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Characteristics of Clonally Propagated *Aconitum charmichaeli* Debx. by Tissue Culture

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The homogeneity of aconitine-type alkaloids among the clonally propagated *Aconitum carmichaeli* Debx. population was confirmed by the qualitative HPLC analysis. It was shown that the quantities of aconitine-type alkaloids in the tubers are related to the harvesting time of tubers, and also the cultivation temperature. The mesaconitine and aconitine contents in tubers cultivated at 25°C were higher than those cultivated at 15°C. When the growth temperature was higher (25°C), the ratio of mesaconitine content to aconitine content was lower and that of mesaconitine content to hypaconitine content was higher than those in the tubers cultivated at lower temperatures.

Keywords——*Aconitum charmichaeli*; Ranunculaceae; clonal propagation; aconitine-type alkaloids; cultivation; phytotron

"Fu-tzu" or "Fu-pen", tubers of *Aconitum carmichaeli* Debx., a perennial herb of the family Ranunculaceae indigenous to China, has been used as one of the most important Chinese drugs, to be prescribed together with other herbal drugs, as an analgesic in the treatment of rheumatism and neuralgia. However, great care must be taken in the use of the crude drug, because the therapeutic dose and the toxic dose are quite near. The crude drug contains various toxic aconitine-type alkaloids such as aconitine, hypaconitine and mesaconitine and other pharmacologically active alkaloids, like higenamine,[1] coryneine,[2] benzoylaconine-type alkaloids[3] and lipoaconitine-type alkaloids,[4] and often, significant differences in the types and the quantities of alkaloids are observed among the tubers grown in different places[5,6] or harvested in different seasons.[7,8] In order to breed a homogeneous strain of *A. carmichaeli* Debx. with respect to the quality and quantity of aconitine-type alkaloids, we have already reported the clonal micropropagation of *A. carmichaeli* Debx. by shoot tip culture, the subsequent restoration of adult plants and short-term-cultivation for the analysis of alkaloids,[9] and also the micropropagation by somatic embryogenesis via anther culture.[10]

In this communication, we describe the homogeneity of alkaloid contents of 3-year-old clonally propagated plants, and the seasonal variation of aconitine-type alkaloids in them. Furthermore, the correlation between the aconitine-type alkaloids contents and the growth environment was studied by cultivating the clonally propagated plants at 15, 20, and 25°C in a phytotron.

Materials and Methods

Plant——Clonal *A. carmichaeli* plants were produced by shoot tip culture as reported previously.[9] The microtubers were stored in a refregerator (4°C) for 175 days before planting.

Cultivation in the field——The stored microtubers were transplanted during the month of May to the herbal garden, Faculty of Pharmaceutical Sciences, Kyushu University in 1986, and cultivated until October 12, 1987. The daughter tubers cultivated by division were repeatedly cultivated in the same field under the same conditions. Part of the tubers were harvested for the alkaloid analysis in October. For the seasonal variation studies, daughter tubers from a single plant were harvested monthly from September to February and assayed for their alkaloid contents. Daughter tubers (6) from one single plant were harvested in 1989 and, each was examined for its aconitine-type alkaloid content (Table I). Six daughter tubers of a single plant were planted in 1988, and from each of the 6 plants, one daughter tuber was harvested individually and each was used for the analysis of aconitine-type

TABLE I. Aconitine-type Alkaloid Contents in Individual Daughter Tubers from One Clonally Propagated Plant of *Aconitum carmichaeli*

Daughter tuber No.	% of dry weight			Fr. Wt. (g)
	Mesaconitine	Aconitine	Hypaconitine	
1	0. 271	0. 0686	0. 0510	2. 4
2	0. 307	0. 0809	0. 0443	12. 1
3	0. 267	0. 0622	0. 0216	12. 7
4	0. 290	0. 0704	0. 0633	13. 2
5	0. 218	0. 0538	0. 0353	16. 3
6	0. 286	0. 0593	0. 0293	21. 2
Average±S.D.	0. 273±0. 028	0. 0659±0. 0087	0. 0408±0. 0139	
(C.V.%)*	(10. 2)	(13. 3)	(34. 0)	

* Coefficient of variation.

TABLE II. Aconitine-type Alkaloid Contents of Individual One Plant Propagated by Division from a Single Plant of *A. charmichaeli*

Strain No.	% of dry weight			Fr. Wt. (g)
	Mesaconitine	Aconitine	Hypaconitine	
1	0. 225	0. 0443	0. 0218	13. 7
2	0. 207	0. 0421	0. 0262	12. 0
3	0. 194	0. 0370	0. 0233	7. 4
4	0. 209	0. 0466	0. 0221	14. 0
5	0. 225	0. 0482	0. 0215	11. 5
6	0. 220	0. 0395	0. 0284	13. 1
Average±S.D.	0. 213±0. 011	0. 0430±0. 0039	0. 0239±0. 0026	
(C.V.%)*	(5. 14)	(9. 05)	(10. 76)	

* Coefficient of variation.

