Seasonal Variation of Essential Oil Components in *Atractylodes lancea* (THUNB.) DC. Propagated by Division of Their Rhizomes[1)]

Osami TAKEDA,*,[a] Eiji MIKI,[a] Susumu TERABAYASHI,[a] Minoru OKADA,[a] Shan-an HE,[b] and Yutaka SASHIDA[c]

Tsumura Central Research Laboratories,[a] 3586 Yoshiwara, Ami-machi, Inashiki, Ibaraki 300–11, Japan, Jiangsu Institute of Botany,[b] Nanjing, Jiangsu 210014, People's Republic of China, Tokyo College of Pharmacy,[c] 1432–1, Horinouchi, Hachioji, Tokyo 192–03, Japan. Received November 15, 1995; accepted December 28, 1995

To elucidate the variation of essential oil components in the rhizomes of *Atractylodes lancea* growing in China we used nine clones of the species, which were propagated by division of the rhizomes, and characterized them by means of GC analyses of the six essential oil components. The seasonal changes in the components were also investigated for 3 years using one of the clones. These clones contained β-eudesmol (4) and hinesol (3) as the main components and the values of the correlation coefficients between 4 and 3 were more than 0.9 in every clone. Although the amount of each component varied in a clone, the value of the content ratio between the components was constant during growth of the plants for 3 years and each clone had a different ratio value. These results indicated that the biosynthetic ratio could be genetically determined. Implications of the variation in the components of this species in its native habitat were also discussed.

Key words *Atractylodes lancea*; seasonal variation; essential oil; hinesol; β-eudesmol; atractylon

Atractylodes lancea (THUNB.) DC. (Compositae) is a perennial plant which is native to central China. Its dried rhizome is used as the Chinese crude drug, "Cangzhu" and it has appeared in the Japanese and Chinese Pharmacopoeia. The crude drug is frequently prescribed for diuretic and analgesic purposes in traditional Chinese medicine. The rhizomes of *Atractylodes* spp. including *A. lancea* contain sesquiterpenes and polyacetylenes as essential oil components and these have pharmacological activities.[2)] In addition, some of the components have been used to distinguish between "Cangzhu" and another crude drug, "Baizhu", which is the dried rhizomes of *A. japonica* KOIDZ. ex KITAM. or *A. macrocephala* KOIDZ. (*A. ovata* DC.) in the Japanese Pharmacopoeia.

Previous studies have demonstrated the existence of three chemotypes of *A. lancea* growing wild in China in the essential oil components,[3a,b)] and these types are supported by the morphological data of the plants.[3c,d)] The plants of these types, however, grow under different geographical conditions.[3e)]

The growth stages and environmental factors of plants significantly affect the chemical constituents.[4,5)] Generally, phenotypic variation consists of genetic, environmental and developmental variations.[6)] In vegetatively propagated plants, the genotype of each plant (ramet) is uniform and, accordingly, variation among the ramets means environmental variance.[7)]

In the present paper, we investigated the variations in the essential oil components of *A. lancea* growing in China, among the vegetatively propagated plants of the species and the seasonal change in an experimental field over a period of three years.

Materials and Methods

Plant Materials Genetically homogeneous plants of *A. lancea* were obtained by division of the rhizome from a single plant. Nine clones: IN, TM, K-1, K-2, K-3, K-4-1, K-4-2, K-4-3 and K-4-4 were used in this experiment. The IN and TM clones were cultivated in the Tsumura medicinal plant garden as the botanical origin of the crude drug, "Sadosoujutsu" (in Japanese) that is thought to have been introduced into Japan from China in the 1700's. The others were derived from rhizomes of the species originally obtained from the People's Republic of China.

The nine clones were characterized by analyses of their essential oil components, and their IN was used to investigate seasonal variations in the components. Characterization of essential oil components contained in the nine clones.

