Differences in Caffeine, Flavanols and Amino Acids Contents in Leaves of Cultivated Species of *Camellia*

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Caffeine, flavanols and amino acids in the first flush shoot leaves of 23 species belonging to the genus *Camellia* were assayed by gas and high performance liquid chromatography. Based on these results, principal component analysis was performed. *Camellia sinensis* and *C. taliensis* of section *Thea* contained caffeine, five flavanols (catechins) and theanine (the most abundant amide in tea), whereas *C. irrawadiensis* of the same section was characterized by the absence of caffeine. Chemically, *C. taliensis* was closely related to *C. sinensis*. Most species in sections other than *Thea* did not contain detectable amounts of caffeine or theanine, except for a clone of *C. kishi* of section *Paracamellia* that contained a small amount of caffeine and *C. furfuracea* of section *Heterogenea* which contained theanine. An approximate correlation of the flavanol patterns with subgeneric sections was made. ECG (epicatechin gallate) and EGCG (epigallocatechin gallate) were present in only the plants of section *Thea*. In most of the species studied, the EC (epicatechin) content was higher than that of (+)-C ((+)-catechin), but in four species, *C. furfuracea* and *C. granthamiana* of section *Heterogenea*, and *C. oleifera* of section *Paracamellia* and *C. japonica* subsp. *hozanensis* of section *Camellia*, (+)-C was the predominant flavanol. The flavanol gas chromatograms of *C. sasanqua*, *C. saluenensis* and *C. japonica* subsp. *hozanensis* showed 4 marked peaks derived from unidentified components.

KEY WORDS : genus *Camellia*, chemotaxonomy, leaf, caffeine, flavanols, amino acids.

Introduction

According to SEALY (1958), the genus *Camellia* includes as many as 82 species which are classified into 12 subgeneric sections. He also noted that it was difficult to determine the interrelationships between the species. *Camellia sinensis* (tea) contains characteristic compounds, such as caffeine, catechins and theanine. The determination of the contents of these compounds in each species of the genus *Camellia* is important not only from the angle of tea breeding with interspecific hybrids, but also as a means of clarifying the metabolic specificity of tea in relation to comparative physiology. ROBERT *et al.* (1958), SAKAMOTO (1962), NAKAGAWA and SAKAMOTO (1967) and KONISHI *et al.* (1969) have studied these compounds in *C. taliensis*, *C. irrawadiensis*, *C. japonica*, *C. sasanqua*, *etc.* We (1981) have also reported specific differences in the composition of caffeine and amino acids in leaves sampled from camellia plants grafted on *C. sinensis* or *C. japonica*. In addition, some compounds are biosynthesized in roots and translocated to leaves; *e. g.*, theanine. Therefore, chemotaxonomical data presented in this paper were derived from rooted cuttings or seedling plants of 21 species in 6 subgeneric sections.

Materials and Methods

The materials were mostly obtained from species collections maintained in Saitama Prefectural Tea Experiment Station, Faculty of Agriculture of Niigata University, camellia collection by "Kodomo-no-kuni" in Yokohama and others in 1980. They were sampled

Received March 12, 1984.

from cuttings in the greenhouse of the National Research Institute of Tea and cultivated in (1/5000)a Wagner pots. Ammonium sulfate 1 g and rapeseed meal 1 g were added to each pot every three months. Some of the data were derived from plants grown in the greenhouse or fields at the Institute.

Young leaves of first flush shoots were sampled from February to May in 1982 and in the spring of 1983. The fresh leaves collected from several plants of each clone were mixed and steamed, then dried and ground. The dried leaf powder was then stored in a refrigerator until analysis. The weight of the leaf powder from each clone ranged from 90 to 3000 mg.

The analyses of the leaves are shown schematically in Fig.1. The limit of detection was about 0.01% for each compound based on the dry weight of the leaves. The values obtained in each sample were estimated by single analytical measurement.

Apparatus

A gas chromatograph (Hitachi 063) equipped with a flame ionization detector was used for the detection of caffeine and flavanols.

A high performance liquid chromatograph (Waters Assoc.) consisting of two Model 45 pumps and a Model U6K injector was used for the detection of amino acids. Fluorescence was detected using a Model 410 fluorescence detector with a 360 nm excitation filter and a 500 nm emission filter.

