To examine the effect of cultivation year on contents of the essential oil compounds, six clones of *A. lancea* were grown in the same experimental field in 2016 and 2017, and contents of the

compounds analyzed using two-way ANOVA. The effects of genotype (G), cultivation year (Y), and G × Y interaction were significant for contents of β-eudesmol, hinesol, and atractylon. However, mean squares of genotypes for each compound's contents were higher than those of cultivation year and G × Y interaction (Table 2.5). In addition, interaction plots between genotype and cultivation year for β-eudesmol, hinesol, and atractylon showed that there were low qualitative interactions (Figure 2.6 A, B, C). Atractylodin contents were not significantly influenced by cultivation year, although there were significant differences for genotypes and G × Y interactions (Table 2.5). Figure 2.6 D illustrates that qualitative interaction between genotype and cultivation year based on atractylodin contents was low, although in line5, the content levels of atractylodin in six clonal lines varied depending on cultivation year. In addition, the correlation coefficients between the two cultivation years for β-eudesmol, hinesol, atractylon, and atractylodin contents were 0.94, 0.94, 1.00, and 0.83, respectively (Figure 2.7).

2.3.5. Effects of cultivation location on the contents of essential oil compounds

To determine the effects of cultivation location on contents of the essential oil compounds, six clonal lines of *A. lancea* were cultivated in different locations. Two-way ANOVA results for β-eudesmol and hinesol contents revealed that there were significant differences in genotypes (G), locations (L), and G × L interactions. In addition, mean squares of genotype were lower than those of location (Table 2.6). However, low qualitative interactions between genotype and location for contents of β-eudesmol and hinesol were observed (Figure 2.8 A, B). In addition, correlation coefficients for contents of β-eudesmol and hinesol between the two cultivation locations were 1.00 and 0.94, respectively (Figure 2.9 A, B). Two-way ANOVA analyses of atractylon and atractylodin contents revealed that there were significant differences in genotypes, locations, and G × L

interaction. However, the mean squares of genotypes were higher than those of locations and G × L interactions (Table 2.6). Figures 2.8 C and 2.8 D show that there were low qualitative interactions between genotype and location for contents of atractylon and atractylodin. In addition, correlation analyses of the contents of atractylon and atractylodin between the two locations revealed high correlations coefficients, 1.00 and 0.94, respectively (Figure 2.9 C, D).

2.4. Discussion

In the present study, broad sense heritability for β-eudesmol, hinesol, atractylon, and atractylodin contents in *A. lancea* was high (Table 2.1), suggesting that the contents of the essential oil compounds in *A. lancea* are highly influenced by genetic factors. In addition, correlations between each compound contents and rhizome yield were low (Figure 2.5), indicating that *A. lancea* strains, which have high contents of essential oil compounds could be selectively bred irrespective of rhizome yields. Therefore, cultivars of *A. lancea* with not only high contents of essential oil compounds but also high yields could be developed through selective breeding.

Correlation coefficients among different compound contents indicated low correlations, except in the case of the correlation between β-eudesmol and hinesol contents (Table 2.4). The biosynthetic pathways of β-eudesmol and hinesol are considered potentially closely associate since the compounds have similar chemical structures with the same molecular mass (Yosioka et al., 1958). In addition, Takeda et al. (1997) observed that the content ratio of β-eudesmol to hinesol keep constant in *A. lancea* clones. The above results could be the reason why the correlation coefficient between β-eudesmol and hinesol contents was high. When selectively breeding for the contents of the compounds, that the contents of β-eudesmol and hinesol could be linked. Conversely, the contents of the other compounds could be selected independently in the course of cultivar development.

I used the six clonal lines to investigate the influence of cultivation year on the contents of the essential oil compounds. Based on the results of the two-way ANOVA analyses, mean squares of genotype for contents of the essential oil compounds were higher than the interannual variability (Table 2.5). Additionally, there were low G × Y qualitative interactions (Figure 2.6), while the correlation coefficients between cultivation years and compound contents were relatively high

(Figure 2.7). The results imply that, regardless of cultivation year, the contents of essential oil compounds in *A. lancea* clonal lines were stable, and genetic factors had greater influence than cultivation year did.

I cultivated six clones in two locations and evaluated the effects of cultivation location on the contents of essential oil compounds. The effect of cultivation location on the contents of essential oil compounds in *A. lancea* was larger than that of cultivation year, especially, the mean squares of cultivation location for β-eudesmol and hinesol contents were higher than those of genotype (Table 2.6). In general, secondary metabolites such as sesquiterpenes are induced by biological and abiotic stress *via* phytohormone signaling (Sudha and Ravishankar, 2002). In *A. lancea*, the contents of essential oil compounds are induced by phytohormones such as jasmonic acid and ABA, *via* symbiosis with endophytes (Ren and Dai, 2012; Wang et al., 2015). In addition, increased soil acidity triggers an increase in β-eudesmol contents (Yuan et al., 2009). Therefore, the influence of cultivation location on the accumulation of β-eudesmol and hinesol in the present study could be induced by plant hormones due to biological or abiotic stress. It has been reported that mean temperature in their natural habitats in China are 11.1-16.0℃, and accumulated rainfall are 850-1560 mm (Takeda et al., 1995a). In the present study, there were differences in climatic and soil conditions between the 2 cultivation locations. Mean temperatures in Ibaraki were within the range of mean temperature for the natural habitats, whereas the average temperature of Hokkaido was lower than that of the natural habitats. It is possible that the contents of essential oil compounds in *A. lancea* could be influenced by these climatic differences. Furthermore, it has been reported that soil type and soil cray content are ecological factors having the high contribution rate against atractylodin contents in *A. lancea* (Shoudong et al., 2017). In the present study, soil type and soil texture were different between the 2 cultivation locations, and the contents of the essential oil

compounds in *A. laneca* might be varied by these soil conditions. It has been reported that *A. lancea* secretes allelochemicals from their roots to rhizosphere, and the allerochemicals have autotoxic effects (Guo et al., 2006). Soil texture and type in Ibaraki prefecture were loam and volcanic ash soil, but soil texture and type in Hokkaido were sandy loam and alluvial soil (Table 2.2). In general, sandy soil is easily leaching allelochemicals from the soil compared with cray soil, and allelotoxic activity is decreased in sandy soil (Salama et al., 2014). In Hokkaido, it is possible that the autotoxic chemicals was easily leaching out from the soil and abiotic or biotic stress on the plantlets was small, so that induction of jasmonic acid and ABA were small. It is known to accumulate the essential oil compounds by phytohormones such as jasmonic acid and ABA in *A. lancea* (Ren and Dai, 2012; Wang et al., 2015). From these, it is possible that the contents of essential oil compounds such as β-eudesmol and hinesol in *A. lancea* cultivated in Hokkaido might tend to be low compared with *A. lancea* cultivated in Ibaraki prefecture. More detailed analyses on the influence of environmental factors are required, particularly on what are the major factors influencing the contents of the essential oil compounds.

Conversely, low qualitative interactions between genotype and location in the contents of β-eudesmol, hinesol, atractylon and atractylodin were observed (Figure 2.8), and correlation coefficients between 2 locations for these componds' contents were high (Figure 2.9). The results suggest that genetic potential of *A. lancea* regarding the contents of β-eudesmol, hinesol, atractylon and atractylodin are stable regardless of environmental conditions, although the contents of the compounds are influenced by environmental factors as absolute values. It has been reported that artemisinin contents in *Artemisia annua* clones grown under different cultivation conditions were highly correlated (Ferreira et al., 1995). The contents of β-eudesmol, hinesol, atractylon, and atractylodin in *A. lancea* exhibited similar results in the present study. The results indicate that the

sesquiterpenoids and polyacetylene content levels among genotypes are not influenced by environmental conditions, although their absolute values varied depending on environments. Therefore, selective breeding could be effective in controlling the contents of essential oil compounds in *A. lancea*. In addition, cultivars exhibiting wide adaptation in the contents of essential oil compounds could be developed. Recent studies have proposed a putative sesquiterpenoid biosynthetic pathway based on transcriptome analyses of different *A. lancea* tissues, and several candidate genes related to sesquiterpenoid biosynthesis have been reported (Chen et al., 2017; Huang et al., 2016). As for sesquiterpenoid biosynthesis, β-eudesmol synthase mainly expressed in rhizomes has been identified in other plants such as *Zingiber zerumbet* (Yu et al., 2008). Furthermore, high levels of SNPs (simple nucleotide polymorphisms) have been reported from leaf, stem, and root cDNA libraries of *A. lancea* (Ahmed et al., 2016). The results suggest the potential of generating genetic markers for the contents of essential oil compounds in *A. lancea* using genetic association analysis.

In conclusion, the present study demonstrates that the contents of β-eudesmol, hinesol, atractylon, and atractylodin in *A. lancea* are influenced mainly by genetic factors, and selective breeding could be an effective strategy for developing *A. lancea* populations that yield high contents of essential oil compounds. In addition, β-eudesmol, hinesol, atractylon, and atractylodin contents could be selected regardless of rhizome yields in the course of *A. lancea* cultivar development. Consequently, *A. lancea* cultivars with high rhizome yields and high contents of each the essential oil compounds could be developed. However, since the contents of the essential oil compounds are influenced by environmental condition, further investigations on the effects of environment factors on the compound contents in *A. lancea* are required.

Table 2.1 Broad-sense heritability for contents of the essential oil compounds and rhizome weight in *A. lancea*.

Essential oil compounds	Factors	Df	Mean Sq	P-value	Effective replication	Genotypic variance	Environmental variance	Broad sense heritability
β-eudesmol	Clonal lines	24	539.1	<2e-16**	16.5	32.2	6.3	0.84
	Residuals	390	6.3					
hinesol	Clonal lines	24	698.2	<2e-16**	16.5	41.5	12.7	0.77
	Residuals	390	12.7					
atractylon	Clonal lines	24	23.5	<2e-16**	16.5	1.4	0.2	0.87
	Residuals	390	0.2					
atractylodin	Clonal lines	24	6.1	<2e-16**	16.5	0.4	0.1	0.86
	Residuals	390	0.1					
rhizome weight	Clonal lines	24	767.0	<2e-16**	16.5	42.9	96.8	0.31
	Residuals	390	96.8					

Df; degree of freedom, Mean Sq; Mean square, **; Probability value for test of significance < 0.01.

Table 2.2 Environmental conditions of each cultivation years and locations.

Attribute	Cultivation location		
	Ibaraki prefecture		Hokkaido
Cultivation year	2016	2017	2017
Geographic coordinates	35°.99’N, 140°.20’E		43°.01’N, 140°.53’E
Soil type	Andosol (volcanic ash soil)		Alluvial soil
Soil texture	loam		sandy loam
Mean temperature (°C)	14.9	15.4	8.3
Maximum temperature (°C)	35.6	37.3	32.0
Minimum temperature (°C)	-4.5	-5.1	-15.6
Accumulated rainfall (mm)	1243	1196	1021
Accumulated sunshine hours (h)	2105	1915	1487

Table 2.3 Correlation matrix of β-eudesmol, hinesol, atractylon, and atractylodin contents.

	β-eudesmol	hinesol	atractylon	atractylodin
β-eudesmol	-	0.59 **	0.29 **	0.09
hinesol	-	-	-0.13 **	0.39 **
atractylon	-	-	-	-0.04
atractylodin	-	-	-	-

**; Probability value for test of significance < 0.01.

Table 2.4 Two-way ANOVA results for β-eudesmol, hinesol, atractylon, and atractylodin contents in six *A. lancea* clones grown in 2016 and 2017.

Essential oil compounds	Factors	Df	MeanSq	Fvalue	P-value
β-eudesmol	Genotype (G)	5	12.3	250.4	2.0.E-16 **
	Year (Y)	1	1.0	19.8	1.4.E-05 **
	G × Y	5	0.2	3.4	6.1.E-03 **
	Residuals	228	4.9.E-02		
hinesol	Genotype (G)	5	23.2	306.9	2.0E-16 **
	Year (Y)	1	0.3	3.6	6.1E-02 *
	G × Y	5	0.2	2.9	1.6E-02 **
	Residuals	228	0.1		
atractylon	Genotype (G)	5	0.6	812.4	2.0.E-16 **
	Year (Y)	1	0.1	88.9	2.0.E-16 **
	G × Y	5	1.4.E-02	17.5	1.1.E-14 **
	Residuals	228	8.0.E-04		
atractylodin	Genotype (G)	5	0.20	550.2	2.0E-16 **
	Year (Y)	1	0.0.E+00	5.0.E-03	0.9
	G × Y	5	2.7.E-03	7.2	2.6E-06 **
	Residuals	228	3.7.E-04		

Df; degree of freedom, Mean Sq; Mean square, *, **; Probability value for test of significance < 0.05 and < 0.01, respectively.

Table 2.5 Two-way ANOVA for β-eudesmol, hinesol, atractylon, and atractylodin contents in six *A. lancea* clones grown in Hokkaido and Ibaraki prefecture.

Essential oil compounds	Factors	Df	MeanSq	Fvalue	P-value
β-eudesmol	Genotype (G)	5	7.8	166.4	2.0E-16 **
	Location (L)	1	16.7	355.7	2.0E-16 **
	G × L	5	0.24	5.1	2.2E-04 **
	Residuals	206	0.05		
hinesol	Genotype (G)	5	9.6	122.9	2.0E-16 **
	Location (L)	1	52.8	674.5	2.0E-16 **
	G × L	5	1.6	20.2	2.3E-16 **
	Residuals	206	0.1		
atractylon	Genotype (G)	5	0.8	699.7	2.0.E-16 **
	Location (L)	1	0.2	192.4	2.0.E-16 **
	G × L	5	1.2.E-02	9.8	1.9.E-08 **
	Residuals	206	1.2.E-03		
atractylodin	Genotype (G)	5	0.16	381	2.0E-16 **
	Location (L)	1	0.013	32	4.6E-08 **
	G × L	5	0.0025	5.9	3.9E-05 **
	Residuals	206	0.00042		

Df; degree of freedom, Mean Sq; Mean square, **; Probability value for test of significance < 0.01.

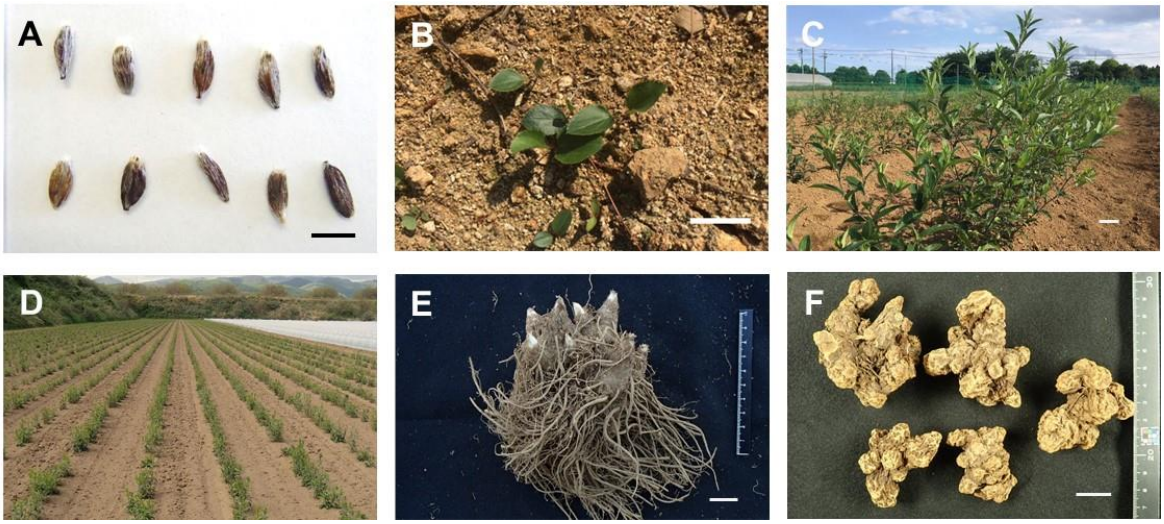

Figure 2.1 Plant characteristics and cultivation of *A. lancea*.

(A) Seeds of *A. lancea*, (B) *A. lancea* seedlings for 2 month after germination, (C) Shoots of *A. lancea* which is grown from rhizomes, (D) Cultivation of *A. lancea*, (E) The rhizome and root of *A. lancea* which is grown for 1 year from a divided rhizome, (F) The crude drugs of *A. lancea* which is dried from harvested rhizomes. (Scale bars = 1 cm)

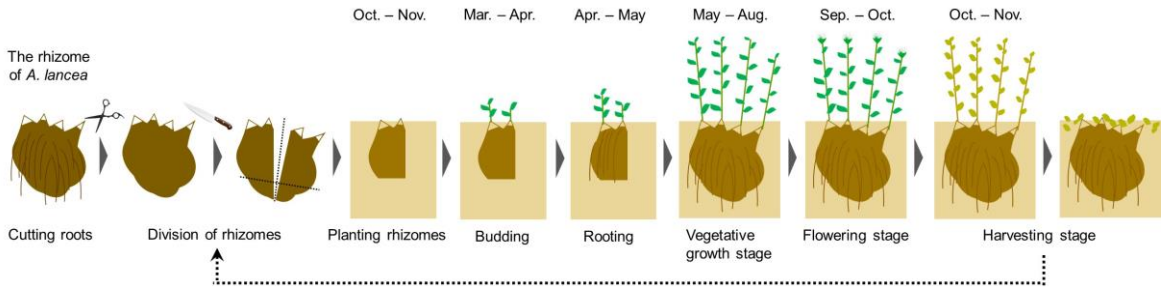

Figure 2.2 Image of cultivation cycle of *A. lancea* by division of rhizomes.