Vegetatively propagated plants were cultivated in an experimental field in the medicinal plant garden located in Ami-machi, Inashiki-gun, Ibaraki prefecture. TM was cultivated for two years and the others for one year. IN was harvested on November 8, 1989, TM on November 25, 1992, and the other clones on January 10, 1994. These rhizomes were dried in an air oven at 30 °C. Six essential oil components: elemol (**1**), atractylon

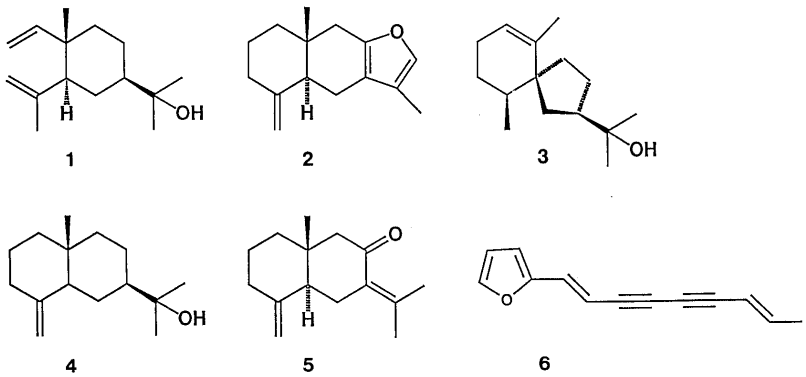

Chart 1

* To whom correspondence should be addressed.

(**2**), hinesol (**3**), β-eudesmol (**4**), selina-4(14),7(11)-dien-8-one (**5**) and atractylodin (**6**) were determined by capillary GC analysis, as previously described (Chart 1).[3a] Compounds **3** and **4** were purchased from Funakoshi but the others were obtained from the crude drug.

The powdered rhizome (0.2 g) was accurately weighed, extracted with hexane (10 ml) for 15 min under sonication, and then centrifuged (3000 rpm, 10 min). The residue was extracted with hexane (4 ml) for 5 min and centrifuged. The two supernatants were combined and phenanthrene (0.6 mg, 500 μl in hexane) was added as an internal standard. The solution was made exactly 20 ml by the addition of hexane, and then a 0.5 μl portion was subjected to capillary GC. The analysis was carried out using a Hewlett-Packard HP5890A gas chromatograph with FID. The component contents were calculated on the basis of the weight of the rhizomes, which were dried in an air-oven at 30 °C until a constant weight was obtained.

Seasonal Change in Essential Oil Components The subterranean parts of IN clone were divided into 37.8±0.6 g ($n=16$) and these were planted at intervals of 30 cm in rows 60 cm wide on May 8, 1984 in another experimental field located in Ohito-machi, Tagata-gun, Shizuoka prefecture. During cultivation, 16 plants were dug up at a time in different developmental stages, *i.e.*, dormancy (January), budding(from late March to early April), elongation of stem (May), setting of flower bud (July), flowering (September) and withering leaves and flowers (from late November to early December) over a three year period. The fresh weight of these rhizomes was measured after washing. These four rhizomes were dried in an air-oven at 105 °C and the dry weight was measured. From these results the mean value of the dry matter percentage of each rhizome was calculated and using this value, we estimated the dry weights of the other rhizomes. For analysis of the essential oil components, the other 12 rhizomes were dried in an air oven at 30 °C until a constant weight was attained. These 12 rhizomes were mixed and powdered, and the essential oil components, **2**, **3**, **4** and **6** contained in them were determined using GC. The powdered rhizomes (1 g) was accurately weighed, and extracted with hexane (20 ml) for 15 min under sonication, and then centrifuged (3000 rpm, 10 min). The procedure was repeated. The supernatants were combined and hexane was added to make exactly 50 ml. A portion (1 μl) of the solution was subjected to GC analysis according to Anetai and Yamagishi.[8] The analysis was carried out using a Shimadzu GC-7AG gas chromatograph with FID. Essential oil contents (ml/50 g of dried rhizome) and amounts of dilute ethanol-soluble extracts in the powdered rhizomes were determined according to the Japanese Pharmacopoeia.

Statistical Analysis To characterize the clones examined, calculations of correlation coefficient, principal component analysis and cluster analysis were carried out using the analytical data on essential oil components.[9] The calculations were done using the Lotus 1-2-3 program with an add-in program for statistical analysis (Lotus 1-2-3 Multivariate analysis v 1.0) provided by Audemain, Tokyo, Japan.