Caffeine analysis

An extraction procedure based on the official methods of analysis of tea (Chemical Laboratory of Tea Research Station, 1970) and a modification of the procedure described by INA and MORISITA for gas chromatography (1969) were applied.

A sample of leaf powder, weighing from 20 to 50 mg depending on the weight of each stored sample, was moistened with 5% sodium carbonate solution for 1 hr, transferred

Fig.1. Analysis of caffeine, flavanols and amino acids in leaves of plants of the genus *Camellia*.

to a caffeine extractor, and extracted with chloroform for 3 hrs. The residue obtained after chloroform evaporation was dissolved in acetone containing methylmyristate as an internal standard. This solution was then injected into the gas chromatograph. Three percent of SE-30 on Chromosorb G packed in a glass column (2 m×3 mm) was used for 30 ml/min of nitrogen carrier gas. Oven, injector and detector temperatures were maintained at 200, 250 and 250°C, respectively.

Flavanol analysis

The gas chromatography of flavanols was described by NAGATA (1981).

A quantity of leaf powder ranging from 20 to 60 mg was extracted with 10 ml of 80% aqueous acetone for 24 hrs. Acetone in 5 ml supernatant liquid was removed by evaporation, and the residue solution brought to 5 ml by addition of water, then extracted with 3×10 ml ethylacetate. The residue obtained after evaporation of ethylacetate was dissolved in acetone. A certain amount of the acetone solution was transferred to a small vial. After removal of acetone under a nitrogen stream, the residue and lignoceric acid as an internal standard were trimethylsilylated with BSTFA-TMCS (10 : 1) at 50°C for 30 min and chromatographed.

Amino acid analysis

In a previous study, the procedure described by TAKEO (1979) had been applied and at least 500 mg of the sample had been required for the automatic amino acid analyzer. In this study, since some of the samples weighed less than 100 mg, the procedure based on OHTUKI *et al.* (1979) was adopted and at least 50 mg of the sample was required for high performance liquid chromatography.

A quantity of leaf powder weighing from 50 to 100 mg was extracted with hot water. The extract was centrifuged and filtrated. After removal of tannins by ethylacetate extraction, equal volumes of sodium dodecylsulfate (2%), twofold volumes of sodium bicarbonate (0.4 M, pH 8.3) and equal volumes of 1% Dns-Cl/acetone were added to the filtrate. After incubation at 50°C for 1 hr, this sample was dried up with a nitrogen stream. The injection solution was prepared by dissolving the residue in the initial mobile phase of chromatography.

This sample was analyzed on a 30 cm×3.9 mm I.D. μ Bondapak C₁₈ column (Waters Assoc.). Resolution of peaks was achieved using gradient elution with mobile phase in pump A consisting of 0.1 M sodium acetate buffer, pH 7.0-acetonitrile (90 : 10) and mobile phase in pump B consisting of the same compounds (30 : 70). The mobile phase started at a flow rate of 1.0 ml/min at 100% A and decreased to 60% A using a linear gradient over 30 min at which time an isocratic hold lasting 10 min was set up. Since the peaks derived from arginine and alanine could not be separated, the value obtained for arginine may contain a few percent of alanine.

Principal component analysis

The principal component analysis was carried out by using the Norin Library (SUZUKI, 1979) in the Computer Center for Agriculture, Forestry and Fisheries Research.

Results

The results of the analysis of caffeine, flavanols and 6 main amino acids are presented

Table 1. Caffeine, flavanol and amino acid contents in leaves of plants from the genus *Camellia* (% per dry weight)