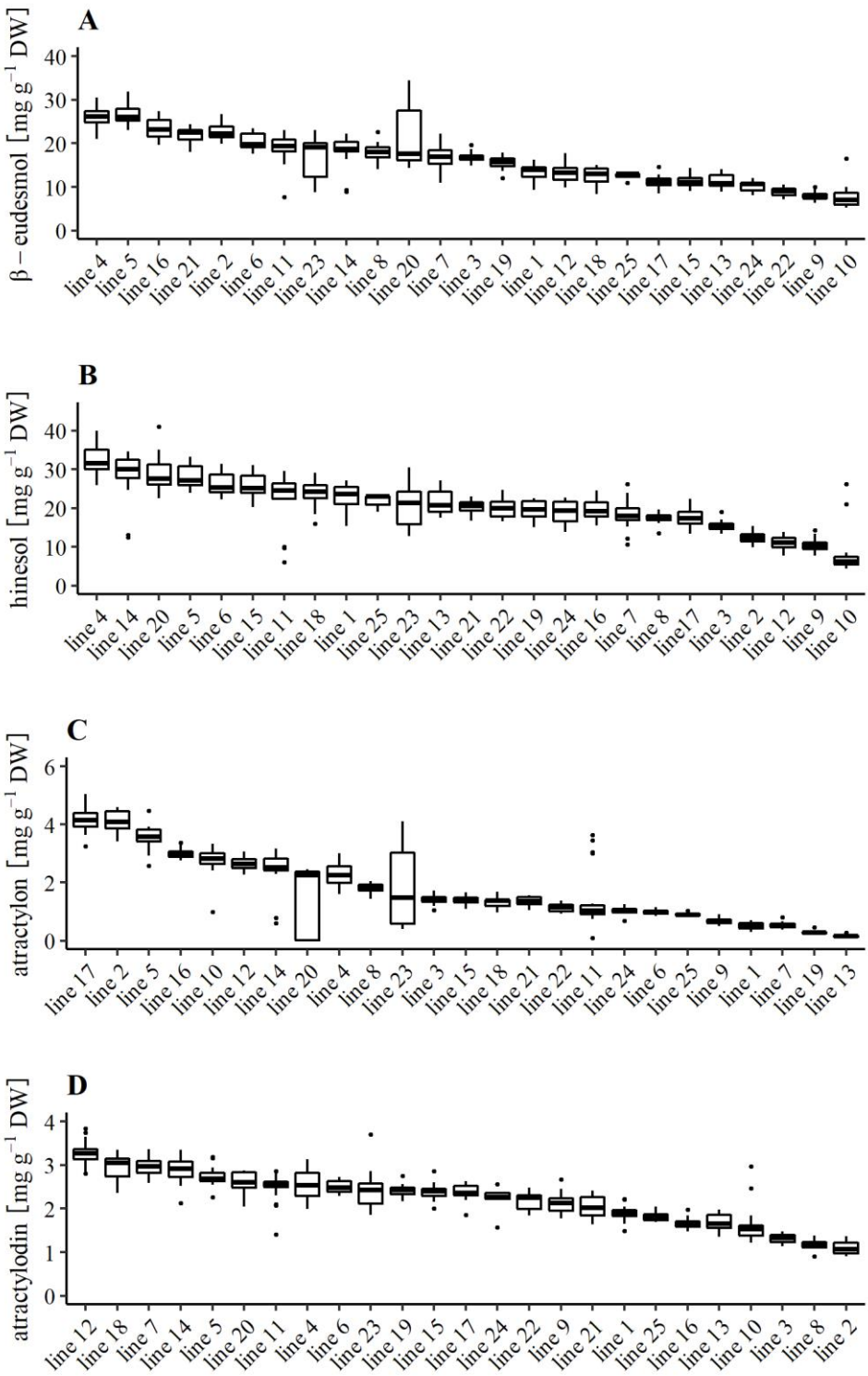

Figure 2.3 Range of variations for contents of the essential oil compounds in *A. lancea*.

The boxes represent the values from the 25th to 75th percentile. The middle lines represent the median. The vertical lines extend from the minimum to the maximum values. Biological replicates within each clonal lines were as follows: line 1–line 17 (n = 20), line 18–line 24 (n = 10), line 25 (n = 5). A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents.

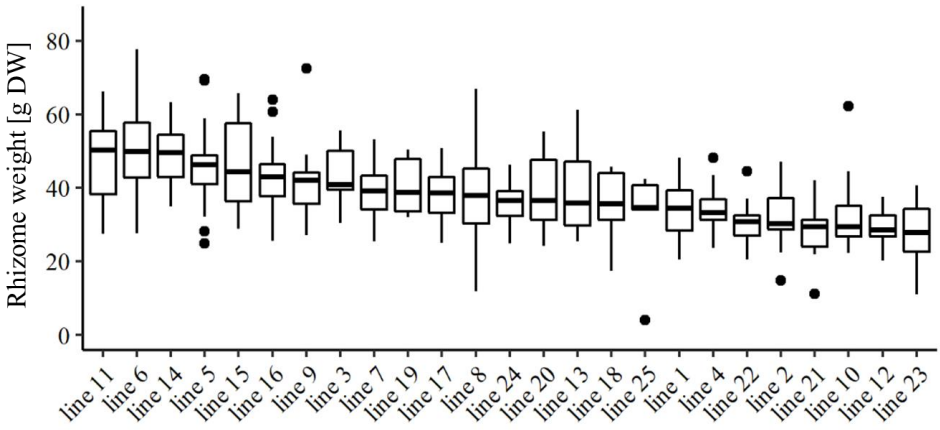

Figure 2.4 Range of variation of rhizome weight in *A. lancea*.

The boxes represent the values from the 25th to 75th percentile. The middle lines represent the median. The vertical lines extend from the minimum to the maximum values. Biological replicates within each clonal lines were as follows: line 1–line 17 (n = 20), line 18–line 24 (n = 10), line 25 (n = 5).

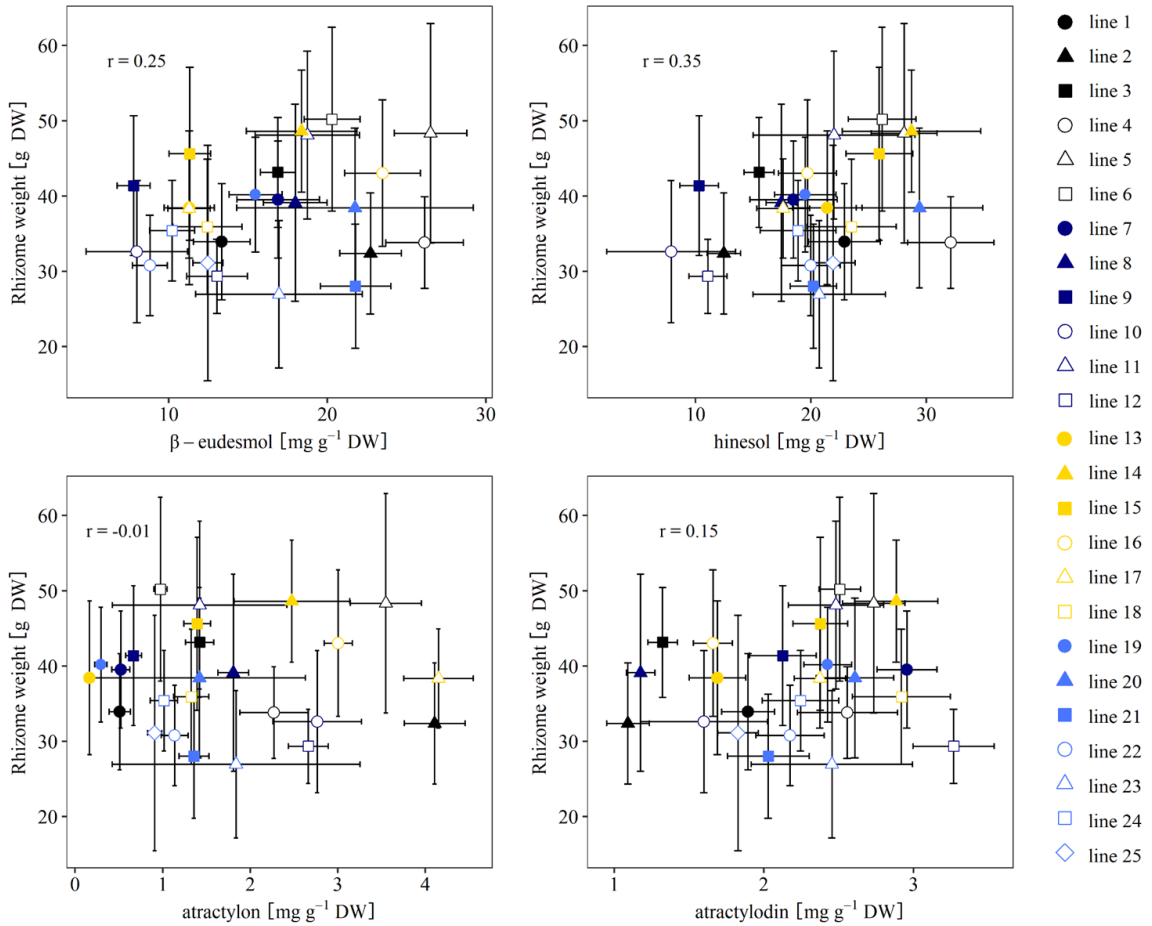

Figure 2.5 Relationships between contents of the essential oil compounds and rhizome weight in *A. lancea.*

A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents. The data are presented as means ± SD.

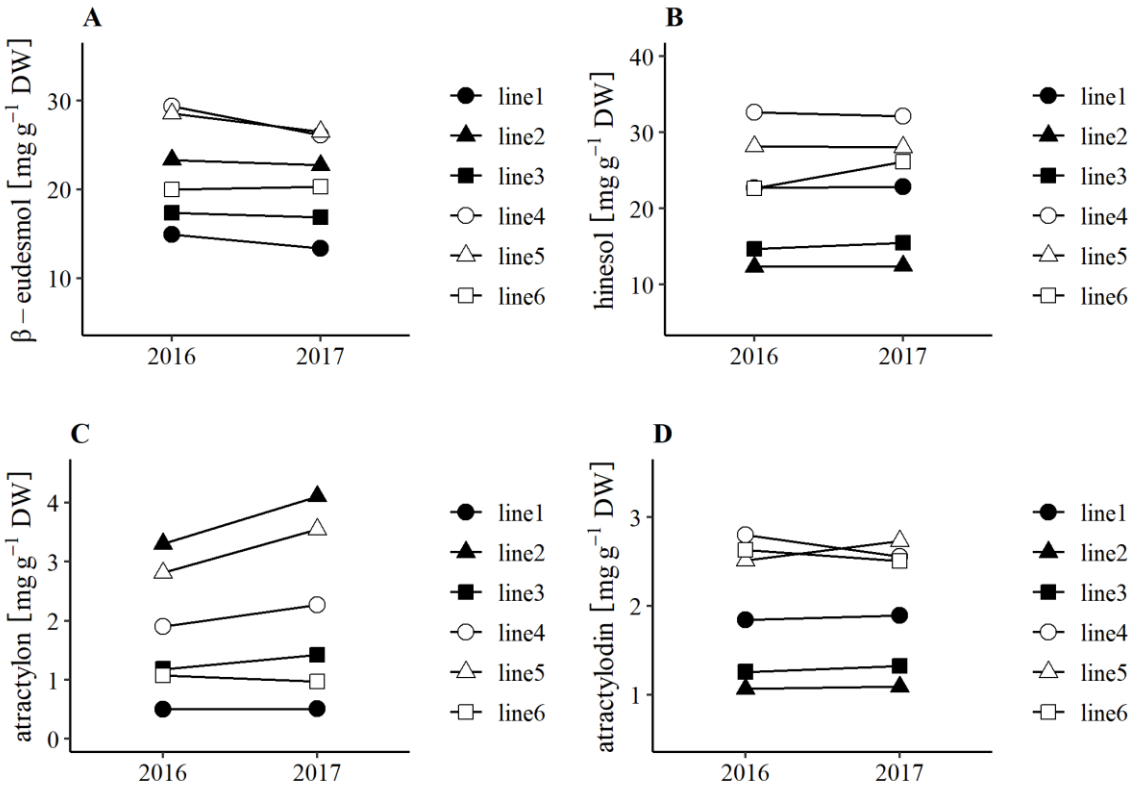

Figure 2.6 Interaction plots for interannual variability of the essential oil compound contents in *A. lancea*.

Each point represents the mean of 20 measurements within clonal lines each in 2016 and 2017. A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents.

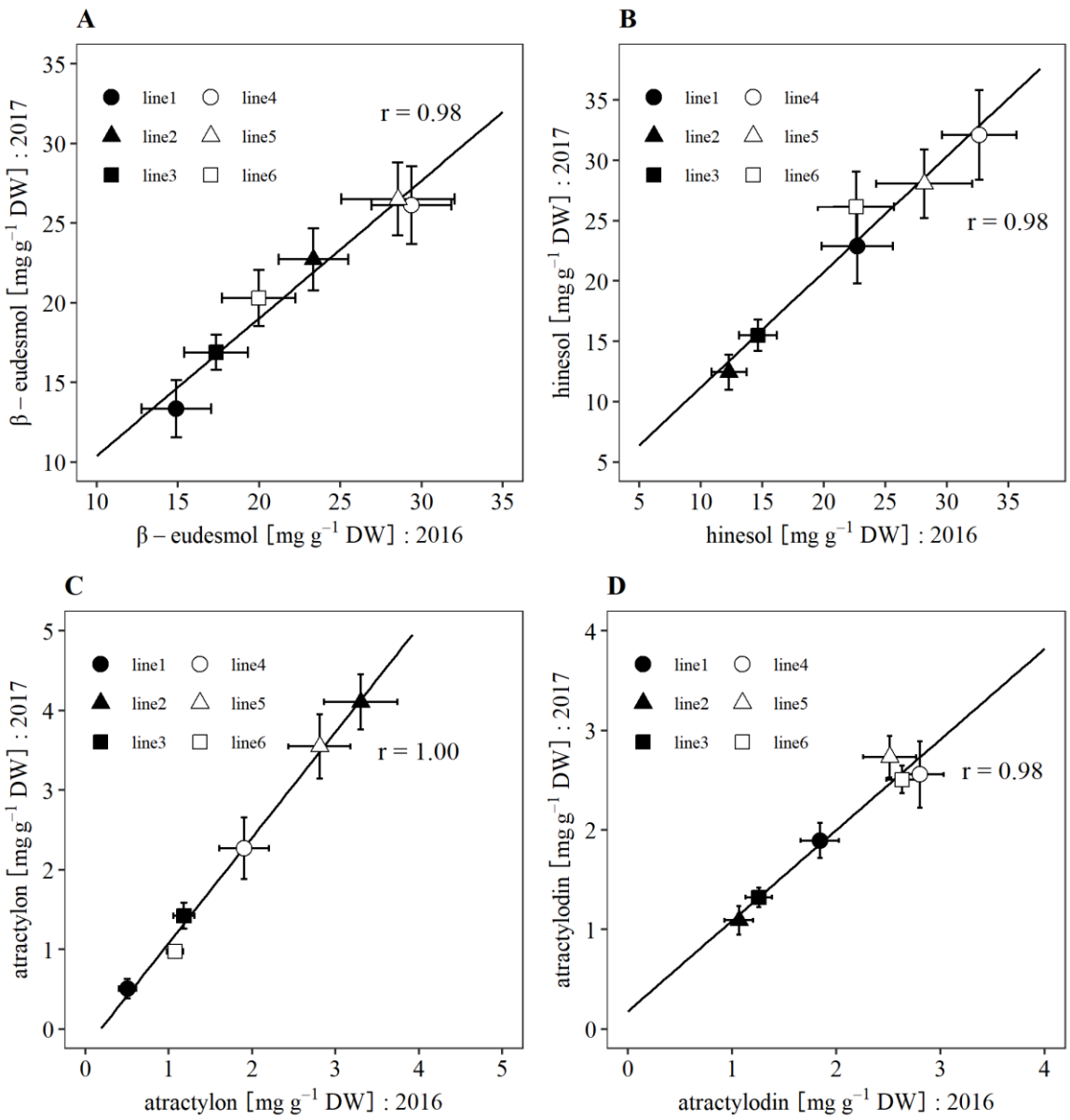

Figure 2.7 Relationships between contents of the essential oil compounds from six *A. lancea* clones grown in 2016 and 2017. Each point represents the mean of 20 measurements each in 2016 and 2017. A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents.