Results

Characteristics of Essential Oil Components of the Nine Clones The contents of the essential oil components are shown in Table 1. The mean contents of **2**, **3** and **4** in the clones showed a wide range of variation. The range of **2** was from 1.36% in TM for the highest to a trace amount in K-4-3 for the lowest; **3** was from 3.11 to 0.73%; and **4** was from 2.64% to 0.86%. Compound **5** was contained in only two clones, TM and K-3. In contrast, the range of **6** was small compared with the other components from 0.37% to 0.15%. The total contents varied from 6.08% in K-4-3 to 2.04% in K-4-2. IN, TM, K-2, K-3, K-4-2, K-4-**4** clones contained **4** as the main component while in the others it was **3**. Thus, each clone differed in content and composition of its constituents. The coefficient of variation for each component showed a different value in each clone; for example, in the IN clone this value of **2** was 200%, **3** was 34%, **4** was 33% and **6** was 16%. The value of each constituent was different among the clones. These values of **3**, **4** and **6** varied from 4 to 41% and **6** was an especially low value of from 4 to 27%. Compared to these constituents, **1**, **2** and **5** were highest in value; for example, the value of **1** varied from 71 to 400%.

Three groups were also recognized in the clones by cluster analysis using the nearest neighbor method on the basis of the analytical data (Fig. 1A). The result based on the cluster analysis was similar to that of principal component analysis (Fig. 1B). As shown in Table 2, the contribution rate of the first principal component (Z1) was 82.2% and that of the second principal component (Z2) was 16.4%, so that the cumulative contribution ratio of Z1 and Z2 was 98.6%. From the coefficient of each variable, Z1 was believed to indicate the total contents of **3** and **4**, namely a degree of deposition of white cotton-like crystal on its new section of the dried-rhizome,

Table 1. Contents of Essential Oil Components Contained in *Atractylodes lancea* Propagated by Division of Rhizomes

Clone	Number of plants examined	Component (% of dry weight) (C.V. %)						
		1	**2**	**3**	**4**	**5**	**6**	Total
IN	58	0.01±0.04 (400)	0.01±0.02 (200)	0.93±0.32 (34)	1.18±0.39 (33)	ND	0.28±0.04 (14)	2.40±0.78 (33)
TM	10	0.05±0.14 (280)	1.36±0.20 (15)	0.41±0.12 (29)	1.89±0.53 (28)	0.44±0.08 (18)	0.36±0.06 (17)	4.50±1.00 (22)
K-1	31	0.12±0.03 (25)	0.17±0.02 (12)	2.87±0.54 (19)	2.58±0.70 (27)	ND	0.15±0.04 (27)	5.88±1.25 (21)
K-2	4	0.08±0.05 (63)	0.12±0.07 (58)	1.98±0.19 (10)	2.02±0.22 (11)	ND	0.24±0.03 (13)	4.44±0.40 (9)
K-3	8	TR	0.22±0.02 (9)	0.91±0.08 (9)	0.92±0.11 (12)	0.06±0.06 (100)	0.23±0.01 (4)	2.34±0.15 (6)
K-4-2	10	0.01±0.03 (300)	0.15±0.03 (20)	0.84±0.27 (32)	0.86±0.35 (41)	ND	0.17±0.03 (18)	2.04±0.67 (6)
K-4-3	8	0.07±0.05 (71)	TR	3.11±0.45 (14)	2.64±0.42 (16)	ND	0.26±0.04 (15)	6.08±0.91 (6)
K-4-4	5	0.04±0.05 (125)	0.29±0.03 (10)	0.73±0.06 (8)	1.32±0.20 (15)	ND	0.37±0.04 (11)	2.75±0.32 (12)

1, elemol; **2**, atractylon; **3**, hinesol; **4**, β-eudesmol; **5**, selina-4(14),7(11)-dien-8-one; **6**, atractylodin; ND, not detected; TR, trace amount; C.V., coefficient of variation. Values represent mean±S.D.