Species	Abbreviation ²⁾	Collection site ³⁾	Caffeine	Flavanols ⁴⁾				unidentified 4 peaks ⁶⁾	Amino Acids ⁷⁾						
				EC	(+)-C	EGC	EGCG		Thea.	Arg.	Gln.	AA	GA	Ser.	
SECTION THEA															
<i>C. sinensis</i> var. <i>sinensis</i>	SS	Kanaya	2.78	1.13	0.07	2.38	1.35	8.59	—	1.21	0.06	0.07	0.13	0.23	0.04
<i>C. sinensis</i> var. <i>assamica</i>	SA	Kanaya	2.44	1.44	0.02	0.35	3.35	12.10	—	1.43	0.24	0.19	0.20	0.28	0.10
<i>C. taliensis</i>	T	Iruma	2.54	0.58	tr	0.80	1.90	6.84	—	0.27	0.15	0.01	0.05	0.06	0.01
<i>C. irrawadiensis</i>	I	Iruma	0	0.72	0.03	0.12	0.67	0.21	—	0.21	0.38	0.02	0.07	0.10	0.02
		Niigata	0	0.79	0.03	0.12	0.83	0.28	—	0.27	0.11	0.05	0.11	0.16	0.02
SECTION PARACAMELLIA															
<i>C. sasanqua</i>	SQ	Kanaya	0	0.02	0	0	0	0	+	0	0	0.01	0.02	0.02	0.01
		Kanaya	0	0.03	0	0	0	0	+	0	0.04	0.02	0.06	0.10	0.01
<i>C. kissi</i> *1)		Makurazaki	0	0.02	0	0	0	0	—	0	0.97	0.10	0.18	0.13	0.02
		Yokohama	0	0.04	0	0	0	0	—						
**1)		Kyoto	0.02	tr ⁵⁾	tr	0	0	0	—						
<i>C. oleifera</i>		Iruma	0	0	0.19	0	0	0	—	0	0.34	0.01	0.01	0.03	0.01
		Kikugawa	0	0	0.14	0	0	0	—	0	0	0	0.01	0.01	0
SECTION CAMELLIA															
<i>C. japonica</i>	J	Kanaya	0	4.81	0.25	0	0	0	—	0	0.05	0.02	0.05	0.10	0.02
<i>C. japonica</i> var. <i>decumbens</i>	JD	Yokohama	0	3.57	2.04	0	0	0	—	0	0.11	0.01	0.03	0.05	0.01
		Niigata	0	3.13	0.27	0	0	0	—						
<i>C. japonica</i> form. <i>macrocarpa</i>		Yokohama	0	2.55	1.90	0	0	0	—						
<i>C. japonica</i> subsp. <i>hozanensis</i>	JH	Niigata	0	0.11	3.38	0	0	0	+	0	0.14	0	0.01	0.02	0
		Yokohama	0	0.10	3.03	0	0	0	±						
<i>C. reticulata</i>		Niigata	0	0.26	0.11	0	0	0	—	0	1.24	0.04	0.08	0.09	0.02
<i>C. saluenensis</i>	SL	Niigata	0	0.35	0.07	0	0	0	+	0	0.54	0.17	0.20	0.24	0.04
		Yokohama	0	1.54	0	0	0	0	±						
<i>C. pitardii</i>	P	Yokohama	0	6.64	0.25	0.46	0	0	—	0	0.02	0.15	0.02	0.05	0.01
SECTION HETEROGENEA															
<i>C. furfuracea</i>	F	Iruma	0	0.16	0.58	0.05	0	0	—	0.02	0.05	0.01	0.06	0.09	0.01
<i>C. granthamiana</i>	G	Niigata	0	0.14	1.37	0	0	0	—	0	0.10	0.01	0.03	0.05	0.01
		Kikugawa	0	0.12	3.10	0	0	0	—	0	0	0	0.01	0.02	0

in Table 1.

The caffeine content of *C. sinensis* and *C. taliensis* of section Thea was identical, while *C. irrawadiensis* of the same section did not contain caffeine. In the plants of the other five sections studied, no caffeine was detected except in one clone of *C. kissi*.

All the flavanols surveyed were detected in the three species of section Thea. Large amounts of both ECG and EGCG were observed in *C. sinensis* and *C. taliensis*, and the content of EGCG exceeded 6%. On the other hand, ECG and EGCG contents in *C. irrawadiensis* were below 1%. WOOD and BARUA (1958) reported that EC and EGC could not be detected by paper chromatography in *C. irrawadiensis*. But in this study the gas chromatogram of *C. irrawadiensis* showed clear peaks at the retention time corresponding to EC and EGC. We considered that these peaks confirmed the presence of EC and EGC in *C. irrawadiensis*.

The plants of the other sections studied showed various patterns of flavanol components. In plants of the genus *Camellia* except for the section Thea, we could not detect ECG or EGCG. *C. pitardii* and *C. furfuracea* contained EGC (epigallocatechin) of a low concentration in their leaves unlike the other species.