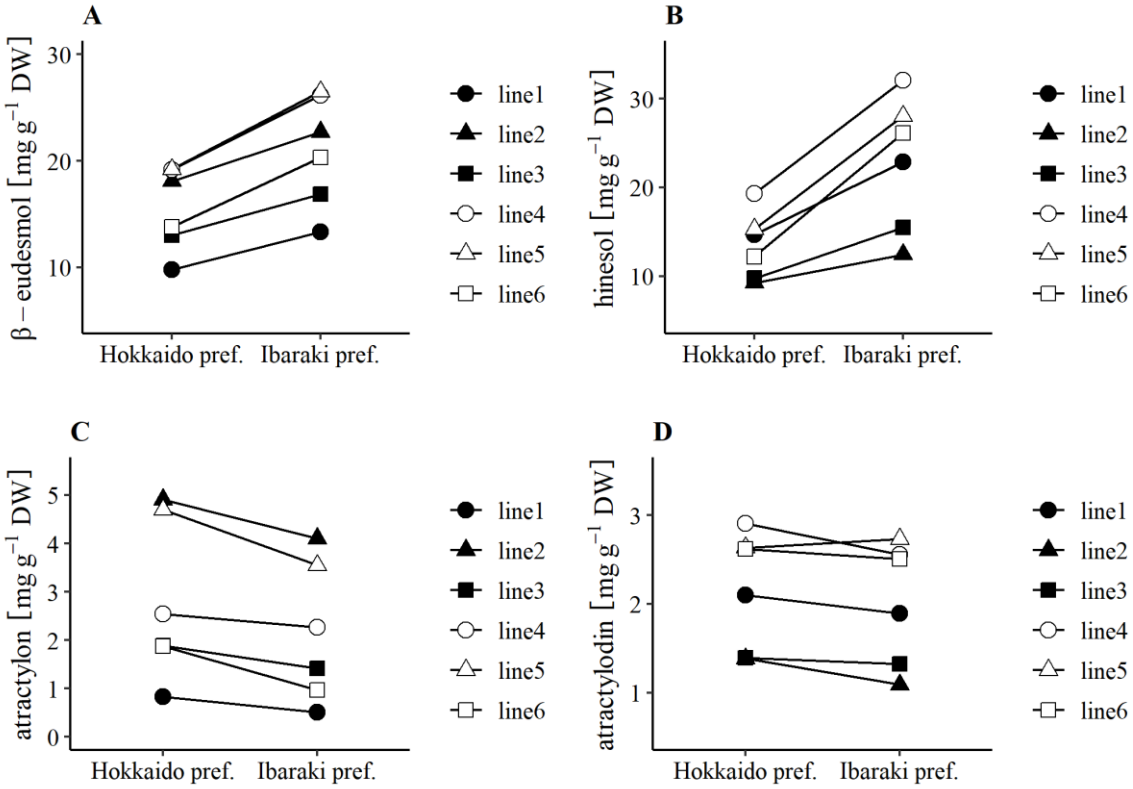

Figure 2.8 Interaction plots for contents of the essential oil compounds in six *A. lancea* clones grown in Hokkaido and Ibaraki prefectures. Data represent mean values of each compounds' contents in *A. lancea* (n = 20 in line 1, n = 12 in line 2, n = 8 in line 3, n = 20 in line 4, n = 18 in line 5, n = 20 in line 6). A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents.

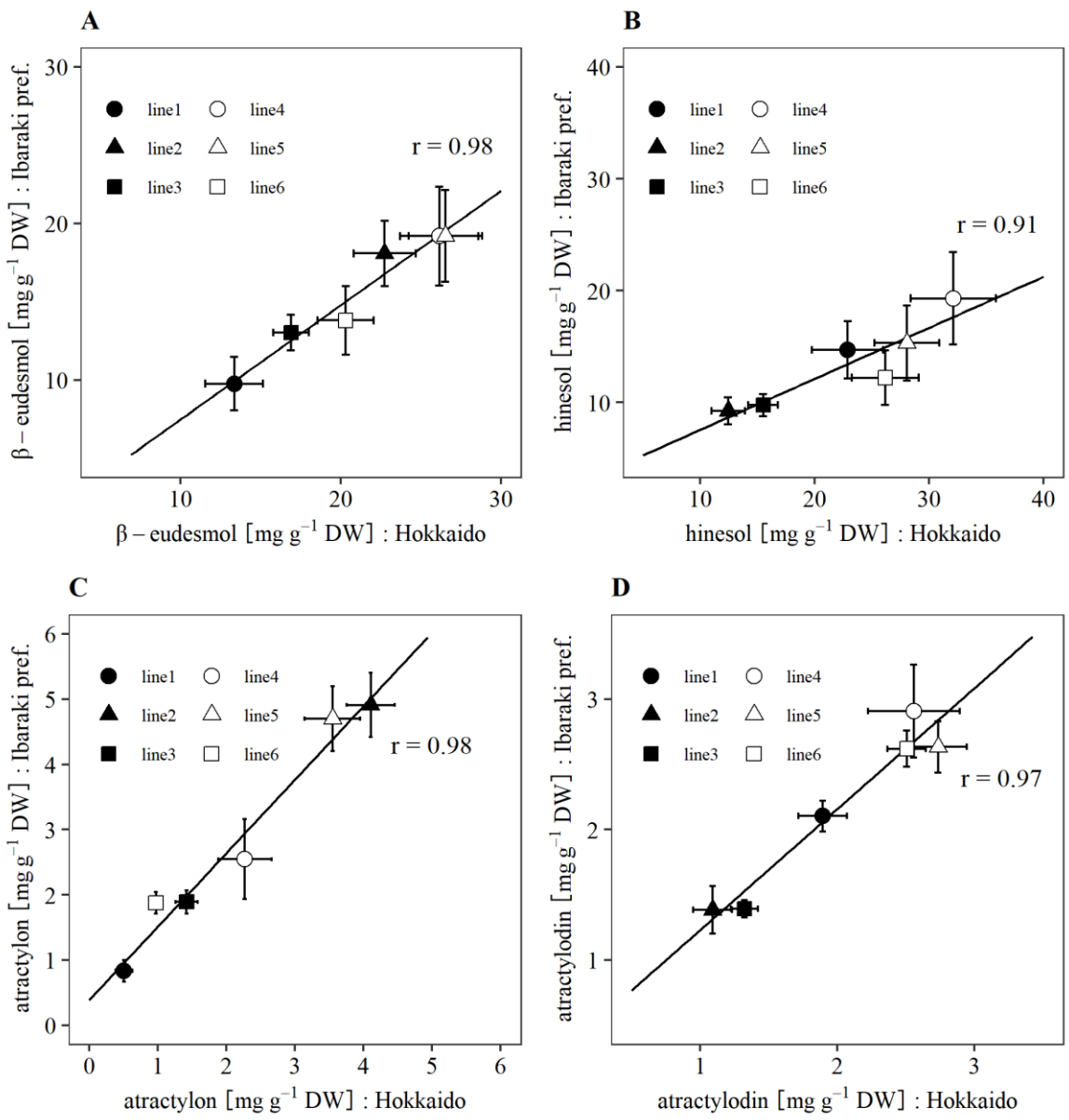

Figure 2.9 Relation for contents of the essential oil compounds from six *A. lancea* clones grown in Hokkaido and Ibaraki prefecture. Data represent means ± SD (n = 20 in line 1, n = 12 in line 2, n = 8 in line 3, n = 20 in line 4, n = 18 in line 5, n = 20 in line 6). A; β-eudesmol contents, B; hinesol contents, C; atractylon contents, D; atractylodin contents.

Chapter 3

Evaluation of heritability of β-eudesmol/hinesol content ratio in *Atractylodes lancea*

3.1. Introduction

Dried rhizome of *A. lancea* has been used as an important crude drug in traditional Chinese and Japanese medicines (Shiba et al., 2006). In these medicines, various decoctions containing the crude drug have been used for a treatment of digestive disorders and body fluid imbalance (Kubo et al., 1983; Wang et al., 2008). In modern pharmacological studies, the major active compounds obtained from *A. lancea* rhizomes have been shown to exhibit pharmacological activities on nervous, cardiovascular, and gastrointestinal systems (Koonrungsesomboon et al., 2014). Additionally, their anticancer, anti-inflammatory, and antimicrobial activities have also been reported (Koonrungsesomboon et al., 2014). Major active compounds in *A. lancea* rhizome are sesquiterpenoids such as β-eudesmol and hinesol, which have closely related chemical structures from each other (Yosioka et al., 1958). Pharmacological activities of these two compounds are resembled, however intensity of pharmacological activity and action mechanism may be different (Nogami et al., 1986). For instance, both of β-eudesmol and hinesol have mitigation effects against gastric ulcer, but their pharmacological action mechanisms are different (Nogami et al., 1986; Satoh et al., 1992; Satoh et al., 2000). Additionally, hinesol has activity to induce apoptosis, suggesting the possibility that hinesol may be useful anticancer drug, while, it is weak pharmacological activity in β-eudesmol (Masuda et al., 2015). In recent years, the demand for mass production of the crude drug with a stable quality has increased (Zhang et al., 2015). Therefore, keeping the contents of β-eudesmol and hinesol constant in *A. lancea* rhizomes is important to stabilize its pharmacological activity (Chen et al., 2017).

In the classical medicine texts, especially in Japan, it has also been mentioned that the crude drug highly suited for medicinal use deposits white cotton-like crystals on a section or epidermis of the dried rhizome (Takeda et al., 1998). Most of the crystal is comprised of β-eudesmol and hinesol

as major constituents (Yosioka et al., 1958). In addition, the formation of the crystal has been shown to be dependent on not only their high absolute contents but also an equivalent ratio of their contents in the dried rhizome (Takeda et al., 1995a). It has been estimated that the crystal tend to deposit on *A. lancea* rhizomes when the β-eudesmol/hinesol content ratio is lower than 1 (Takeda et al., 1995b). Consequently, the β-eudesmol/hinesol content ratio is also assumed as an important quality criteria of *A. lancea* rhizomes as the crude drug suited for medicinal use (Takeda et al., 1995a; Takeda et al., 1996).

The quality of *A. lancea* rhizome is closely related to environment of its natural habitats, and β-eudesmol and hinesol content vary across geographical regions (Takeda et al., 1995a; Zhen et al., 2012). I demonstrated that β-eudesmol and hinesol content are strongly influenced by genetic factors in the chapter 1 of this study. In addition, Takeda *et al.* (1996) suggested that geographical differences in terms of the β-eudesmol/hinesol content ratio are mainly caused by genetic differences. Hence, detailed genetic analyses of broad-sense heritability, genotype–environment (G × E) interactions, and the effects of environmental factors on the β-eudesmol/hinesol content ratio are warranted.

In this study, I analyzed data from the chapter 1 on the β-eudesmol/hinesol content ratio in *A. lancea* rhizomes and investigated the heritability of this ratio. In particular, a total of 25 clonal lines of *A. lancea* were grown in an experimental field, and broad-sense heritability of the β-eudesmol/hinesol content ratio was estimated. Additionally, to investigate stabilities of the traits in annual variability and cultivation locations, I evaluated G × E interactions between genotype and cultivation year or location. Six clonal lines were grown under different years and locations, and two-way ANOVA and correlation analysis between different cultivation years or locations on the β-eudesmol/hinesol content ratio were performed. In the present study, I attempted to determine the

relative effects of genetic factors and environmental factors on the β-eudesmol/hinesol content ratio in *A. lancea*.

3.2. Materials and Methods

3.2.1. Plant materials

Twenty-five *A. lancea* clones (lines 1–25) were propagated from rhizomes of a single plant as described in the chapter 1 of this study. In brief, original seed of *A. lancea* were obtained from another previous study (Takeda et al., 1996). These seeds were originated from China. According to morphological characteristics, the plants were identified as *A. lancea* by the first author. Voucher specimens (THS33885) were deposited at the herbarium stock room of the Botanical Raw Materials Research Laboratories, Tsumura & Co. Japan.

3. 2. 2. Cultivation of *A. lancea*

Plants were cultivated following the method described in Chapter 1. To estimate the broad-sense heritability of the β-eudesmol/hinesol content ratio in *A. lancea* rhizomes, 25 clonal lines were grown in an experimental field in Ami Town, Inashiki-gun, Ibaraki Prefecture (35°.99'N, 140°.20'E), Japan, in 2017. Rhizomes of these *A. lancea* clonal lines were cut into 50-g sections; planted on November 25, 2016; and harvested on November 23, 2017. The number of biological replicate plants for each clonal line was as follows: lines 1–17 (n = 20), lines 18–24 (n = 10), line 25 (n = 5).

To evaluate G × E interaction and the effects of environmental factors, 6 clonal lines were grown in different years and in different locations. To examine the effects of cultivation year, 6 clonal lines (lines 1–6) were cultivated in 2016 in an experimental field located in Ibaraki Prefecture. I planted the six clones on November 25, 2015 and harvested them on November 23, 2016. These experiments were performed with 20 biological replicates for each clonal line. In addition, to evaluate the effects of location, six clonal lines were grown in 2017 in another experimental field at Kyowa-Town, Hokkaido (43°.01'N, 140°.53'E), Japan. Rhizomes of the 6 clonal lines were divided into 50 g

sections and I cultivated the 6 clonal lines from October 21, 2016, to October 19, 2017, in Hokkaido. This experiment was performed with 8–20 biological replicates for each clonal line as follows: line 1 (n = 20), line 2 (n = 12), line 3 (n = 8), line 4 (n = 20), line 5 (n = 18), and line 6 (n = 20).

3.2.3. Extraction of sesquiterpenoids and Gas chromatography-mass spectrometry (GC-MS) analysis

β-Eudesmol and hinesol content in *A. lancea* rhizomes were determined as described in the chapter 1. The *A. lancea* rhizomes were dried in a convection drying oven (RY-120HG, ALP Co., Ltd., Japan) at 50 °C for 7 days and pulverized using a vibrating rod mill (TI-200, Cosmic Mechanical Technology, Co. Ltd., Japan) for GC-MS analysis. The powder samples of *A. lancea* rhizomes were accurately weighed at 0.5 g, and extracted with *n*-hexane (25 mL) using a recipro shacker (SR-1, TAITEC Co., Japan) for 15 min, followed by centrifugation (1660 × *g*, 10 min). After collection of the supernatant, the residues were re-extracted with *n*-hexane (20 mL) in a same manner. An internal standard (I.S.), phenanthrene (1.5 mg, 1 mL in *n*-hexane), was added to the combined supernatants in 50 ml-volumetric flask, and the solutions were made up by adding *n*-hexane to a total volume of 50 mL. The analyses were conducted using an Agilent 7890A gas chromatograph (GC) coupled to a 5975C mass spectrometer (MS) (Agilent Technologies, Palo Alto, CA, USA). Sample solutions (1 μL) were injected into a DB-WAX capillary column (polyethylene glycol, 30 m × 250 μm *i.d.*, 0.25 μm film thickness; Agilent J&W Scientific, Folsom, CA) in a split ratio of 50:1. Helium was used as the carrier gas at a flow rate of 1 mL /min. The injector temperature was set at 240°C. The column oven temperature was initially held at 160 ºC for 2 min after injection, and programmed to increase from 160 to 200 ºC at a rate of 5 ºC /min, then increase from 200 to 240 ºC at a rate of 8 ºC /min, and hold at 240 ºC for 5 min. The interface temperature was set at

240 °C. The MS was operated in an electron impact ionization at 70 eV and the temperatures of the ion source and the quadrupole mass spectrometer were set at 230 °C and 150 °C, respectively. Total ion current (TIC) chromatograms were acquired in a mass range of 40-500 amu using an Agilent MSD Chemstation software (version E.02.00.493).

The quantitative analyses of β-eudesmol and hinesol were calculated on the basis of peak-area ratio to the I.S. in TIC chromatogram and regression analyses were performed. Standards of β-eudesmol (14.08 mg) and hinesol (20.16 mg), provided by Tsumura & Co. (Japan), were initially dissolved in each 10 mL hexane, and then stepwise-diluted with hexane followed by adding the I.S. (1.5 mg, in 1 mL *n*-hexane) to make series of the standard solutions ranging in concentration from 0.005 to 0.7 mg/mL of each compounds. The correlation coefficients for the standards of β-eudesmol and hinesol were 0.99 and 1.00, respectively. The contents of the sesquiterpenoids were expressed based on the dry weight of the powdered sample.

3.2.4. Statistical analysis

Statistical analyses were performed using R (version 3.5.0). To evaluate the effects of genetic factors on the β-eudesmol/hinesol content ratio, one-way ANOVA was performed using the data of *A. laneca* clonal line grown in an experimental field. Broad-sense heritability (h_B) was calculated from variance components in ANOVA as follows: $h_B = \sigma^2_G / (\sigma^2_G + \sigma^2_E)$, where σ^2_G is genotypic variance and σ^2_E is environmental variance (Ukai, 2002; Burton and Devane, 1953). Effective numbers of replicates (r) for estimating σ^2_G were calculated as follows: $r = (\sum_{i=1}^{a} r_i - \sum_{i=1}^{a} r_i^2 / \sum_{i=1}^{a} r_i) / (\mathrm{a} - 1)$, where a is the number of clonal lines (Yoshida, 1998).

To assess G × E interaction and the effects of environmental factors, I performed analyses of the β-eudesmol/hinesol content ratio with two-way ANOVA and Pearson's correlation tests as well as

compared *A. lancea* grown in different years and at different locations. Further, broad-sense heritability was estimated from two-way ANOVA values using the following formula: $h_B = \sigma^2_G / [\sigma^2_G + (\sigma^2_{G\times E} / e) + (\sigma^2_E / re)]$, where $\sigma^2_{G\times E}$ is the G × E interaction variance and e is the number of experiments (cultivation year or location) (Toker, 2004).

3.3. Results

3.3.1. Estimation of broad sense heritability on the β-eudesmol/hinesol content ratio

In order to evaluate the heritability of the β-eudesmol/hinesol content ratio in *A. lancea*, twenty-five clonal lines were cultivated in an experimental field located in Ibaraki Prefecture (Japan), and the contents of the compounds were determined. Figure 3.2 shows that the results of correlation analysis between β-eudesmol and hinesol content in each clonal line. The range of β-eudesmol contents in all clonal lines were 5.3－34.5 mg/g, and the range of hinesol contents were 4.4－41.1 mg/g. As seen, β-eudesmol and hinesol content are significantly and positively correlated, with high r-values of 0.73－0.99 in all clonal lines, suggesting β-eudesmol/hinesol content ratio is stable within a clonal line.