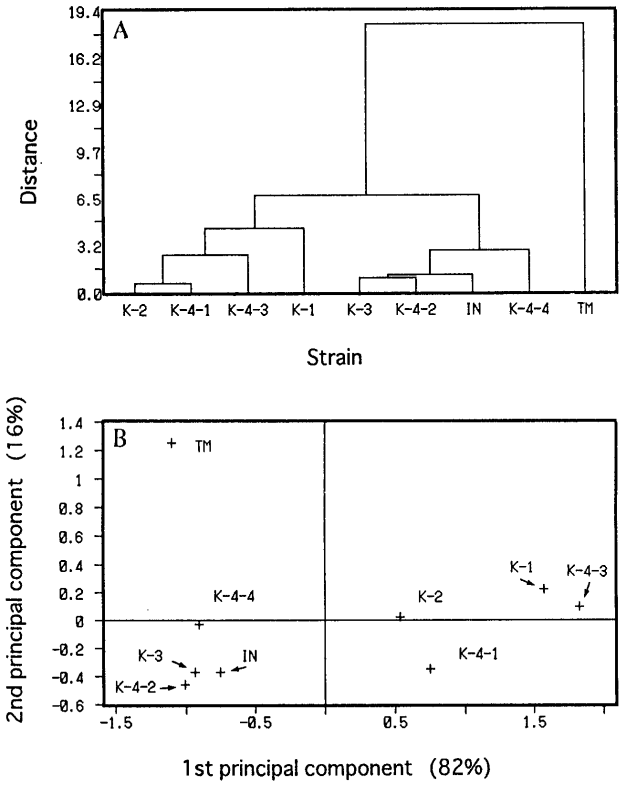

Fig. 1. Cluster Analysis and Scatter Diagram of Principal Component Score (Z1, Z2) for Clones of *Atractylodes lancea* Calculated on the Basis of Essential Oil Components by GC Analyses

A is a cluster analysis using nearest neighbor method based on standardized squared Euclidian distance. B is a scatter diagram of principal component analysis based on variance-covariance matrix.

Table 2. Eigenvectors and Eigenvalues from the Data of GC Analyses for Clones of *Atractylodes lancea* Propagated by Division of Their Rhizomes

Variable	Z1	Z2	Z3
1: elemol	0.029	0.025	−0.016
2: atractylon	−0.159	0.704	−0.580
3: hinesol	0.859	−0.209	−0.446
4: β-eudesmol	0.482	0.632	0.588
5: selina-4(14),7(11)-dien-8-one	−0.048	0.237	−0.288
6: atractylodin	−0.027	0.067	0.189
Eigenvalues	1.236	0.248	0.017
Cumulative contribution ratio	0.822	0.986	0.998

$\sum_{i=1}^{p} V_{ii}/p = 0.251$ (p: number of variables, V: variance).

and Z2 to mean the content of **2** and a content ratio of **4** to **3**. The three groups based on the statistical analysis were as follows: K-3, K-4-2, K-4-4 and IN clones belonged to the first group; K-1, K-2, K-4-1 and K-4-3 to the second one; TM to the third one. The first group contained **2** in low than trace amounts, the second group had a low content and the third group had a high content, while the content of **3** showed a significant positive correlation with that of **4** and the correlation coefficients between the two components were more than 0.9 (Fig. 2). The values of the content ratio of **4** to **3** were as follows: IN belonging to the first group was 1.27; K-1 belonging to the second one was 0.96; and TM belonging to the third one was 4.61. The coefficients of variation of these ratios

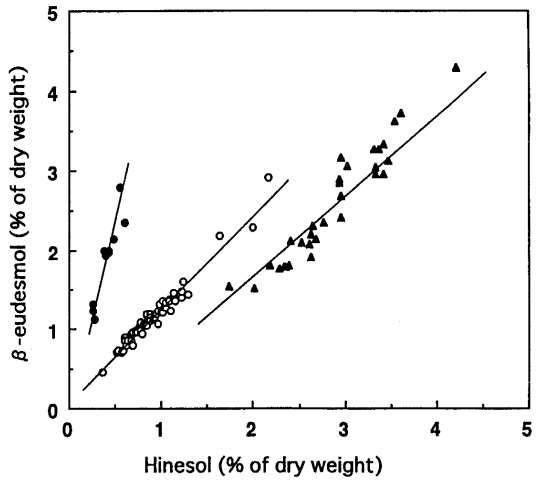

Fig. 2. Correlation between β-eudesmol and Hinesol in Clones of *Atractylodes lancea*

The clones examined (coefficients of correlation and number of ramets) were as follows: IN clone (○, $r=0.98^{**}$, $n=58$), TM (●, $r=0.94^{**}$, $n=10$), K-1 (▲, $r=0.96^{**}$, $n=31$). $^{**}p<0.01$.

showed low values (7, 11 and 9%, respectively).