TABLE III. Aconitine-type Alkaloid Contents in Clonally Propagated Plants of *A. charmichaeli*

Cultivation year	N	% of dry weight (C.V.%)*		
		Mesaconitine	Aconitine	Hypaconitine
1987	17	0. 195±0. 028 (14. 5)	0. 0546±0. 0072 (13. 2)	0. 0337±0. 0111 (32. 9)
1988	13	0. 198±0. 029 (14. 7)	0. 0382±0. 0067 (17. 5)	0. 0224±0. 0066 (29. 3)
Parental strain[9]	70	0. 127±0. 032 (26. 3)	0. 020±0. 009 (43. 1)	0. 041±0. 017 (41. 7)

* Coefficient of variation.

alkaloids (TABLE II). In order to determine the homogeneity of aconitine-type alkaloids, 30 tubers (one tuber from each of the 17 plants (1987) and from each of the 13 plants (1988) were harvested in October and assayed for their alkaloid contents (TABLE III).

Cultivation in phytotron——The stored tubers were cultivated in a mixture of clay and compost (3:1) in the phytotron from May 8 to September 30 in 1986, and from May 6 to October 12 in 1987, as indicated previously.[9]

Quantitative analysis of aconitine-type alkaloids——Aconitine-, hypaconitine- and mesaconitine-contents were analyzed by HPLC as previously described.[9]

Results and Discussion

The aconitine-type alkaloid contents and the fresh weight of individual daughter tubers from a single plant of clonally propagated *A. carmichaeli* are given in TABLE I. The coefficients of variation (C.V.) of the mesaconitine, aconitine and hypaconitine contents were 10.2, 13.3 and 34.0%, respectively, although the weights of the daughter tubers analyzed were quite different (betweem 2.1 g to 21.2 g). This suggests that the aconitine-type alkaloid production was not related to the size of the daughter tubers.

To find out whether the aconitine-type alkaloid contents vary among the clonal progeneis, the aconitine-type alkaloid contents of each set of daughter tubers from six plants which had been derived from a single parent plant and which had been grown under the same conditions were determined. Tle results are shown in TABLE II. The C.V. of mesaconitine, aconitine and hypaconitine were 5.1%, 9.1% and 10.8%, respectively. From these results it became evident that the aconitine-type alkaloid content was about the same among the plants of clonal strains cultivated under the same growth conditions. Some quantitative differences were observed, however, when the plants were grown in different conditions (Compare the results of TABLE I with those of II).

As reported previously, in the case of aconitine-type alkaloid contents of the conventional strain, which was the parent of the present clonally propagated plants, the C.V. of the mesaconitine, hypaconitine and aconitine contents were 26.3%, 41.7% and 43.1%, respectively.[9] On the other hand, the C.V. of mesaconitine and aconitine were 14.5 and 13.2%, respectively and that of hypaconitine was much larger, 32.9% in the clonally propagated plants (TABLE III). Therefore, compared with them, the aconitine-type alkaloid contents may be said to be quite homogeneous among the clonally propagated plants. This homogeneity of alkaloid contents was maintained during the continuous cultivation as indicated in TABLE III. It is theoretically possible to obtain more than 1.2×10^7 clornal plants from a single shoot in a year as reported previously.[9] In the case of normal cultivation of the plant, it propagates itself only by 6 times by division in a year. Therefore, it is evident that by clonal propagation of *A. carmichaeli* through tip tissue, we can rapidly obtain an enormous number of the plants with homogeneous alkaloid contents. Quantitative differences in aconitine-type alkaloids were observed between the tubers of different planting years as shown in TABLE III. This might be due to the effect of different environmental factors and/or the climatic variation. Hikino *et al.*[7] and Yoshikawa *et al.*[8] presented the seasonal variation of aconitine-type alkaloid contents of *A. carmichaeli*. However, the plants used were of the conventional strain cultivated in the usual manner. In this work, we investigated the seasonal variation of aconitine-type alkaloid contents using the daughter tubers from one single plant during the period from September to February (just before germination). The results are given in Fig. 1. The mesaconitine, aconitine and hypaconitine contents were the lowest in September and then, they gradually increased to reach their maxima in February. This result shows that etc. aconitine-type alkaloid contents of tubers may vary greatly depending upon the harvesting time of tubers, as previously reported.[7,8]

There are several other factors, such as growth temperature, soil fertility, humidity, light *etc.*, which may possibly affect the aconitine-type alkaloid contents, of which the most important factor seems to be the growth temperature. Therefore, the clonally propagated plants were cultivated in a phytotron at 15, 20, and 25°C for 2 years. The results are given in TABLE IV. The plant height was proportional to the cultivation temperature and the daughter tuber weight was inversely proportional to it. The mesaconitine and aconitine contents were higher when the plants were grown at higher temperatures. The ratio of the mesaconitine content to aconitine content (M/A) decreased but the ratio of the mesaconitine content to hypaconitine content (M/H) increased as the growth temperature was higher. Similar results were obtained in the repeated 2-year-cultivation experiment, indicating that the growth temperature clearly affects the biosynthesis of aconitine-type alkaloids and suggesting that to produce homogeneous crude drugs the clonally propagated plants of *A. carmichaeli* should be grown under the same conditions. Moreover, the total aconitine-type alkaloid content per daughter tuber was the highest when the plant was grown at 20°C, as indicated in TABLE IV, suggesting that the most suitable growth temperature is around 20°C.