Figure 3.3 shows variations in the β-eudesmol/hinesol content ratio across different *A. lancea* clonal lines. The ranges of variation for the β-eudesmol/hinesol content ratio within an *A. lacnea* clonal line were smaller than varietal differences. One-way analysis of variance (ANOVA) identified significant differences ($P > 0.01$) in the β-eudesmol/hinesol content ratio among the *A. lancea* clonal lines (Table 3.1). From the ANOVA result, the broad-sense heritability of the β-eudesmol/hinesol content ratio was also revealed to be high at 0.92 (Table 3.1).

3.3.2. Effects of cultivation year on the β-eudesmol/hinesol content ratio

To determine the effects of cultivation year on the β-eudesmol/hinesol content ratio in *A. lancea* rhizome, 6 clonal lines were grown and analyzed in 2016 and 2017 under the same experimental field located in Ibaraki Prefecture. In two-way ANOVA of the β-eudesmol/hinesol content ratio, the effects of genotype (G) and cultivation year (Y) were significant, while mean square value for cultivation year was lower than that for genotype (Table 3.2). No significant differences in G × Y

interactions were identified (Table 3.2). In addition, the broad-sense heritability of the β-eudesmol/hinesol content ratio, which was calculated using variance components from two-way ANOVA, was high at 1.00 (Table 3.2). Few qualitative interactions were observed between genotype and cultivation year (Figure 3.4), and the correlation between the two cultivation years on the β-eudesmol/hinesol content ratio was high (r = 1.00; Figure 3.5).

3.3.3. Effects of cultivation location on the β-eudesmol/hinesol content ratio

In order to evaluate the effect of cultivation location on the β-eudesmol/hinesol content ratio, 6 clonal lines were grown at two different locations. Two-way ANOVA of the β-eudesmol/hinesol content ratio identified significant differences for the cultivation location in terms of genotypes (G), cultivation locations (L), and G × L interaction; however, mean square for cultivation location and G × L interaction were smaller than that for genotype (Table 3.3). Additionally, broad-sense heritability of the β-eudesmol/hinesol content ratio was high at 0.98 in two-way ANOVA of variance components (Table 3.3). Furthermore, a minimal qualitative interaction was observed between genotype and cultivation location (Figure 3.6), and the correlation coefficient for the β-eudesmol/hinesol content ratio between the two cultivation locations was 0.97 (Figure 3.7).

3.4. Discussion

In this study, β-eudesmol content was positively correlated with hinesol content in all *A. lancea* clonal lines (Figure 3.2). Takeda et al. (1996) similarly showed a strong correlation between β-eudesmol and hinesol content in *A. lancea* clonal lines. Additionally, variations of the β-eudesmol/hinesol content ratio within an *A. lancea* clonal line were smaller than those among varietals (Figure 3.3), and broad-sense heritability of the β-eudesmol/hinesol content ratio was high (Table 3.1), suggesting strong effects of genetic factors. These data indicate that selective breeding is an effective strategy for stabilizing the β-eudesmol/hinesol content ratio.

In analyses of the β-eudesmol/hinesol content ratio, I found no interactions between genotype and cultivation year, and mean square for genotype was higher than that for cultivation year (Table 3.2). In addition, I demonstrated high broad-sense heritability of the content ratio (Table 3.2), and a high positive correlation between the two cultivation years (Figure 3.5). These results indicate that proportion of genetic variation in total variance is higher than annual variation, and the β-eudesmol/hinesol content ratio remains stable irrespective of interannual differences in environmental conditions.

The two-way ANOVA result for the comparative study of cultivation location identified significant differences in not only genotypes but also cultivation locations and G × L interaction (Table 3.3). Also, variations of cultivation location and G × L interaction were higher than that of cultivation years and G × Y interaction (Table 3.3). These results might be caused by variation of environmental conditions such as average temperature and soil conditions. The environmental conditions of two cultivation locations were more varied than that of two cultivation years (Table 2.2). However, the broad-sense heritability and correlation coefficient between the two cultivation locations was exhibited high-values (Table 3.3 and Figure 3.7), suggesting that the ratio of genetic

variation in total variance is relatively larger than that of the environmental variation (cultivation location). These data indicate that the β-eudesmol/hinesol content ratio has wide adaptability in *A. lancea*.

In Chapter 1 of study, I determined the effects of environmental factors on β-eudesmol and hinesol content. I showed that the broad-sense heritability of this ratio was higher than that of absolute β-eudesmol and hinesol content, and that the effects of cultivation year and location on the β-eudesmol/hinesol content ratio were smaller than on β-eudesmol and hinesol content (Table 3.1, Table 3.2, Table 3.3).

In general, sesquiterpenoids are induced by plant hormones produced in response to biotic and abiotic stresses (Sudha and Ravishankar, 2002). In *A. lancea*, β-eudesmol and hinesol production is reportedly induced by plant hormones, such as jasmonic acid and absisic acid, through symbiosis with endophytes (Ren and Dai, 2012; Wang et al., 2015). Soil acidity was shown to induce β-eudesmol accumulation in a previous study on *A. lancea* rhizomes (Yuan et al., 2009). These investigations show that absolute β-eudesmol and hinesol content vary with environmental factors. In contrast, I showed limited effects of environmental factors on the β-eudesmol/hinesol content ratio, corresponding to high broad-sense heritability of this ratio. β-Eudesmol and hinesol have the same chemical structure and molecular weight; therefore they likely have closely related biosynthetic pathways (Yosioka et al., 1958). Hence, it is possible that the β-eudesmol/hinesol content ratio is genetically controlled in *A. lancea*. Recent transcriptome analyses of *A. lancea* identified several candidate genes related to sesquiterpenoid biosynthesis (Chen et al., 2017; Huang et al., 2016). Additionally, high levels of single- nucleotide polymorphisms have been detected in cDNA libraries of *A. lancea* leaf, stem, and root tissues (Ahmed et al., 2016). These findings highlight the possibility of developing a marker-assisted selection strategy based on the β-

eudesmol/hinesol content ratio using genetic association analysis.

Herein, I indicate that the β-eudesmol/hinesol content ratio in *A. lancea* rhizomes is highly dependent on genetic factors and suggest that clonal propagation is effective for stabilizing this ratio in *A. lancea*. *A. lancea* tissues have been propagated *in vitro*, particularly tip tissues have been cultured extensively (Hiraoka et al., 1984). In particular, Shoyama et al. indicated that 6-benzylaminopurine facilitates shoot propagation, and gibberellin stimulates root enlargement in *A. lancea* (Shoyama et al., 1987). Thus, *A. lancea* strains with a stable β-eudesmol/hinesol content ratio could be propagated through mass propagation.

In conclusion, I demonstrate that the heritability of β-eudesmol/hinesol content ratio is high and that the effects of genetic factors were stronger than that of environmental factors such as cultivation location and year. My findings suggest that selective breeding and clonal propagation are effective strategies for the production of *A. lancea* with stable qualities for use in the production of crude drugs. Further molecular biological analyses such as isplation of genes related to biosynthesis of sesquiterpenoids such as β-eudesmol and hinesol are warranted.

Table 3.1 Broad-sense heritability of the β-eudesmol/hinesol content ratio in *A. lancea*.

Factors	Df	Mean Sq	P-value	Effective replication	Genotypic variance	Environmental variance	Broad sense heritability
Clonal lines	24	1.7	<2e-16**	16.3	0.1	0.0	0.92
Residuals	390	0.0					

Df, degree of freedom; Mean Sq, Mean square; **P < 0.01.

Table 3.2 Two-way ANOVA of the β-eudesmol/hinesol content ratio in six *A. lancea c*lonal lines grown in 2016 and 2017.

Factors	Df	MeanSq	P-value	Variance components	Broad sense heritability
Genotype (G)	5	7.6	<2e-16**	0.19	1.00
Year (Y)	1	0.4	<2e-16**		
G × Y	5	0.0	0.70	-0.0001	
Residuals	228	4.0.E-03		0.004	

Df, degree of freedom; Mean Sq, Mean square; **P < 0.01.

Table 3.3 Two-way ANOVA of the β-eudesmol/hinesol content ratio in six *A. lancea* clonal lines grown in Hokkaido and Ibaraki prefectures.

Factors	Df	MeanSq	P-value	Variance components	Broad sense heritability
Genotype (G)	5	6.4	<2e-16**	0.17	0.98
Location (L)	1	1.2	<2e-16**		
G × L	5	0.12	<2e-16**	0.01	
Residuals	206	0.01		0.01	

Df, degree of freedom; Mean Sq, Mean square; **P < 0.01.

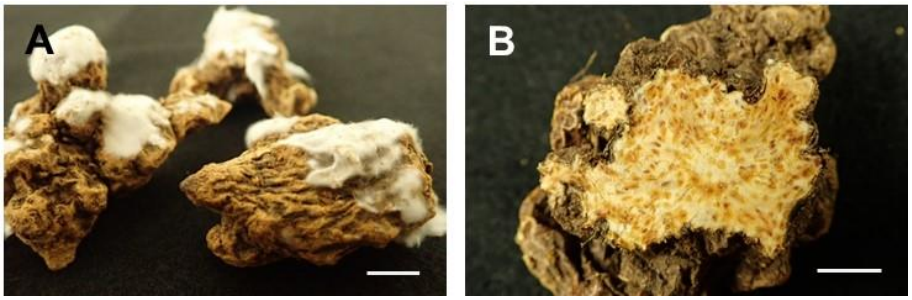

Figure 3.1 The cotton-like crystal of *A. lancea* (A) and oil sac distributed in section of *A. lancea* rhizome (B). (Scale bars = 1 cm)

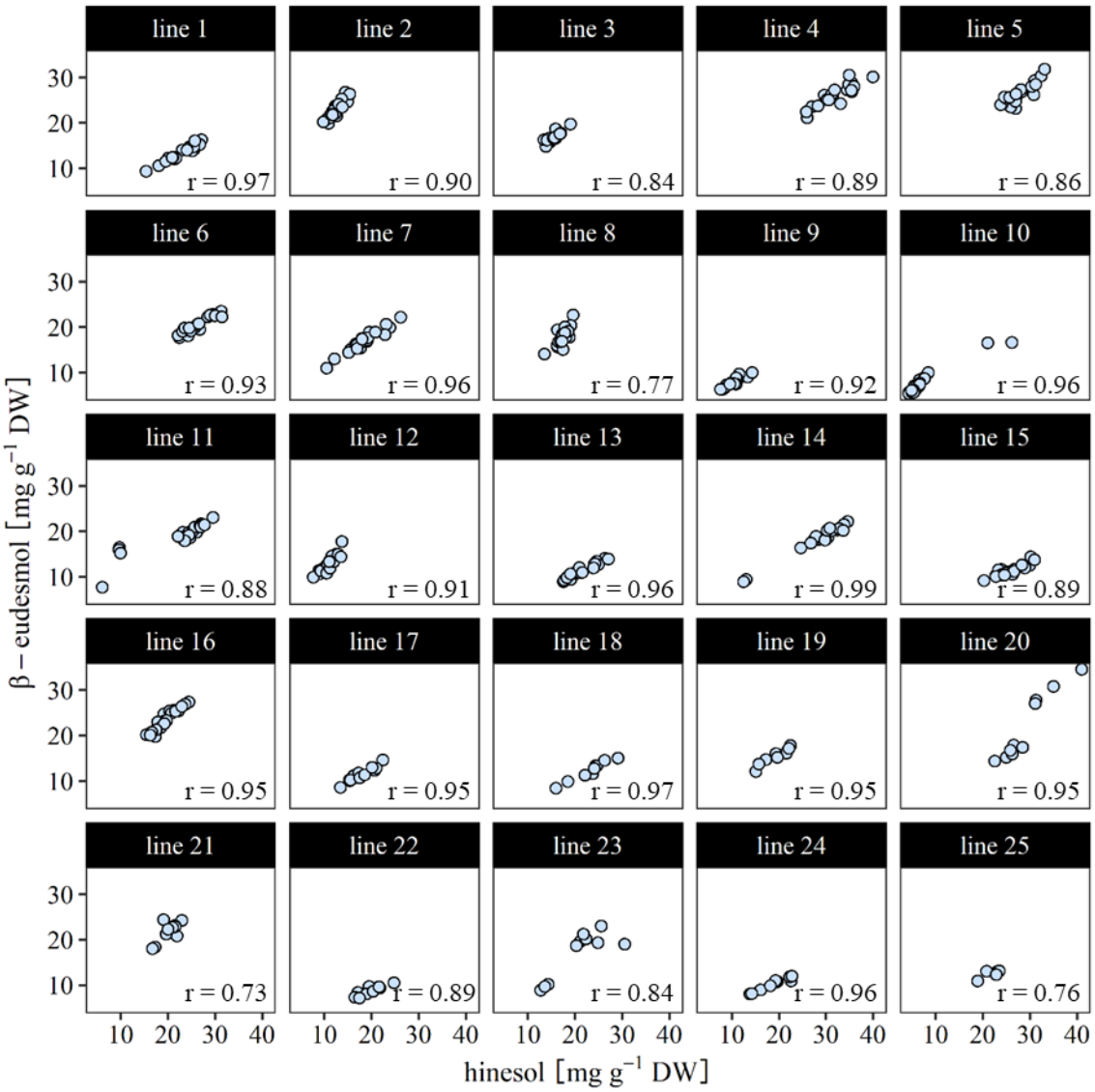

Figure 3.2 Correlation between β-eudesmol and hinesol contents in *A. lancea* clonal lines. Pearson's correlation coefficients (r) were calculated for each clonal line. The number of replicates for each clone is as follows; lines 1–17 (n = 20), lines 18–24 (n = 10), and line 25 (n = 5).

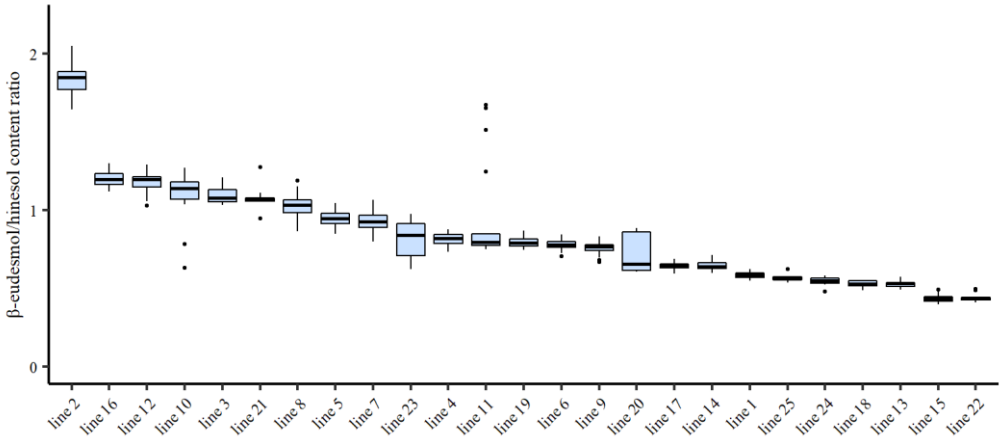

Figure 3.3 Range of variations in the β-eudesmol/hinesol content ratio in *A. lancea*.

Boxes represent 25th–75th percentiles and middle lines represent medians. The vertical lines extend from minimum to maximum values. The numbers of biological replicates for each clonal line is as follows: lines 1–17 (n = 20), lines 18–24 (n = 10), and line 25 (n = 5).

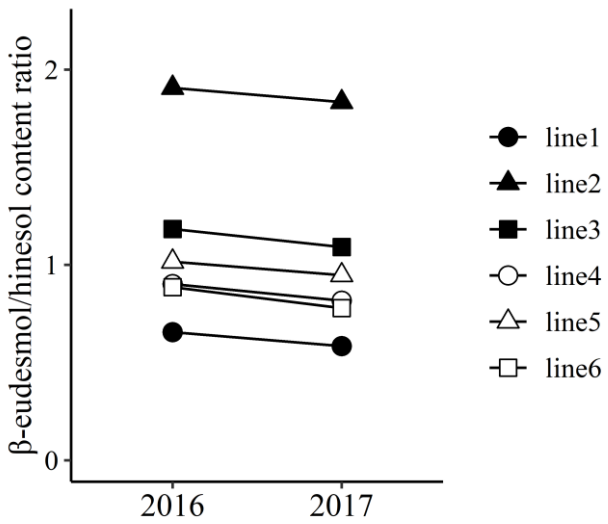

Figure 3.4 Interaction plots for interannual variability in the β-eudesmol/hinesol content ratio in *A. lancea*.

Each point represents the mean of 20 measurements for all *A. lancea* clonal lines in 2016 and 2017.

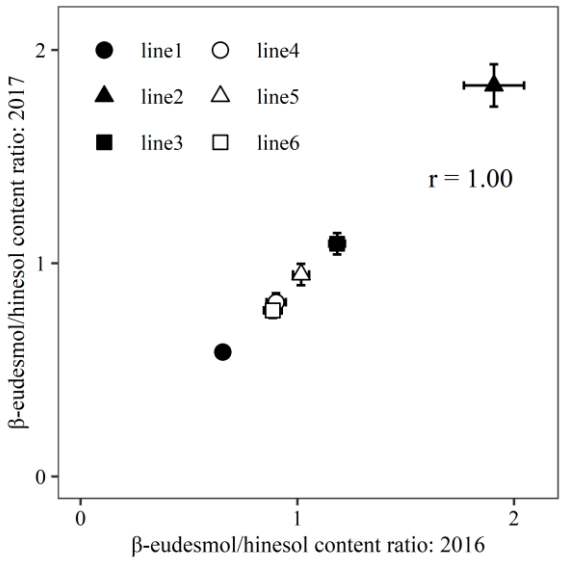

Figure 3.5 Correlation of the β-eudesmol/hinesol content ratio in *A. lancea* clonal lines grown in 2016 and 2017.