Seasonal and Yearly Changes in Rhizome Yield and the Amount of the Dilute Ethanol Aqueous Extract The dry weight of the rhizome of the IN increased together with a marked elongation of the shoot and increases in the number of leaves in May until the leaf withered in November, and then decreased toward dormancy (Fig. 3). After budding, the decrease in the weight continued through elongation of the shoots in May of the next growing season. This growth pattern was repeated. The mean values of rhizome weights increased from 25.0 g in November in the first growing season to 46.9 g in September of the second season. In the third growing season, the weight was 54.0 g in November.

The dry weights of rhizomes had a maximum value at withering time of the aerial parts (from November to December), and then decreased gradually during dormancy. This tendency of the dry weight continued from March (budding) to May (elongation of shoot). In contrast, the amounts of the dilute ethanol aqueous extracts increased during dormancy and then decreased in March (budding). The amounts of dilute ethanol-soluble extracts varied from 57.8% to 37.5% (Fig. 3). In the period of dormancy, the amounts increased and then decreased during budding. Although such a tendency in the change became weak after the second growing season, the increase in the amount during dormancy correlated with the decrease in the dry weight of the rhizome. Accordingly, it is believed that the increase in the amount was a result of cold acclimation because of the increase in fructose from degraded inulin[10] and that the decrease was caused by utilization of the reserve substances to develop shoots. Mino *et al.* report that inulin, which is a major polysaccharide contained in the rhizome of *A. japonica*, varies with growth and development, and is degraded into low molecular substances for an energy resource.[11] Such a metabolic pathway caused the decrease in the rhizome dry weight. The decrease in the dry weight of the subterranean parts during dormancy was also reported for the root of *Scutellaria baicalensis* Georg. (Labiatae).[12]