It becomes clear that the clonal propagation of *A. carmichaeli* through shoot tips and axillary buds rapidly provided us with a strain having homogeneous alkaloid contents and a constant growth rate.

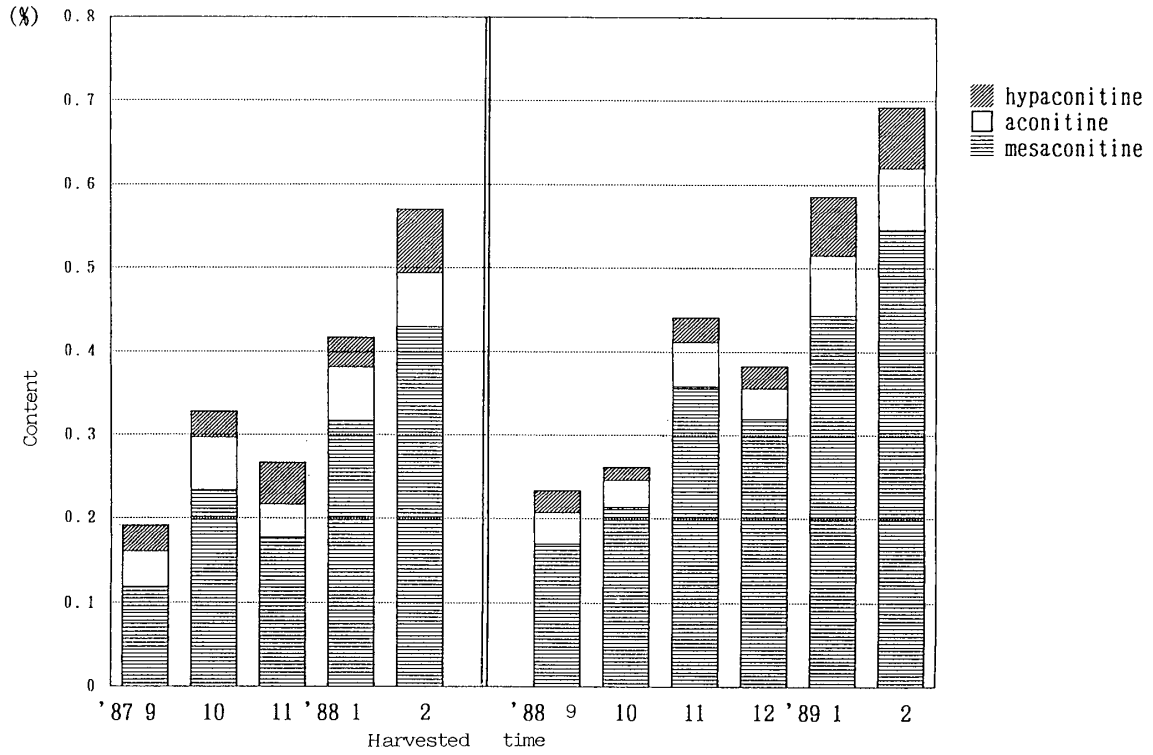

Fig. 1. Seasonal Variation of Aconitine-type Alkaloid Contents in Single Plant of *Aconitum carmichaeli*

TABLE IV. Effect of Cultivation Temperature on Aconitine-type Alkaloid Contents and Growth of Clonally Propagated Plants of *Aconitum carmichaeli*

Temp. (°C)	*N*	% of dry weight (c.v.%)*			M/A**	M/H***	Fresg wt. of tuber (g)	Plant height (cm)
		Mesaconitine	Aconitine	Hypaconitine				
15	10	0.158±0.016 (9.8)	0.0145±0.0046 (31.7)	0.0499±0.0147 (29.4)	10.9	3.2	14.51±2.38 (16.4)	19.3±2.5 (13.0)
20	10	0.241±0.028 (11.5)	0.0372±0.0088 (23.6)	0.0580±0.0180 (31.0)	6.5	4.2	9.97±1.50 (15.0)	24.7±6.4 (26.0)
25	10	0.256±0.036 (14.0)	0.0605±0.0109 (18.0)	0.0326±0.0066 (20.2)	4.2	7.8	6.72±1.17 (17.4)	34.7±6.1 (17.6)

* Coefficient of variation. ** Ratio of mesaconitine and aconitine content. *** Ratio of mesaconitine and hypaconitine content.

This is the first evidence to elucidate the relation between the aconitine-type alkaloid contents of *A. carmichaeli* and the growth or environmental factors.

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