Each point represents the mean of 20 measurements in 2016 and 2017, whereas bars indicate standard deviations.

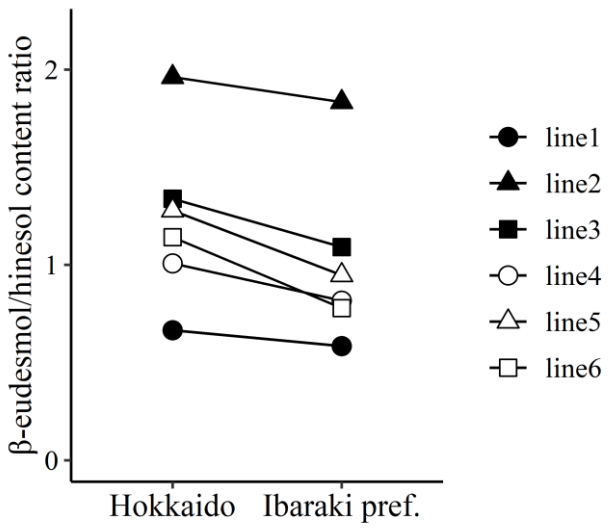

Figure 3.6 Interaction plots of the β-eudesmol/hinesol content ratio in *A. lancea* clonal lines grown in Hokkaido and Ibaraki prefectures.

Data are presented as the mean of biological replicates for *A. lancea* clonal lines grown in Hokkaido; lines 1, 4, and 6 (n = 20), line 2 (n = 12), line 3 (n = 8), and line 5 (n = 18). Twenty replicates were generated for each *A. lancea* clonal lines grown in Ibaraki prefecture.

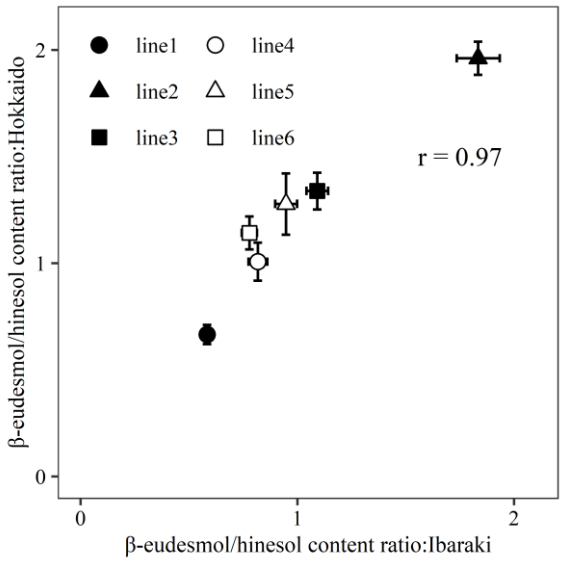

Figure 3.7 Correlation of the β-eudesmol/hinesol content ratio in *A. lancea* clonal lines grown in Hokkaido and Ibaraki prefectures; data are presented as mean and standard deviation.

The number of biological replicates for all *A. lancea* clonal lines grown in Hokkaido is as follows: lines 1, 4, and 6 (n = 20); line 2 (n = 12); line 3 (n = 8); and line 5 (n = 18). Twenty replicates were generated for each *A. lancea* clonal line grown in Ibaraki prefecture.

Chapter 4

In vitro shoot propagation by liquid culture in *Atractylodes lancea*

4.1. Introduction

Atractylodes lancea is a medicinal plant that is a member of the *Asteraceae* (Shiba et al., 2006). Essential oil compounds, such as β-eudesmol, hinesol, atractylon, and atractylodin, are the main medicinal ingredients in *A. lancea*, and contents and content ratio of the compounds are important quality criteria in *A. lancea* production (Koonrungsesomboon et al., 2010; Jun et al., 2018; Takeda et al., 1996b). In the previous chapters, I have indicated that the content and content ratio of these essential oil compounds are largely influenced by genetic factors, and clonal propagation is an effective method for producing *A. lancea* plants of consistent quality.

A. lancea is perennial, and it can be propagated by the division of rhizomes. However, the reproductive rate of this method is inefficient, and only a few individual plants can be propagated in a few years (Hiraoka et al., 1984). *A. lancea* plants can be either female or bisexual, but there are relatively few bisexual plants. In Japan, only female plants are generally available, and they were brought from China during the Edo era (1603–1868 CE). The lack of male gametophytes makes seed production of *A. lancea* difficult, requiring alternative reproductive methods for stable seedling production of *A. lancea* (Hiraoka, 1993).

In general, *in vitro* propagation is regarded as an effective method for efficient mass propagation of clonal seedlings (Takayama, 1986). In medicinal plants, tip tissue culture, shoot multiplication, cell culture, and hairy root culture have been reported in species such as *Panax ginseng*, *Aconitum carmichaelii*, *Pinellia ternata*, and *Rehmannia glutinosa* (Chang and Hsing, 1980; Shoyama et al., 1983; Hatano et al., 1988; Sang et al., 2009). Clonal multiplication methods have also been evaluated in *A. lancea*. Hiraoka et al. reported that *in vitro* culturing methods of flower buds with ABA are effective for shoot propagation of *A. lancea* (1984). Methods using shoot tips of *A. lancea* have also been reported, and the effects of the other plant hormones, such as zeatin, kinetin, IAA, 1-

naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP), and gibberellin in shoot propagation, were evaluated. Previous research indicates that BAP is the most effective plant hormone for shoot propagation (Shoyama et al., 1987).

However, previous research examined the efficacy of BAP using solid media, such as agar and gellan gum, and whether it is effective when used in liquid culture has not been examined. Liquid tissue culture has been shown to be an effective method for mass propagation of plant seedlings (Takahyama. 1986). Generally, plant liquid tissue culture shares the following characteristics: (1) sufficient oxygen supply due to stirring and aeration induces remarkable plant growth; (2) the uptake of nutrients to the plant tissues is facilitated by close contact of plant tissues with the media, which stimulates shoot and root growth; and (3) the apical dominance disappears due to the continuous shaking conditions, which allows numerous axillary buds to be propagated at the same time. In this study, to identify whether or not *A. lancea* could be efficiently produced using liquid tissue culture and what the optimal concentration of BAP for shoot production, I investigated the effect of BAP concentration in liquid culture in *A. lancea* shoot propagation, aiming to develop *A. lancea* seedling mass production methods.

4.2. Materials and methods

4.2.1. Plant materials

In this study, two strains of *A. lancea*, “strain 1” and “strain 2,” were used as plant materials. Rhizomes of the strains were maintained in soil (Nursey soil; Takii & Co., Ltd., Kyoto, Japan) in pots (TO-150S nursery pot; Tokai Kasei Co., Ltd.), and shoot tips were obtained from one individual plant of strains 1 and 2. The shoot tips were sterilized with a 4% solution of sodium hypochlorite for 10 min and 70% ethanol for 30 seconds. Then, the shoot tips were washed with sterile water three times. Apical shoot tips were excised from the sterilized shoot tips under a stereo microscope (SMZ; Nikon) and cultured in basal media containing Murashige and Skoog (MS) (MS Plant Salt Mixture; FUJIFILM Wako Pure Chemical Corporation), MS vitamins (MS Modified Vitamin Solution 1000X; FUJIFILM Wako Pure Chemical Corporation), 3% sucrose and 0.2 % gelrite (Table 4.1). The cultured shoot tips were propagated in the basal medium with 1% BAP. The propagated shoots were used for the comparative study between solid and liquid media.

4.2.2. Investigation of the effect of BAP concentration using either Liquid or Solid media

The propagated shoots of strains 1 and 2 were cultured on liquid and solid media with BAP concentrations of 0, 0.1, 0.5, 1.0, 3.0, and 5.0 ppm for 8 weeks. The basal media contained MS salts, MS vitamins, and 3% sucrose, and only the solid media contained 0.2% gelrite. For the solid media, 100 mL was added into a plant box (CUL-JAR300; AGC TECHNO GLASS Co., Ltd.), and for liquid culture, 100 mL was added to a plant culture flask (CUL-FK100; AGC TECHNO GLASS Co., Ltd.). Four plantlets were cultured in each container, and the media were changed every 4 weeks. The cultured plants were incubated in a growth chamber (MLR-352H; PHC Holdings Corporation) at 22℃ with a photoperiod of 16 h light/8 h dark, and the liquid culture was rotated

on a shaker (MK201D; Yamato Scientific Co., Ltd.) at 100 rpm. Biological replicates included 12 plants for each treatment.

After 8 weeks, survival rate, shoot weight, number of leaves, number of shoots, and vitrification rate were evaluated. Following the investigation, the plants grown under each condition were transplanted into rooting media. The rooting media used hormone-free basal solid media (Table 4.1). They were cultured for 4 weeks, and rooting rate, number of roots, and root weight were evaluated.

4.2.3. Statistical analysis

Statistical analyses, such as t-test, ANOVA, and Tukey-Kramer, were performed for all measured traits using R (version 3.5.0).

4.3. Results

4.3.1 The effect of BAP in liquid and solid tissue culture on *in vitro* shoot propagation

Atractylodes lancea shoots were cultured with different BAP concentrations of liquid and solid media, and shoot growth was compared among varying treatments. Shoot size tended to be larger in liquid culture than solid agar culture (Figure 4.2 A, B, Figure 4.3 A, B). The BAP concentration also significantly affected propagation results. Survival rate of the two strains decreased as BAP concentration increased, especially in strain 1, and the survival rate in liquid culture was significantly lower than that of solid media at high BAP concentrations (Table 4.2). Furthermore, high BAP concentrations in liquid media induced plant death or decreased growth and significantly increased the mortality rate (Figure 4.2 B, Figure 4.3 B, Table 4.2).

Shoot weight was affected by the different types of media and BAP concentration. The shoot weights of strain 1 varied by BAP concentration on solid media, and the shoot weights reached a maximum at a BAP concentration of 3.0 ppm (Figure 4.4). In liquid media, there were no significant differences among the shoot weights of strain 1 at different BAP concentrations (Figure 4.4). When comparing liquid and solid media, the shoot weight of strain 1 was higher on liquid than on solid media at BAP concentrations 0 to 1.0 ppm (Figure 4.4). For strain 2, the shoot weights varied by BAP concentration under both liquid and solid media. The maximum shoot weight values on solid media were obtained at a BAP concentration of 5.0 ppm and 1.0 ppm on liquid media (Figure 4.5). In addition, as with shoot size, shoot weights were higher with liquid media compared to solid media with all BAP treatments (Figure 4.5).

The number of leaves of strain 1 also varied by BAP concentration on solid media, and the maximum number of leaves was produced at 0.5 ppm BAP (Figure 4.6). Conversely, the number of leaves did not vary in liquid culture regardless of BAP concentration (Figure 4.6). When strain 1 in

liquid and solid media were compared, there was a significant difference in leaf number at 0.5 ppm BAP, but not under other BAP concentrations (Figure 4.6). In contrast, the number of leaves of strain 2 tended to vary by BAP concentration under both liquid and solid media conditions, and the maximum values obtained occurred at 5.0 ppm BAP for solid media and at 1.0 ppm BAP for liquid media (Figure 4.7).

The addition of BAP to the solid media induced multiple shoot complexes, which produced three shoots at 1 ppm BAP in strain 1 and at 3.0 ppm BAP in strain 2 (Figure 4.8, Figure 4.9). Multiple shoots were also induced by BAP in liquid media. Three shoots per segment were produced at 0.1 ppm BAP in strain 1 and four shoots at 1.0 ppm BAP in strain 2 (Figure 4.8, Figure 4.9). In particular, shoot propagation of strain 2 in liquid culture was more efficient than with solid media (Figure 4.9). The addition of BAP also tended to induce leaf vitrification, especially with liquid media (Table 4.3).

4.3.2 Rooting rate with the rooting medium

After the shoot propagation using either liquid or solid media, shoots of strains 1 and 2 were transplanted to hormone-free rooting medium and incubated for 4 weeks, after which rooting rate was investigated for both strains. For strain 1, root length, number of roots, and root weights were also investigated. The rooting rate was 100 % for all treatments with the exception of strain 2 grown in liquid media at 3.0 ppm BAP (Table 4.4). The number of roots, root length, and root weights tended to be higher from shoots cultured in liquid media than solid media (Figure 4.10, Figure 4.11, Figure 4.12).

4.4. Discussion

In this study, addition of BAP did improve *A. lancea* shoot growth compared to the basic media control in both liquid and solid media, although the optimal BAP concentration differed between the liquid and solid media (Figure 4.2, Figure 4.3). The addition of BAP has been reported as an effective method for the promotion of shoot growth in *A. lancea* on solid media (Hiraoka et al., 1984), and the results of the present study indicate that the addition of BAP is also effective with liquid media. However, the optimal concentration of BAP in liquid media was lower than that of solid media. For strain 1, the optimum was at 0.1 ppm BAP with liquid media, while it was optimum 1.0 ppm BAP for solid media (Figure 4.8). Because liquid media is in close contact with the cultured tissue, it is likely that the uptake of plant hormones and nutrients is better facilitated (Sandal et al., 2001).

Because tissues react differently to different types of media, the optimal BAP concentration for *A. lancea* shoot growth understandably varies between liquid and solid media. The optimal BAP concentration for shoot growth also differed between strains of *A. lancea*. The optimal BAP concentration of strain 1 grown in liquid media was at 0.1 ppm, whereas it was 1.0 ppm for strain 2 (Figure 4.4, Figure 4.5, Figure 4.8, Figure 4.9). In general, there are varietal differences in response to cultural practices in many crop species and to phytohormone exposure (Yamamoto and Matsumoto, 1992; Tanikawa et al., 1998; Hoshi et al., 2011). There are few studies on the breeding of *A. lancea*, but it is considered to have high genetic diversity (Takeda et al., 1996; Huang et al., 2016). In fact, shoot multiplication has already been reported as a variable trait among strains of *A. lancea* (Hiraoka, 1993). In addition to validating previous findings with regard to shoot multiplication, this study also indicates that there might be varietal differences in response to phytohormones in *A. lancea*.

When the shoot growth under liquid and solid media conditions were compared, shoots grown on solid media produced three shoots for propagation in 8 weeks, whereas shoots grown in liquid media produced 3–4 shoots for propagation in the same timeframe (Figure 4.8, Figure 4.9). The efficient propagation of *A. lancea* shoots in liquid culture in this study may be due to several reasons. The liquid media, likely due to an efficient uptake of nutrients and plant hormones, induced better shoot and root growth at optimal BAP concentrations. In addition, the loss of apical dominance in liquid culture may have promoted the formation of numerous axillary buds, allowing for the production of several shoots (Mehrotra et al., 2007).

Rooting rate was evaluated after cultured shoots were moved to a rooting medium, and it was high for all shoot culture conditions (Table 4.4). In a previous study, similarly high rooting rates were reported using rooting media that were either hormone-free or had added IAA (Hiraoka et al., 1984). Overall, it appears that rooting of cultured *A. lancea* shoots is consistently high, and the development of an efficient shoot propagation method is the key factor in creating a method for the mass propagation of *A. lancea* seedlings.

In the present study, I demonstrated that the propagation rate of *A. lancea* shoot was higher in liquid culture compared to solid media. For instance, a single shoot of strain 2 on average was used to generate 3.917 shoots in 8 weeks at an optimal BAP concentration with liquid culture (Figure 4.9). Based on these results, rough calculations can be made to estimate annual production of *A. lancea* shoots. Under similar conditions, it may be possible to produce as many as 7144 plantlets from a single shoot of a responsive variety in liquid culture in a year; however, upscaling such a process has its own unique set of issues. For shoot propagation in solid media, rough calculations based on the results in this study estimate that about 1000–1500 plantlets from a shoot may be produced in a year (Figure 4.8 and Figure 4.9). Hiraoka et al. (1984) also reported that annual production of *A.*

lancea shoots with solid media ranged from 2000 to 4000 plantlets per single shoot. In contrast, when *A. lancea* clonal propagation is performed by conventional methods, namely, division of rhizomes, it can only be propagated 2–4 times a year resulting in 4–16 rhizomes (Hiraoka et al., 1984).

Based on these results, liquid tissue culture and shoot propagation is the most efficient method for plantlet production, especially when compared to conventional methods. However, it is necessary to select strains responsive to tissue culture conditions, since shoot propagation rate varies depending on the strains (Figure 4.8, Figure 4.9). In addition, the vitrification of leaves was easily induced in the liquid media (Table 4.3), which is a common issue and a known disadvantage of plant tissue culture (Kevers et al., 1984; Debergh et al., 1992). The main causes of vitrification are related to the culture media, such as the absence of agar for support and asphyxiation due to the lack of oxygen in the liquid media (Hakkaart and Versluijs, 1983; Kevers et al., 1987; Mehrotra et al., 2007). It has been reported that a reduction in or lack of NH_4NO_3 reduced vitreous plants in *in vitro* culture of petunia (*Petunia* × *atkinsiana*) (Thomas et al., 1991). To reduce vitrification and increase shoot production, further development and optimization of tissue culture methods of *A. lancea* that leverage tissue culture practices of the other plant species should be pursued.