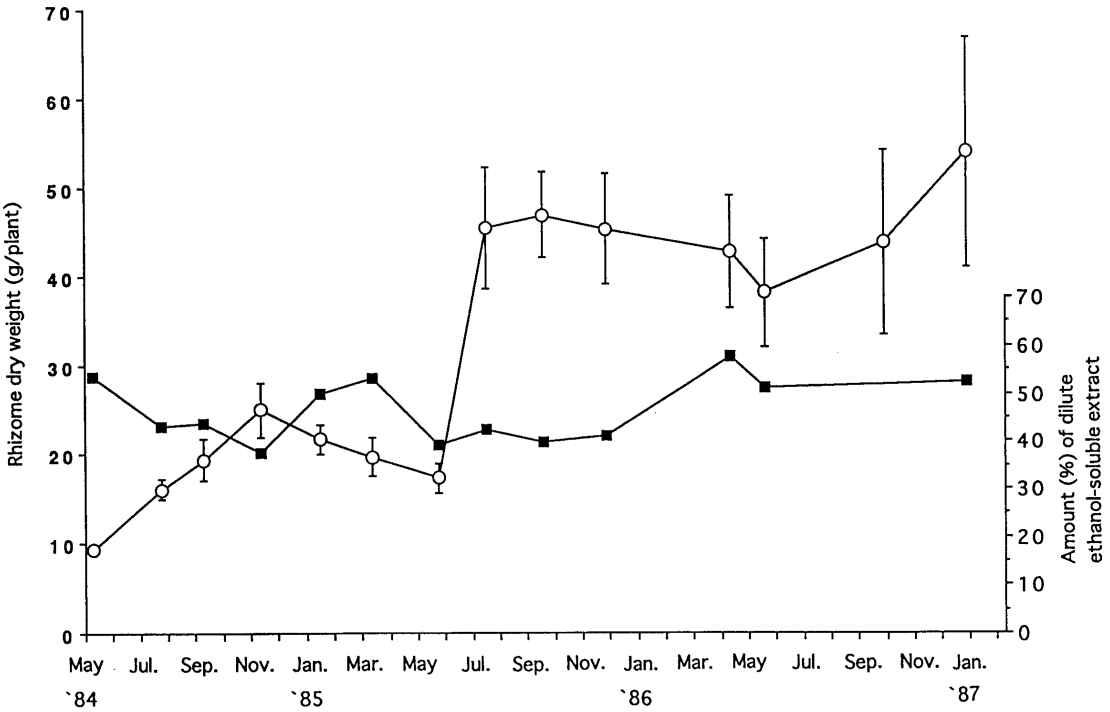

Fig. 3. Changes in Rhizome Dry Weight and Amount of Diluted Ethanol-Soluble Extract in *Atractylodes lancea* during Growth and Development

Vertical bar represents standard deviation from 16 plants of IN clone. ○, rhizome dry weight; ■, amount of diluted ethanol-soluble extract.

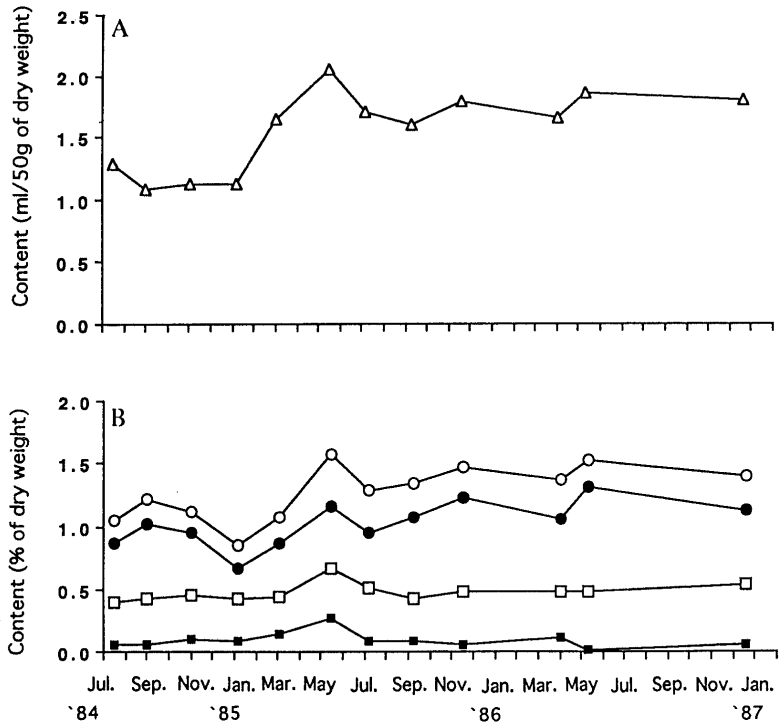

Fig. 4. Changes in Essential Oil and the Components Contained in Rhizomes of *Atractylodes lancea* during Growth and Development

A: contents (ml/50 g of dried rhizomes) of essential oil, B: contents (% of dried rhizomes) of essential oil components. ○, β-eudesmol; ●, hinesol; □, atractylodin; ■, atractylon.

Seasonal and Yearly Changes in the Content of Essential Oil of the Rhizome The mean values of the contents (ml/50 g of dried rhizome) of essential oil were 1.22 ml/50 g in the first growing season (Fig. 4A) and then increased in the second and third seasons (1.77 and 1.78 ml/50 g, respectively). The interannual variation in the contents of essential oil was similar to that of the components (Fig. 4B). The contents of **3** and **4** were higher in the second and the third growing seasons than in the first season. The values of the correlation coefficient between the contents of essential oil and those of **3** or **4** were 0.69, and 0.82, respectively, and these values were significant at the 0.1% level. The contents of **3** or **4** increased through September in the first growing season, and then decreased until

January. In May (budding), the contents increased again through May (elongation of shoot), and then decreased until July (setting of flower bud) and increased again. Thus, the contents decreased during the dormancy period. Although the contents of **3** and **4** changed during growth and development, the mean value of the content ratio of **4** to **3** were 1.25 and the coefficient of variation was 5% through the three growing seasons. Similarly, seasonal and yearly changes in the contents of **2** and **6** were small compared with those of **3** and **4**.