In the case of the sections Paracamellia, Camellia and Heterogenea, EC or (+)-C or both were detected in all leaves. EC content of section Camellia was high, comparatively. However, the content of EC and other catechins was low or negligible in plants of sections Camelliopsis and Theopsis.

In most of the species studied, EC content was higher than that of (+)-C. However, in two species of section Heterogenea, *C. oleifera* and *C. japonica* subsp. *hozanensis*, (+)-C was the main component of the flavanols.

Gas chromatography of flavanols in a few species such as *C. sasanqua*, *C. japonica* subsp. *hozanensis* and *C. saluenensis*, revealed the presence of four peaks derived from unidentified components in a considerable amount. These results are shown in Table 1.

Theanine which was the most abundant and characteristic amide in tea was detected in all the leaves of plants of the section Thea. Though the theanine content in *C. sinensis* was above 1%, that of *C. taliensis* and *C. irrawadiensis* ranged from 0.2 to 0.3%. Theanine was not detected in the other five sections, except for *C. furfuracea* of section Heterogenea which contained 0.02% theanine. KONISHI *et al.* (1969) reported that *C. sasanqua* contained theanine and we (1981) also reported trace amounts of theanine below 0.01% in this species. However, in this study the presence of theanine in *C. sasanqua* could not be confirmed, since its content might be below the detectable level.

The content of other amino acids such as arginine, glutamine, aspartic acid, glutamic acid and serine varied among the leaves examined. There was no clear correlation between the contents of these five amino acids and the taxonomical classification of the species or the subgeneric sections. It was evident from a large number of investigations that the content of amino acids in leaves was influenced by a variety of environmental factors and by the leaf age. In this study although the age of the trees from which the leaf samples were collected and the method of fertilizer application were uniform, and care had been exercised to minimize variations due to leaf age or environmental factors, the variations in the contents of amino acids may still have been caused by these factors.

Furthermore, principal component analysis was applied to 27 samples using both the contents of 13 compounds in Table 1 and those of amino acids except arginine, glutamine, glutamic acid, aspartic acid and serine. The results of the latter group were more significant than those of the former presumably because the principal component analysis of the former group was correlated to the contents of the 5 amino acids. As regards the principal component analysis of the latter group, factor loadings and contributions of the first, second and third principal components are shown in Table 2. Total contributions of the first three components were 82.4%. The scatter diagram of each sample in the first and second principal component plane is also shown in Fig. 2. The first principal component was considered to be a factor concerned with the difference between the plants of section Thea and those of the other five sections studied, while the second principal component corresponded to the factor concerned with the presence of four unidentified peaks and contents of EC and (+)-C.

Discussion

In this study, three species of section Thea were characterised by the presence of EGC, ECG, EGCG and theanine, especially ECG and EGCG. *Camellia taliensis* showed phytochemical similarities with *C. sinensis*, which was also confirmed by the principal component analysis, as shown in Fig. 2. But *C. irrawadiensis* of this section did not contain caffeine. These results support the data reported by ROBERT *et al.* (1958) who showed that the paper chromatogram of *C. taliensis* was very similar to that of tea, and also that the presence

Table 2. Factor loadings and contributions of first (Z_1), second (Z_2) and third (Z_3) principal components computed from the contents of caffeine, flavanols, theanine and the presence of four peaks derived from unidentified components (4 P)

Compound	Principal component		
	Z_1	Z_2	Z_3
Caffeine	-0.97	-0.10	-0.01
EC	-0.13	0.56	-0.71
(+)-C	0.22	-0.42	-0.73
EGC	-0.79	0.03	-0.14
ECG	-0.92	-0.08	0.05
EGCG	-0.98	-0.11	0.00
4 P	0.22	-0.79	-0.11
The.	-0.95	-0.09	-0.01
Contribution	54.6	14.4	13.4

Abbreviations : see Table 1.

Fig. 2. Scatter diagram of 27 *Camellia* plants in the first and second principal component plane.