In conclusion, the effect of liquid media in *A. lancea* shoot production was examined for the first time, and I demonstrated that liquid culture is an effective method for clonal propagation of *A. lancea* plantlets. I also showed that clonal propagation of *A. lancea* is an effective method for producing plants with consistent qualities, including essential oil compound contents and content ratio (see previous chapters). In this chapter, preliminary work demonstrates that a high-quality strain of *A. lancea* could be clonally propagated on a massive scale. However, survival rate and vitrification are issues that have not yet been fully resolved, and further investigations and

optimization of efficient propagation methods of *A. lancea* shoots are required.

Table 4.1 The recipe for the basic liquid media used for tissue culture.

	Basal Salts	mg/L
Major essential elements	KNO_3	1,900
	NH_4NO_3	1,650
	$CaCl_2 \cdot 2H_2O$	440
	$MgSO_4 \cdot 7H_2O$	370
	KH_2PO_4	170
Minor essential elements	Na_2-EDTA	37.3
	$FeSO_4 \cdot 7H_2O$	27.8
	$MnSO_4 \cdot 4H_2O$	22.3
	$ZnSO_4 \cdot 7H_2O$	8.6
	H_3BO_3	6.2
	KI	0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.25
	$CuSO_4 \cdot 5H_2O$	0.025
	$CoCl_2 \cdot 6H_2O$	0.025
Vitamines and Amino acids	Glycine	2.0
	Myo-inositol	100
	Nicotinic acid	0.5
	Pyridoxine hydrochloride	0.5
	Thiamine hydrochloride	0.1
Sugar	Sucrose	30 g/L
Gelling agent	Gelrite	2.0 g/L

Table 4.2 Survival rate of shoots cultured using liquid and solid media at different BAP concentrations.

meduim	strain	Survival rate (%)					
		BAP concentration (ppm)					
		0.0	0.1	0.5	1.0	3.0	5.0
Liquid medium	strain 1	100.0	100.0	50.0	0.0	0.0	0.0
	strain 2	100.0	100.0	87.5	100.0	100.0	50.0
Solid medium	strain 1	81.3	100.0	87.5	87.5	87.5	68.8
	strain 2	100.0	100.0	100.0	100.0	100.0	100.0

The numbers of biological replicates for each strain are as follows: strains 1 and 2 were grown in liquid media (n = 12), strain 1 was grown on solid media (n = 16), and strain 2 was grown on solid media (n = 12).

Table 4.3 Rate of vitrification in leaves of shoots cultured using liquid and solid media.

meduim	strain	Vitrification rate (%)					
		BAP concentration (ppm)					
		0.0	0.1	0.5	1.0	3.0	5.0
Liquid medium	strain 1	62.4±39.7	47.3±29.5	42.5±35.6	—	—	—
	strain 2	17.4±16.3	18.7±8.2	28.5±11.6	28.8±11.6	27.3±28.9	95.2±10.5
Solid medium	strain 1	0	0	0	2.8±4.4	21.1±30.5	54.1±39.8
	strain 2	0	0	0	0	0	0

[a]Different letters indicate significant differences ($P < 0.05$) among BAP media concentrations.

Table 4.4 Rooting rate of shoots cultured using liquid and solid media following transfer to rooting media.

meduim	strain	Rooting rate (%)					
		BAP concentration (ppm)					
		0.0	0.1	0.5	1.0	3.0	5.0
Liquid medium	strain 1	100.0	100.0	100.0	—	—	—
	strain 2	100.0	100.0	100.0	100.0	83.3	—
Solid medium	strain 1	100.0	100.0	100.0	100.0	100.0	100.0
	strain 2	100.0	100.0	100.0	100.0	100.0	100.0

Biological replicates were 12 plantlets for each treatment.

Figure 4.1 Flowers of *A. lancea*. A: Female flowers; B: bisexual flowers. (Scale bars = 1 cm)

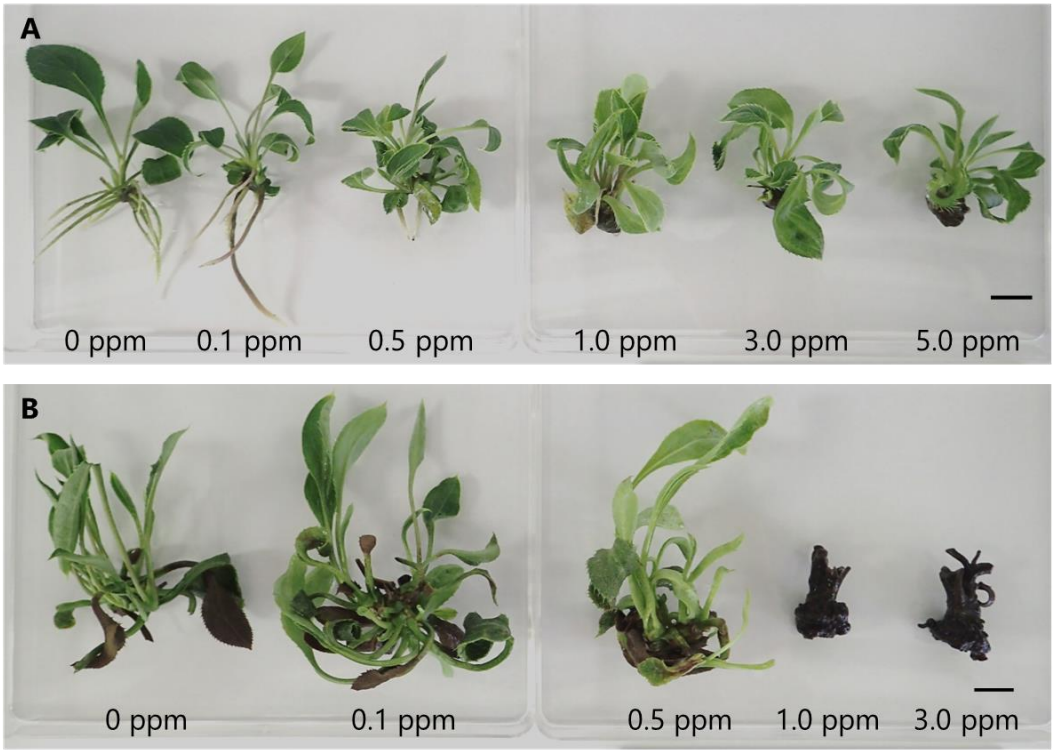

Figure 4.2 Shoots of strain 1 grown at different BAP concentrations on liquid and solid media. (A) Plantlets cultured on solid media, (B) plantlets cultured on liquid media. BAP concentrations are indicated under each representative plantlet. (Scale bars = 1 cm)

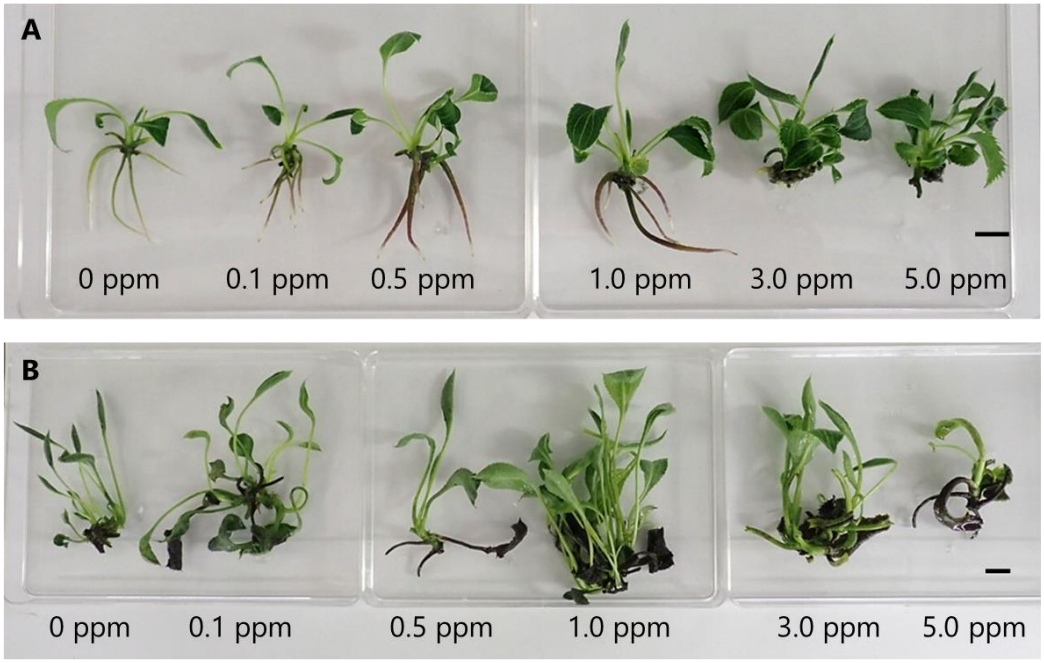

Figure 4.3 Shoots of strain 2 grown at different BAP concentration on liquid and solid media. (A) Plantlets cultured on solid media, (B) plantlets cultured on liquid media. BAP concentrations are indicated under each representative plantlet. (Scale bars = 1 cm)

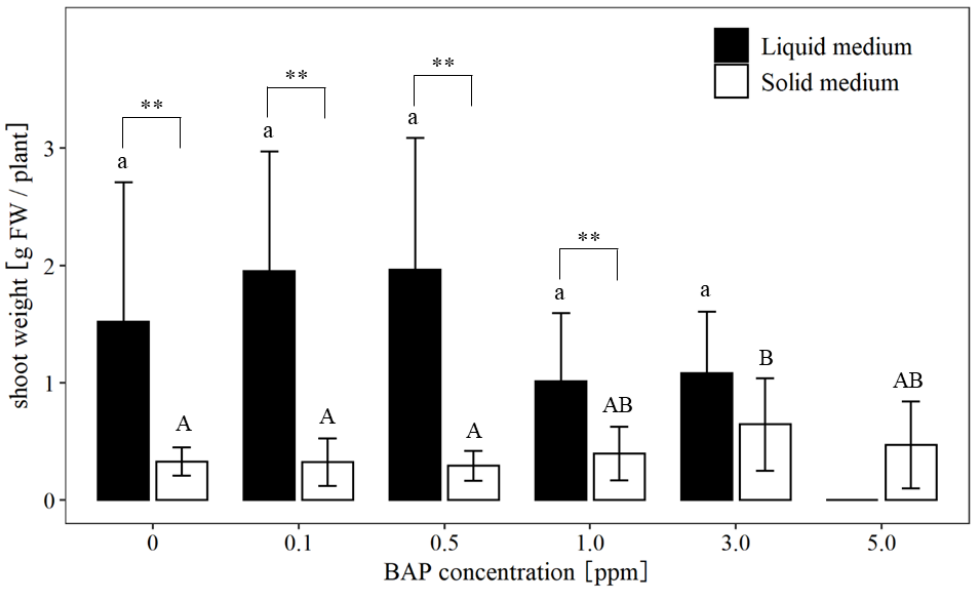

Figure 4.4 Shoot weight of strain 1 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

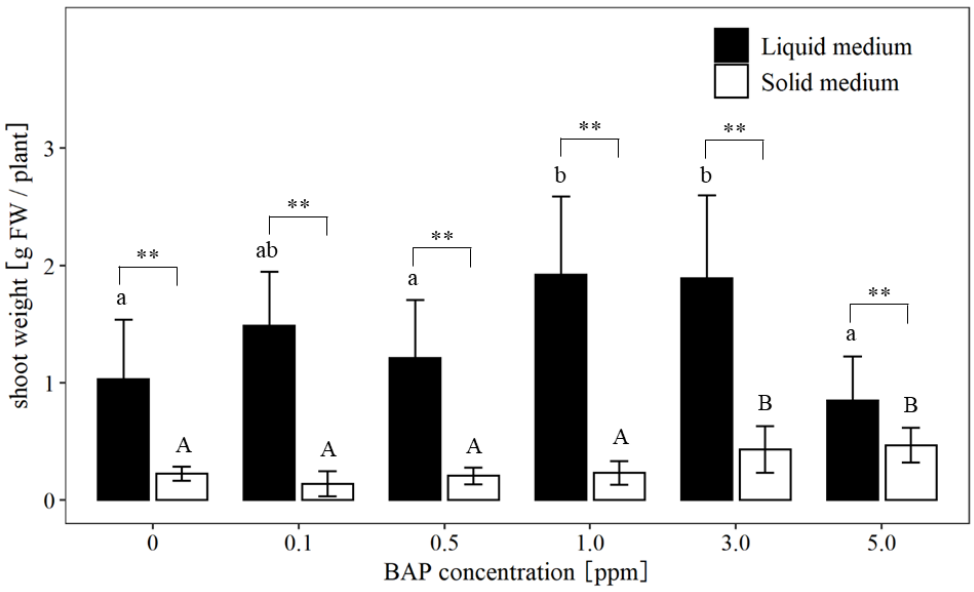

Figure 4.5 Shoot weight of strain 2 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

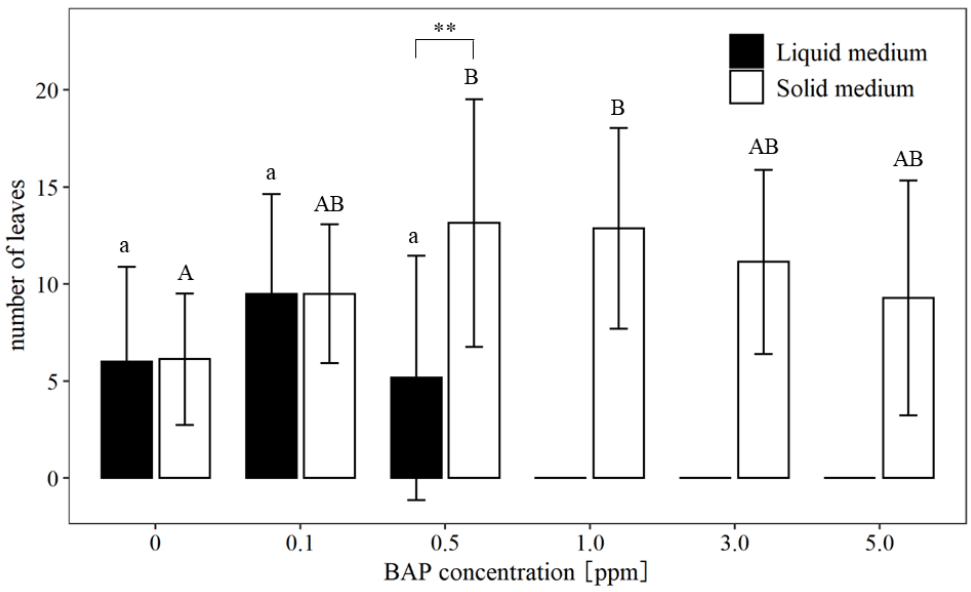

Figure 4.6 Number of leaves of strain 1 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

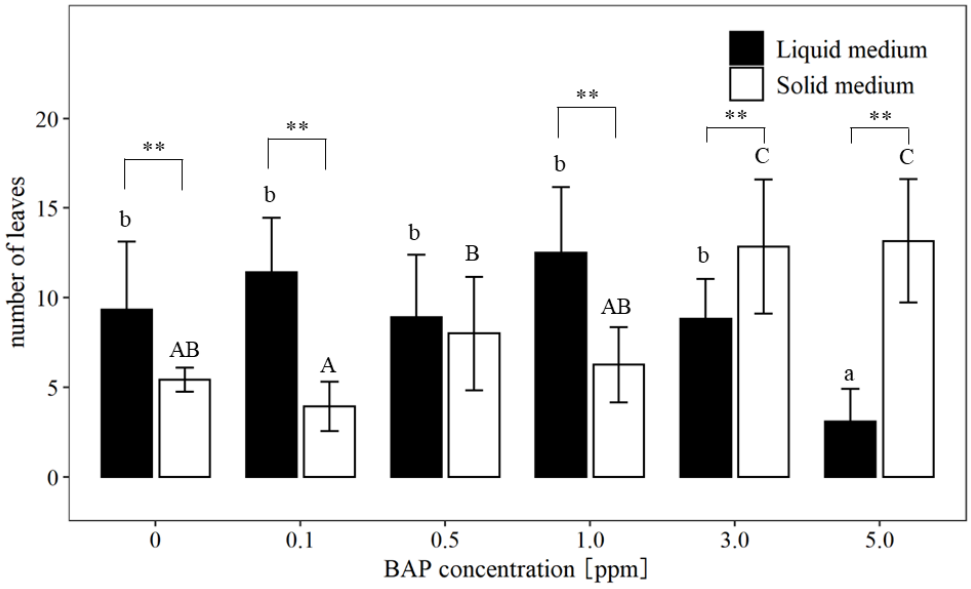

Figure 4.7 Number of leaves of strain 2 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