Discussion

Three types of *A. lancea* growing in China are recognized as having large individual variation of essential oil components.[3a,b] Although the nines clones used in this investigation were classified into three groups, these were included in the variation observed in the species. The plants of TM in the third group were similar to those of Mt. Maoshan type in Jiangsu province, one of the three types, in characteristics of the essential oil components, and the other plants in the first and second groups were of the Hubei–Anhui type.[3a,b]

The values of coefficient of variation of **3**, **4** and **6** were similar to those of components in *A. lancea*,[13] *A. japonica*, *A. macrochephala*[14] and *Aconitum carmichaeli* DEBEAUX (Ranunculaceae),[15] while the coefficient of variation of **1** showed the largest value among the analyzed components. This component seemed to be greatly influenced by environmental factors because it was present in low quantity in the examined clones. Thus, some of the components in the examined clones showed a large coefficient of variation; to characterize the clones more exactly, many more ramets must be used.

Using the IN clone, with which we were able to prepare many ramets, we investigated the seasonal and yearly changes of essential oil components. The content (ml/50 g) of essential oil was higher in the second and third growing seasons than in the first season, especially, the contents of **3** and **4** correlated to the growth of the plants. Kanamori *et al.* reported that the contents of the essential oil components are larger in the second growing season than in the first season during 2 years of cultivation.[16] In contrast, according to Kawanishi *et al.* the contents decreased in the second growing season and then increased in the third season.[17] Consequently, the yearly change in the contents appears to be mainly affected by cultivation conditions of the plants or climate factors. *A. lancea* in the wild has irregularly curved, cylindrical rhizomes and the contents of the middle positions of an individual are constant compared with those of the youngest or the oldest parts, which irregularly vary in content.[3a] According to Hiraoka, *A. lancea*, propagated by division of the rhizomes, has no clear correlation between the position of an internode in a rhizome and the contents of essential oil components.[18]

The dry weight of the rhizomes increased more than two-fold from the first to the second growing season. Compared to this, changes in the essential oil component contents were small, and the gas chromatogram pattern was constant. *Aconitum carmichaeli*,[19] *Datura metel* L. (Solanaceae)[20] and *Scopolia japonica* MAXIM. (Solanaceae),[21] vegetatively propagated by division of their roots or rhizomes, show a large seasonal variation in their major constituents in both parts. Essential oils of *A. lancea* are known to accumulate in their lysigenous oil-sacs and these oil-sacs are found in the cortex, xylem and pith.[22] This might be responsible for a small variation in the essential oil components during the growth of the plants. From these results, it is thought that variation of the constituents of in the species is due mainly to genetic variation.

Especially, a significant positive correlation was observed among **3** and **4** in the IN clone, and the value of the content ratio of the two components was constant during three years of cultivation. The other eight clones also showed a significant positive correlation between the two components and the mean value of the content ratio differed. These results indicate that the biosynthetic ratio of **3** and **4** is genetically determined in *A. lancea*.

One of the three types recognized in *A. lancea* which grows at a hight of 1000 m on Mt. Dabieshan in Hubei province in China is characteristic of the content ratio of **3** and **4**, and the individual variation is smaller than other types.[3b] Another type in Mt. Maoshan has the largest variation of content ratio, and **2** and **6** are its main constituents.[3a,b] Accordingly, the three types in the species growing in China are believed to be responsible for the difference in genotype. It also seems that a large variation in the components of the species in the native habitats is maintained by sexual reproduction. Guan *et al.* reported that in one native habitat, the head-flowers of the species set many seeds and the germination percentage is more than 90%.[23]

According to Hiraoka and Tomita, *A. lancea* propagated by micropropagation reaches a level of the mother plant year by year in content and this delayed accumulation of the essential oil might be related to insufficient growth of the oil-sacs in the rhizome.[13] To clarify the variation in the components of species in the wild, the results obtained in this investigation should be compared with those of seed propagation.

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