● : section Thea, ■ : section Paracamellia, ▼ : section Camellia, ▽ : section Heterogenea, ○ : three plants of section Camelliopsis and two plants of section Theopsis, ◆ : three plants of section Theopsis. Abbreviations : see Table 1.

of caffeine in *C. irrawadiensis* could not be confirmed. Recently KONDO (1979) reported that *C. sinensis* and *C. irrawadiensis* showed some degree of interspecific variations in their karyotypes, even though each species had its own characteristic karyotype. Chemically, *C. irrawadiensis* could be distinguished from *C. sinensis* or *C. taliensis* due to the absence of caffeine or the low content of EGCG. In view of the pathway of catechin biosynthesis in the plants of the genus *Camellia*, it was suggested that only the members of the section *Thea* had some specific enzymes for the formation of ECG and EGCG from gallic acid.

In most species of the section *Camellia* EC content was above 1%. Whereas in all the species of the section *Paracamellia* studied EC content was below 0.02%. (+)-C content of the two species of section *Heterogenea* was higher than that of EC. In none of the species of section *Camelliopsis* studied and two species of section *Theopsis* were EC and (+)-C detected. In view of the flavanol patterns mentioned above, it is evident that there are considerable intrasubgeneric variations in flavanol contents. This finding suggests that the pathway of catechin biosynthesis corresponds to some extent to the subgeneric sections with a few exceptions. However, flavanol characteristics alone were not always sufficient to identify a subgeneric section.

A flavanol profile with high (+)-C content compared with that of EC was a characteristic feature of the section *Heterogenea*. It was interesting to note that similar phenomena occurred in *C. oleifera* and *C. japonica* subsp. *hizanensis*. Moreover, *C. furfuracea* of section *Heterogenea* characteristically contains EGC and theanine, and phytochemically, this species is unique in its constituents. The specific metabolism of *C. furfuracea* may require further investigations.

Flavanol analysis showed the presence of considerable differences between *C. japonica* subsp. *hizanensis* and the other species of section *Camellia* studied. Although it was difficult to assign this subspecies to a section, it was possible to assume on the basis of the flavanol pattern that this subspecies resulted from a cross between *C. japonica* and an unknown species which does not belong to the section *Camellia*.

In our previous report (NAGATA and SAKAI, 1981), a caffeine content of 0.06% was detected in grafted *C. kissi*. In the present study, however, such value was detected only in a clone of *C. kissi*. Caffeine is the most common component in stimulant plants and crops such as tea, coffee, cocoa and mate. NAKAO (1976) reported that *C. kissi* has been used for a local beverage in Southeast Asia. According to our study, *C. kissi* may contain traces of caffeine.

Acknowledgements

We would like to thank Professor K. HAGIYA of Niigata University, Dr. Y. FUCHINOUE of Saitama Prefectural Tea Experiment Station, Mr. S. KIRINO of "Kodomo-no-kuni" and Mr. M. HIRANO of Kyoto Prefectural Tea Research Institute for providing research facilities and plant materials for this work. We would also like to thank the members of the First Tea Laboratory of the Makurazaki Branch of the National Research Institute of Tea for supplying plant materials. Acknowledgements are also due to Dr. K. OHOTUKI, Associate Professor of Kyoto Prefectural University for his valuable suggestions and Dr.

T. TAKEO of the National Research Institute of Tea for correcting the manuscript.

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カメリア属栽培種における葉内カフェイン、フラバノール、 アミノ酸含量の種間変異

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カメリア属各種植物 23 種の第一次伸育葉中のカフェイン、フラバノール、アミノ酸含量を、ガスクロマトグラフィーと高速液体クロマトグラフィーをもちいて調査し、主成分分析を行った。チャ節に属するチャ、タリエンス、イラワジエンスは 5 種のカテキン類とテアニンを含むしたが、イラワジエンスにはカフェインが検出されなかった。葉中成分組成からみるとタリエンスがチャと最も密接な関係があった。テアニン、カフェインは他節の植物中からはほとんど検出されなかった。しかしフルフラセアからテアニンが、またキッシー 1 系統からカフェインが少量検出された。

フラバノール組成は各節ごとに一定の特徴が認められた。フラバノールのうちエピカテキンガレートとエピガロカテキンガレートはチャ節 3 種のみから検出された。また大部分の種はエピカテキンが(+)-カテキンより高濃度であるのに対し、ヘテロジェニア節 2 種とオレイフェラ、鳳山椿は(+)-カテキン含量がエピカテキンを上まわった。さらにサザンカほか 2 種のガスクロマトグラム上に未同定の 4 つのピークが現われた。