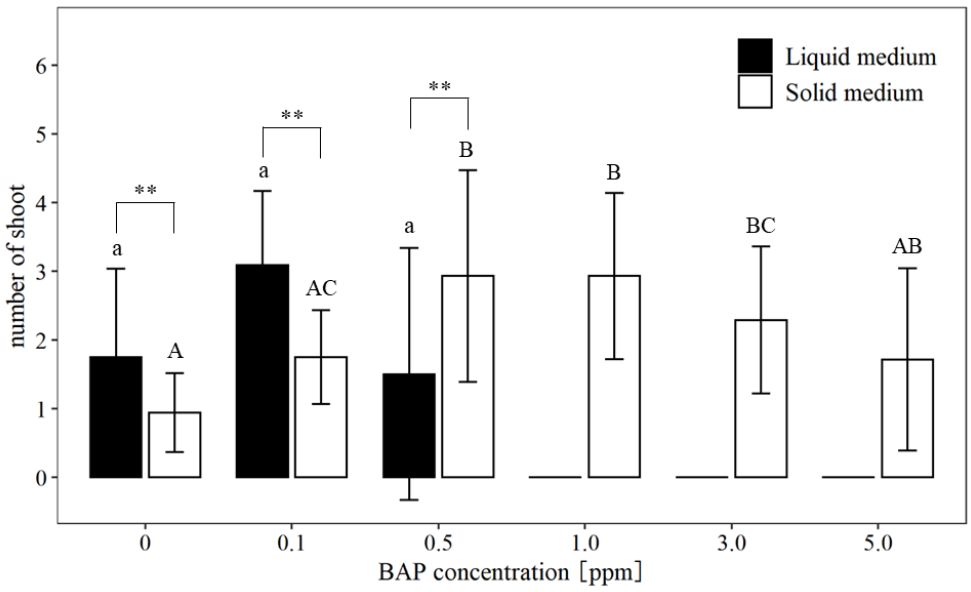

Figure 4.8. Number of shoots of strain 1 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

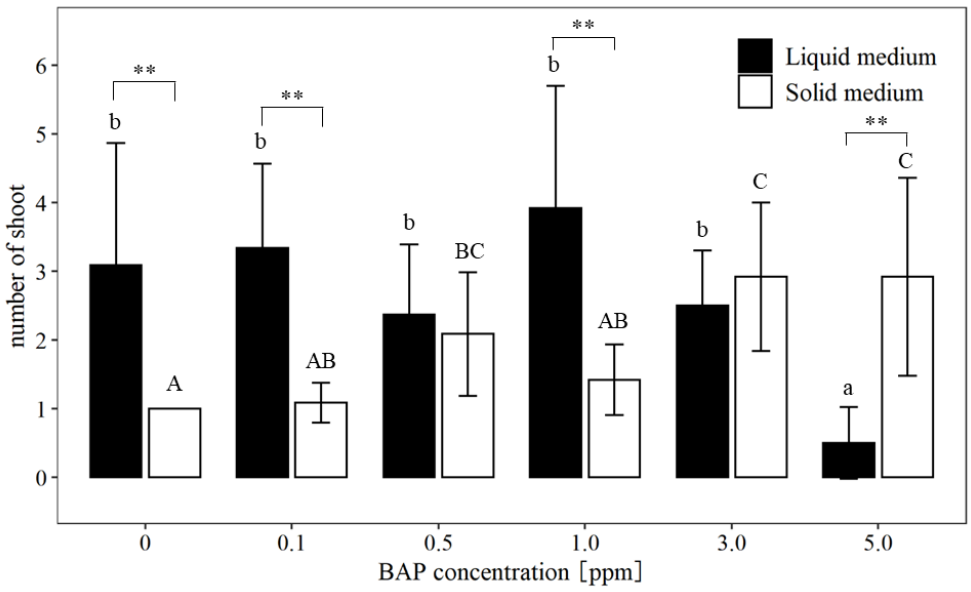

Figure 4.9 Number of shoots of strain 2 cultured on liquid and solid media at different BAP concentrations. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

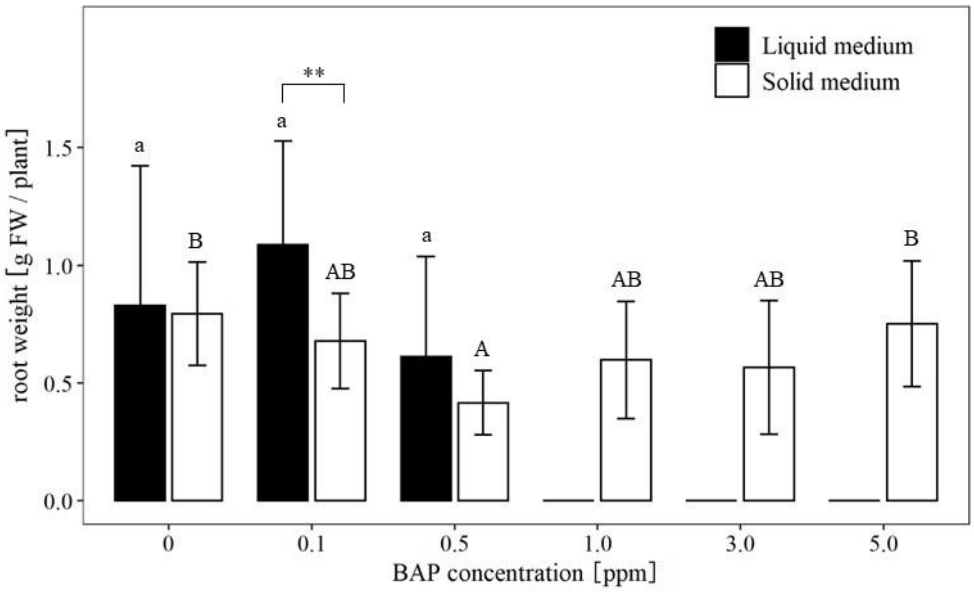

Figure 4.10 Root weight of strain 1 following transfer to rooting media from liquid or solid media. Different letters indicate significant differences among BAP concentrations and * indicate significant differences between solid agar and liquid media (**P<0.01).

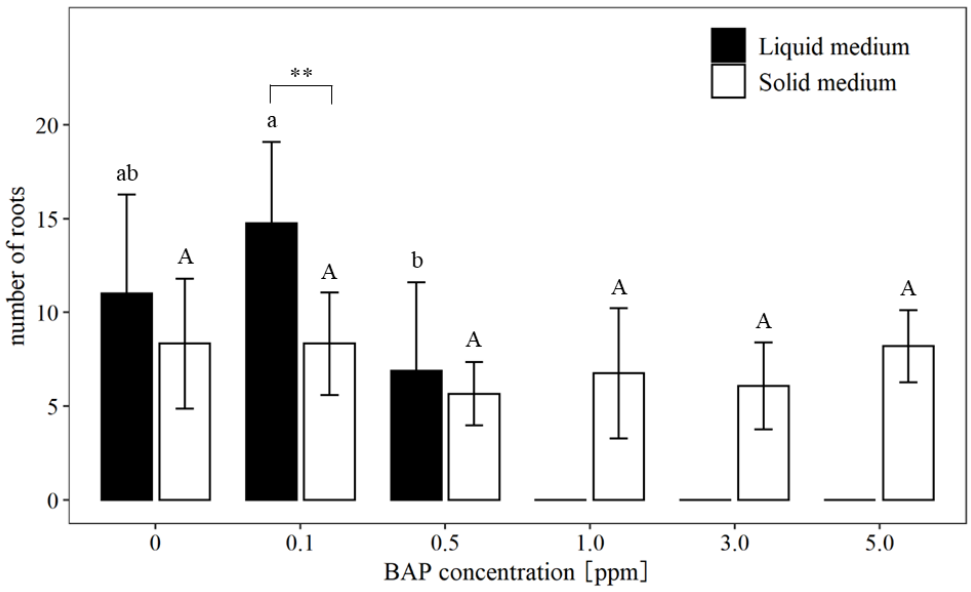

Figure 4.11 Number of roots of strain 1 following transfer to rooting media from liquid or solid media. Different letters indicate significant differences among BAP concentrations and * indicates significant differences between solid agar and liquid media (**P<0.01).

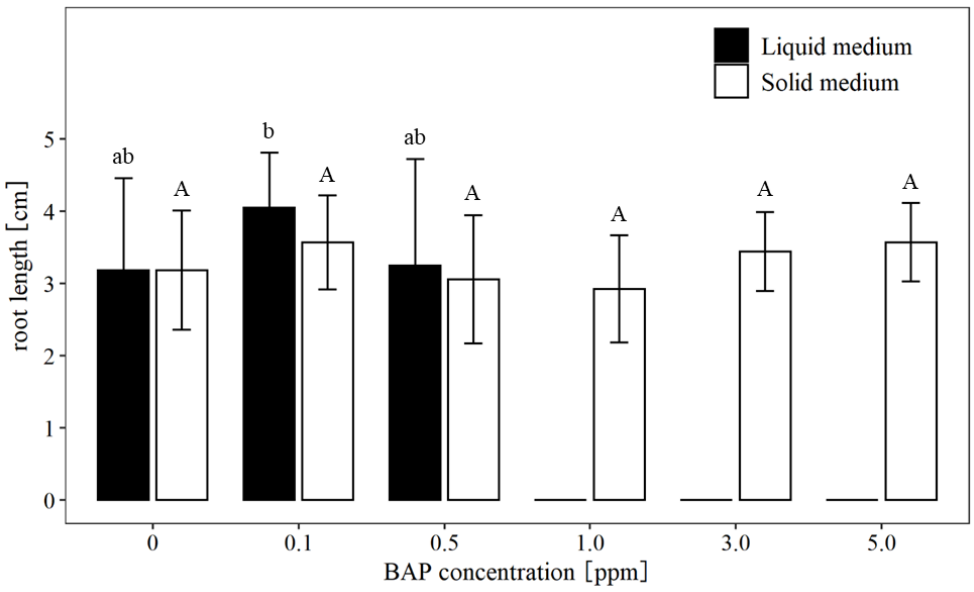

Figure 4.12 Root length of strain 1 following transfer to rooting media from liquid or solid media. Different letters indicate significant differences among BAP concentrations. There were no significant differences between solid agar and liquid media.

Chapter 5

General Discussion and Summary

5.1. General Discussion

Atractylodes lancea is an important medicinal plant in traditional Chinese and Japanese medicines, and the domestication and selection of *A. lancea* suited to Japanese cultivation practices are essential to maintain a stable supply of raw materials needed to prepare traditional medicines (Kubo et al., 1983; Wang et al., 2008). *A. lancea* has been cultivated in China since ~1990; however, research on breeding has yet to be published. Furthermore, one of the issues in the production of *A. lancea* is that the rhizome quality is unstable (Chen et al., 2017; Zhen et al., 2012; Takeda et al., 1995). High-quality rhizomes are mainly evaluated on two criteria: their essential oil content and the ratio of the essential oil active compounds (Takeda et al., 1996b; Takeda et al., 1998a). In this study, I acquired basic knowledge that will help in breeding high-quality *A. lancea*. *A. lancea* is a perennial plant with the ability to be propagated by division of rhizomes, so clonal lines were generated by division of rhizomes for experimental use.

To better understand quality traits, I performed genetic analyses and were able to evaluate heritability and G × E interactions in the present study. Analytical models for vegetatively propagated plants, such as herbages, were used to calculate heritability (Hiraoka et al., 1984; Kalton et al., 1952; Burton et al., 1953). The broad-sense heritability for the contents of essential oil compounds and β-eudesmol/hinesol content ratio in *A. lancea* was high (Table 2.1, Table 3.1), suggesting that these traits are highly influenced by genetic factors. It also indicates that selective breeding could be effective in reducing the variation in essential oil content and content ratio of essential oil compounds in *A. lancea*. In other medicinal plants, the heritability of artemisinin content, which is classified as a sesquiterpene lactone in *Artemisia annua*, exhibited high values (Ferreira et al., 1994). Unfortunately, there is limited information as regards the heritability of secondary metabolites in the other medicinal plants. However, I report that the heritability of three

sesquiterpenoid compounds and a polyacetylene compound in *A. lancea* were high under these study conditions, suggesting that the heritability of other secondary metabolites in medicinal plants may be high. In short, selective breeding could have wide ranging effects on reducing the variation of secondary metabolites in medicinal plants.

In contrast, secondary metabolites are generally considered to be induced by environmental conditions, such as abiotic or biotic stress (Bennett and Wallsgrove, 1994; Ramakrishna and Ravishankar, 2011). Therefore, six clonally derived lines of *A. lancea* were cultivated in different years and locations, and the effects of environmental factors and G×E interactions on the contents of essential oil compounds and the β-eudesmol/hinesol content ratio in *A. lancea* were evaluated. Based on the results of the two-way ANOVA, mean squares of genotypic background for essential oil compound content and the content ratio were higher than the interannual variability (Table 2.3, Table 3.2). In addition, the correlation between cultivation year and the compound contents or the content ratio were high (r = 0.98–1.00) (Figure 2.5 A, B, C, D, Figure 3.5), and a few G×Y interactions were observed (Figure 2.6 A, B, C, D, Figure 3.4). The results suggest that the contents of essential oil compounds and the content ratio were stable regardless of cultivation year, which leads me to expect that selective breeding in similar environmental conditions could be transferrable.

The influence of cultivation location on the contents of essential oil compounds was greater than that of cultivation year, and mean squares of cultivation location for β-eudesmol and hinesol contents were higher than those of genotype (Table 2.5). However, correlation between two locations for the contents of essential oil compounds was high (Figure 2.8). These results suggest that the genetic potential with regard to the contents of essential oil compounds in *A. lancea* is consistent across environments, though the realized contents are influenced by environmental factors as absolute content values. It has been reported that the accumulation of essential oil compounds is

induced by environmental factors, such as soil acidity, soil type, and soil clay content (Yuan et al., 2009; Shoudong et al., 2017). It has also been reported that phytohormones, such as ABA and JA, increase in the contents of essential oil compounds in *A. lancea* (Ren and Dai, 2012; Wang et al., 2015), which is generally induced by abiotic or biotic stress to plants (Sudha and Ravishankar, 2002). The environmental conditions of the two cultivation locations in this study differed in soil conditions and air temperatures (Table 2.1). The environmental differences may have caused the changes in the absolute value of the contents of the essential oil compounds in *A. lancea*. More detailed analysis on the influence of environmental conditions on the contents of the essential oil compounds in *A. lancea* is required. In contrast, the content levels among genotypes do not appear to be significantly influenced by environmental factors, suggesting that selective breeding would be effective for decreasing the variability of the content of essential oil compounds in *A. lancea* and that these traits have wide adaptability.

It has been reported that the essential oil compound contents of *A. lancea* are closely related to their natural habitat and vary greatly based on geographical factors (Takeda et al., 1995a). Takeda et al. (1996a) also indicated that *A. lancea* could be classified into three types based on the contents of essential oil compounds and depending on their natural habitat. In the present study, I showed that the heritability of the content of essential oil compounds was high and that the genetic potential of the compound contents did not vary significantly in response to environmental conditions. Therefore, these three types of *A. lancea* defined by the content of essential oil compounds are likely determined mainly by genetic factors. The differences among these traits within *A. lancea* may be attributable to several processes, such as adaptation to different environments, geographic isolation, genetic drift, or founder effects. In addition, the influence of cultivation location on β-eudesmol/hinesol content ratio was smaller than that on the compound contents (Table 3.3, Figure 3.4, Figure 3.5). The results

suggest that the content ratio is highly influenced by genetic factors compared to the essential oil content, which also indicates that the selective breeding effect on β-eudesmol/hinesol could be great.

In the present study, I demonstrated that the contents and content ratio of essential oil compounds in *A. lancea* are mainly affected by genetic factors. However, it remains unclear why the contents and content ratio of essential oil compounds are under such strong genetic control in *A. lancea*. Genetic analysis on the contents of essential oil compounds in *A. lancea* has not been performed using molecular techniques in the past. However, genetic analysis has been conducted on artemisinin content, which is a sesquiterpene lactone having antimalarial activity in *Artemisia annua*, and a part of the molecular mechanism of artemisinin accumulation in *A. annua* has been elucidated (Abdin and Alam, 2015). Although sesquiterpene compounds like artemisinin are biosynthesized from farnesyl pyrophosphate (FPP) in the mevalonate pathway, there are several metabolic shunting points from FPP to artemisinin or other sesquiterpenes. β-Caryophyllene synthase (CPS) and squalene synthase (SQS) are some of the metabolic shunting points in the artemisinin biosynthesis pathway in *A. annua* (Abdin and Alam, 2015). Chen et al. indicated that artemisinin accumulation is induced by suppressing the expression of CPS using RNA interference (RNAi) in *A. annua* (2011). It was also reported that downregulation of SQS gene expression induces increasing artemisinin content in *A. annua* (Zhang et al., 2009). These results imply that high accumulation of artemisinin in plants may be caused by mutations in the enzymes of the metabolic shunting points of artemisinin biosynthesis.

I indicated that the accumulation of the essential oil compounds, including sesquiterpene compounds such as β-eudesmol and hinesol in *A. lancea*, is mainly controlled by genetic factors in this study. As with artemisinin in *A. annua*, the high accumulation of the essential oil compounds in *A. lancea* may also be caused by mutations in enzymes at metabolic shunting points of the

biosynthesis pathway for the compounds (Figure 5.1). In addition, transcription factors in *A. annua*, which regulate expression of key enzymatic genes in artemisinin biosynthesis, have been identified. Transcription factor genes, *AaERF1* and *AaERF2*, are positive regulators of amorpha-4,11-diene synthase (ADS) and CYP71AV1, and the overexpression of these two transcription factors causes increased artemisinin accumulation in *A. annua* (Yu et al., 2012). It has also been reported that *AaWRKY1* is a positive regulator of *ADS*, and this transcription factor induces artemisinin accumulation in *A. annua* (Gaobin et al., 2009). Conversely, it has been reported that gene silencing of these transcription factors causes a decrease in artemisinin content in plants (Yu et al., 2012). These complex results suggest that mutations in the transcription factors or promoter region sequence of key enzymes regulated by the transcription factors induce decreasing artemisinin content in plants. In *A. lancea*, it is possible that the content of essential oil compounds is regulated by transcription factors as with *A. annua*, and clonal lines with low essential oil content may differ in transcription factor sequence. However, determining sequence differences will require further investigation.

It has been reported that the accumulation of essential oil compounds is induced by ABA and JA in *A. lancea* (Ren and Dai, 2012; Wang et al., 2015). In addition, ABA receptor orthologue, *AaPYL9*, was identified in *A. annua*, and it increased artemisinin content and gene expression of the biosynthesis enzymes through overexpression of *AaPYL9*. It has been suggested that mutations in ABA receptors could cause decreasing artemisinin content in *A. annua* (Zhang et al., 2013). These results suggest that hormone receptors may be involved in genetic control of the content of essential oil compounds in *A. lancea* as with *A. annua*. Further investigations, especially using molecular biological methods, into why the contents of essential oil compounds are largely controlled by genetic factors are required to elucidate these mechanisms in better detail.

Generally, secondary metabolite accumulation is induced by abiotic and biotic stresses, and the accumulation of secondary metabolites has been linked to growth inhibition in plants. Therefore, it is possible that secondary metabolite accumulation may be inversely proportional to plant biomass (Bennett and Wallsgrove, 1994; Ramakrishana and Ravishankar, 2011; Chen et al., 2011). However, correlations between the content of essential oil compounds and rhizome yield were rarely observed in *A. lancea* (Figure 2.5). My results indicate that the strain of *A. lancea* used in this study had high essential oil content, which could be selected for independent of rhizome yields. In fact, *A. lancea* line 5 showed relatively high content of essential oil compounds (β-eudesmol, hinesol, and atractylodin) and rhizome yields compared to the other clonal lines (Figure 2.5). The mean values of β-eudesmol, hinesol, and atractylodin in line 5 were 26.5, 28.1, and 2.7 mg/g DW, respectively, while the mean values of those compound contents in all 25 lines were 16.4, 20.2, and 2.2 mg/g DW, respectively. If line 5 is selected and propagated, the average contents of β-eudesmol, hinesol, and atractylodin could be expected to increase by 61%, 39%, and 12%, respectively, in a clonally derived population compared to an unselected population. In addition, the mean value of the rhizome weight of line 5 was 48.3 g DW, whereas the mean value of the rhizome weight of all 25 clonal lines was 39.1 g DW. If line 5 is selected and used as a cultivar in production of *A. lancea*, rhizome yields can be expected to increase by 24% in a clonally derived population compared to an unselected population. Thus, it is highly possible that the content of essential oil compounds and rhizome yields of *A. lancea* could be increased and made less variable by selective breeding and clonal propagation.

The results are valuable for industrial use not only crude drugs as materials for traditional medicines but also food or cosmetic raw materials, because the compounds have bioactivities and they have values themselves as useful compounds. It has been reported that β-eudesmol increase cool feeling at meals in human through activation of transient receptor potential ankyrin 1 (TRPA1)

(Ohara et al., 2015). Additionally, β-eudesmol has bioactivities such as autonomic nervous regulation (Ohara et al, 2016). Therefore, it is possible that β-eudesmol play a role as raw materials for functional foods. It has also applied a patent, which hinesol is used as accelerator of hyaluronic acid synthase, suggesting that hinesol might be used as cosmetic raw materials (Nakamura, 2018). In this study, I demonstrated that the contents of essential oil compounds in *A. lancea* were genetically controlled. It suggests the possibility that the compounds could be produced efficiently by development of elite cultivars contained high contents of the essential oil compounds, and it might be used for food or cosmetic raw materials.

In recent studies, plant genomics has been used in the analyses of several species due to the development of NGS technology (Kersey, 2019). Also, whole genome sequencing and assembly have been accelerated in several plant species due to long-read sequencing technology developed by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (Belser et al., 2018; Li and Harkess, 2018). In medicinal plants, draft genome assemblies have been generated for *Glycyrrhiza uralensis* and *Panax ginseng*, and these fundamental tools have been instrumental in the isolation of novel genes related to medicinal metabolites (Mochida et al., 2017; Kim et al., 2018). Other technologies that take advantage of genotyping-by-sequencing, such as restriction-associated DNA sequencing (RAD-seq) and genotyping by random amplicon sequencing (GRAS-Di), to detect polymorphisms across whole genomes have facilitated progress in plant breeding, genetics, and other biological sciences (Poland and Rife, 2012; Hosoya et al., 2019).

In *Oryza sativa*, a metabolome genome-wide association study was conducted with natural accessions, and several quantitative trait loci (QTL) associated with various secondary metabolites were identified, and many secondary metabolites were associated with a few intense QTL (Matsuda et al., 2015). It is possible that metabolome quantitative trait loci (mQTL) analysis could be utilized

to identify QTL associated with essential oil compounds in *A. lancea*. Based on transcriptome analyses using RNA-seq with different *A. lancea* tissues, a putative sesquiterpenoid biosynthetic pathway has been proposed, and several candidate genes related to sesquiterpenoid biosynthesis have been reported (Chen et al., 2017; Huang et al., 2016). In addition, high numbers of simple nucleotide polymorphisms have been detected from *A. lancea* leaf, stem, and root tissue cDNA libraries (Ahmed et al., 2016). These findings are the initial steps in developing a marker-assisted selection strategy for the content and content ratio of essential oil compounds in *A. lancea* using genetic association analysis. Furthermore, since there were few correlations among the content of essential oil compounds and rhizome yields (Figure 2.5, Table 2.3), it might be possible to develop DNA markers for each trait independently and create cultivars with both high compound contents and high rhizome yields.

In the present study, I demonstrated that clonal propagation is effective for reducing the variability of the content and content ratio of essential oil compounds in *A. lancea*. However, using the conventional method, *A. lancea* can only be propagated 2–4 times a year (Hiraoka et al., 1984). In this study, I examined efficient propagation methods of *A. lancea*, especially *in vitro* shoot propagation under liquid culture. My results indicated that under optimal BAP conditions, as many as 3.9 shoots could be propagated from a single shoot in 8 weeks using liquid culture, with the caveat that strains suitable for liquid culture conditions need to be selected (Figure 4.9). Instead of a handful of clones produced conventionally, thousands of plantlets could be produced with liquid culture from a single initial selection. However, I confirmed that liquid culture increased the tendency for vitrification of leaves (Table 4.3), and optimization to suppress the vitrification of leaves in liquid culture is required.

The rooting rate after transplant to a rooting medium was highly consistent in *A. lancea* (Table

4.4). Although hormone-free media were used as a rooting medium in this study, it has been reported that the addition of IAA and GA increased the induction of rooting and root growth (Shoyama et al., 1987). As the number of selections of *A. lancea* run through liquid tissue culture increases, optimizing the rooting conditions of cultured shoots will be necessary. After rooting *in vitro*, acclimation to soil is required for seedling production. Conditions for transfer of plantlets to soil have been determined, and a mixture of soil, sand, and peat containing 1 % slaked lime had the highest (82%) success rate for acclimation of *A. lancea* (Hiraoka et al., 1984). Further investigation into optimal condition for transfer of plantlets to soil is needed for large-scale production of *A. lancea* seedlings.

When annual production of *A. lancea* seedlings are estimated based on the results in this study, it may be possible to produce as many as 7144 plantlets from a single shoot in a year under optimal liquid culture condition. When *A. lancea* clonal propagation is performed by conventional methods, namely, division of rhizomes, it can only be propagated 2–4 times a year resulting in 4–16 rhizomes (Hiraoka et al., 1984). It has been also reported that annual production of *A. lancea* shoots with solid media ranged from 2000 to 4000 plantlets per single shoot (Hiraoka et al., 1984). Therefore, clonal propagation of *A. lancea* with liquid culture in this study are quite efficient methods compared with conventional propagation methods. Currently, *A. lancea* cultivation is performed with propagation with division of rhizomes. If liquid culture is applied as propagation methods for *A. lancea* cultivation, it is expected that the efficiency of propagation will increase dramatically.

In brief, this study contributes to the basic knowledge for breeding in *A. lancea* with specific focus on the contents and content ratio of essential oil compounds. While *A. lancea* is currently produced mainly in China, domestic cultivation is of keen interest due to the depletion of wild resources and increasing price. However, environmental conditions differ between Japan and China,

possibly altering the content of the medicinal ingredients. Therefore, I explored how breeding could reduce the variability of the content of essential compounds in *A. lancea*. As a result, I demonstrated that the compound contents and content ratio are largely controlled by genetic factors, and an *A. lancea* cultivar with high content of essential oil compounds could be developed and selected independent of rhizome yields. In short, I demonstrated that selective breeding could be highly effective for the domestication and improvement of desirable traits of *A. lancea*. It is also necessary to mass propagate and supply seedlings of elite lines for stable *A. lancea* cultivation to meet demands for traditional medicine raw materials and *So-jutsu* production. I examined the effect of *in vitro* shoot propagation by liquid culture as a high-throughput method for production of plantlets. The results suggested that *in vitro* shoot propagation by liquid culture is an efficient method for clonal propagation of *A. lancea* seedlings. I believe that these findings will contribute to the fundamental knowledge of *A. lancea* and play a key role in the development of elite cultivars and mass production of plantlets for stable production and supply of high-quality *A. lancea* plants in Japan.

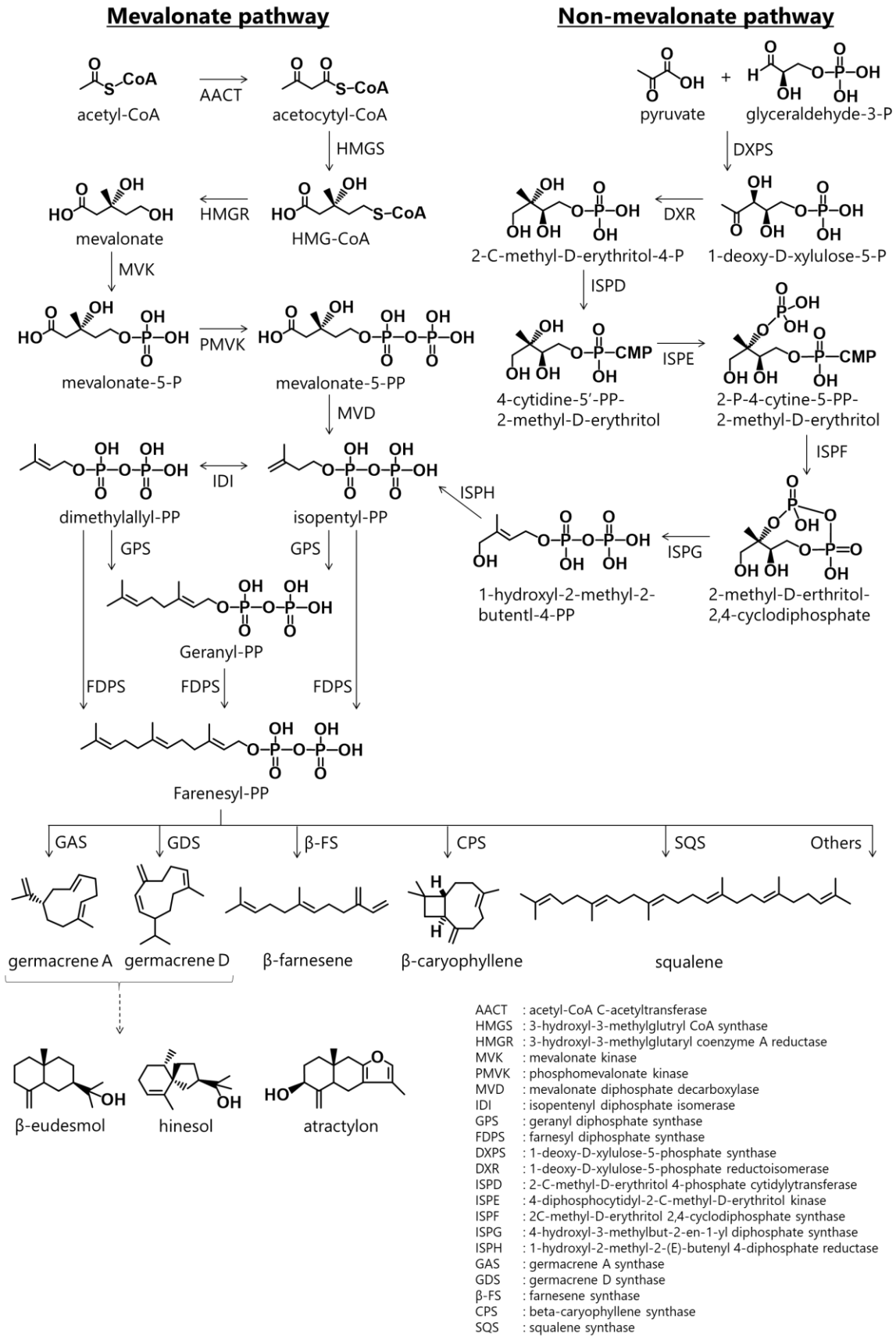

Figure 5.1 Putative biosynthesis pathway of sesquiterpenoids in *A. lancea*

5.2. Summary

Atractylodes lancea is a medicinal plant distributed across East Asia. Its rhizomes are used in traditional Japanese medicines (Kampo) and Chinese medicines to treat a lot of diseases and disorders because they contain many pharmacologically active compounds. *A. lancea* is one of the most important botanical raw materials for Kampo medicine due to its overwhelming use. In recent years, the demand for mass production of *A. lancea* with consistent quality has increased as the use of Kampo has increased.

The major active compounds in *A. lancea* are essential oil compounds, namely, β-eudesmol, hinesol, atractylon, and atractylodin, and the contents of these compounds are important quality criteria in *A. lancea* production. Moreover, important quality criteria for pharmacognosy are not only limited to the contents of essential oil compounds but also include the content ratio between β-eudesmol and hinesol. The quality of *A. lancea* rhizomes is closely related to their natural habitat. Furthermore, content and content ratio of essential oil compounds in *A. lancea* reportedly vary based on geographical factors. However, it is not clear whether the geographical variations are based on genetic or environmental factors. Therefore, this study aims to (1) evaluate the influence of genetic and environmental factors on the content of essential oil compounds in *A. lancea* and (2) evaluate heritability of β-eudesmol/hinesol content ratio in *A. lancea*.

A. lancea is a perennial plant, and it is propagated by division of rhizomes. A clonal line was propagated from a rhizome of a single plant, and 25 clonal lines were used in this study. The clonal lines were cultivated in different years (2016, 2017) and different locations (Hokkaido, Ibaraki), and heritability, G×E interactions, and influence of environmental factors on the content and content ratio of essential oil compounds in *A. lancea* were evaluated. To estimate the broad-sense heritability, calculations were performed using a model for vegetatively propagated crops like herbage.

In this population, broad-sense heritability of β-eudesmol, hinesol, atractylon, and atractylodin contents were 0.84, 0.77, 0.86, and 0.87, respectively. The effects of interannual variability on the contents of the compounds were lower than those of genotype. In addition, the cultivated environmental factors were assessed by different locations, and the correlations between Hokkaido and Ibaraki grown plants based on β-eudesmol, hinesol, atractylon, and atractylodin contents were 0.94, 0.94, 1.00, and 0.83, respectively. Furthermore, the broad-sense heritability of the β-eudesmol/hinesol content ratio was revealed to be high at 0.92. The effects of cultivation year and location were smaller than that of genotype, and few G × E interactions were observed. The results suggest that the content and content ratio of essential oil compounds in *A. lancea* are largely influenced by genetic factors, indicating that selective breeding and clonal propagation could be an effective strategy for obtaining populations with high content of essential oil compounds. Furthermore, the contents of β-eudesmol, hinesol, atractylon, and atractylodin in *A. lancea* exhibited few correlations with rhizome yields. *A. lancea* cultivars with not only high content of essential oil compounds but also high rhizome yield could be developed through selective breeding.

Breeding elite cultivars is only useful if they can be distributed for cultivation, and current propagation techniques are limited in how efficiently they can increase *A. lancea* seedlings. To increase *A. lancea* seedlings efficiently, *in vitro* propagation has been explored, but previous studies have only investigated *in vitro* propagation on solid media, not in liquid media. In general, liquid culture is an effective method for mass propagation of plant seedlings. In this study, the effect of liquid culture for *A. lancea* shoot propagation was investigated with the goal of developing mass production methods for *A. lancea* seedlings.

The shoot propagation rate of *A. lancea* was higher in liquid culture than on solid media, and the rate of shoot propagation was 3.9 shoots per initial shoot in liquid media with an optimal BAP

concentration after 8 weeks. A caveat of this method is that it is necessary to select a suitable strain for liquid culture. Under optimal culture condition, it is possible to produce thousands of plantlets from a single shoot in 1 year depending on the selected line. In addition, the rooting of shoots from *in vitro* culture on a rooting medium was consistent. However, liquid culture tended to induce the vitrification of leaves, warranting further investigation and optimization to reduced leaf vitrification. In this study, I demonstrated that the content and content ratio of essential oil compounds in *A. lancea* were largely influenced by genetic factors, indicating that selective breeding and clonal propagation are effective methods for reducing the variability of *A. lancea* quality. Furthermore, I showed that *in vitro* shoot propagation by liquid culture is an effective method for mass clonal propagation of *A. lancea*. I believe that the knowledge acquired in the present study will provide basic information for the development of elite cultivars and seedling production, and contribute to a stable production and supply of high quality *A. lancea*.

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