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Variation in the Quality and Quantity of Flavonoids in the Leaves of Coastal and Inland Populations of *Adenophora triphylla* var. *japonica*

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羽柴　敬子[1]・岩科　司[2]・松本　定[2]：海岸型および内陸型ツリガネニンジンのフラボノイド組成と相対量の変異

The coastal environment is attributed to various stresses. Salinity and wind are two of the major stresses in the coastal region, where limited plant species tolerable to those stresses become dominant (Miyawaki 1983). Such limitations in terms of viable species result in relatively low vegetative cover, and consequently increase exposure to excessive light, accompanied with high-level ultraviolet radiation.

Ultraviolet-B radiation (280〜315 nm; UV-B) is known to be a strong factor that can destroy living organisms (Shibata 1985, Rozema *et al.* 1997, Ichihashi and Sasaki 2000). It is known that an excessive amount of UV-B causes distortion of photosynthetic organs and decreases plant growth (Tevini *et al.* 1983, Teramura *et al.* 1984, Tevini and Teramura 1989).

Plants that occupy the maritime areas are thought to be adaptive to excessive sunlight and UV-B. Epicuticular wax and hairs play an important role for avoiding damage caused by excessive light (Karabourniotis and Bornman 1999, Laakso *et al.* 2000, Holmes and Keiller 2002, Manetas 2003). It is observed in some species that the coastal individuals acquire physiological and morphological traits that are different from those of inland individuals. Such ecotypic divergence may occur as a consequence of adaptation to the harsh environment.

Flavonoids are effective UV-B screening compounds that are synthesized in plants (Caldwell *et al.* 1983, Cokell and Knowland 1999). Flavonoids, especially those accumulated in the epidermal layers of leaves, act as effective UV-B screens. Experimental studies report that the induction of flavonoid synthesis, especially increase of chalcone synthase (CHS) activity, occurs under enhanced UV-B radiation, confirming its protective function against UV-B (Kakegawa *et al.* 1991, Tevini *et al.* 1991). Recently, the UV-absorbing substances in translucent bracts of an alpine plant, Himalayan *Rheum nobile* were characterized as some flavonol glycosides such as quercetin 3-[6″-(3-hydroxy-3-methylglutaroyl)-glucoside] (Iwashina *et al.* 2004)

Adenophora triphylla var. *japonica* is a perennial herbaceous species that has intermittent distribution from the coastal to the inland areas (Chibaken-shiryo-kennkyu-zaidan 2003, The Flora-Kanagawa Association 2001). Morphological differences are observed between coastal and inland populations. Coastal populations have relatively small, thick and glaucous leaves, and characteristically have decumbent stems. Inland populations have large, thin, and non-glaucous leaves, and the stems usually grow vertically. Not only such morphological differences but also physiological heterogeneity may occur within the species.

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The first objective of this study was to identify and compare the flavonoid compositions of coastal and inland populations. Amounts of common flavonoids of each population were also measured. Based on these qualitative and quantitative analyses of flavonoids, UV-B effects on composition and content of flavonoids in plants originating from different habitats was discussed.

Materials and methods

Study area and sampling procedure

Plants were collected from Suzaki Peninsula located in the southern rim of Central Japan (Fig. 1). The peninsula extends into the Pacific Ocean, so that the area is located in a warm oceanic climate with an average annual temperature of 16.5℃ and annual precipitation of 2,000 mm (Japan Meteorological Agency 2004).

Four populations were selected for chemical analyses. Three populations (**S1, S2** and **S3**) with thick and glaucous leaves were located on the hill that was faced with the ocean. One population (**TBG**) in the Tsukuba Botanical Garden (Ibaraki, Japan) was sampled as a representative of inland population. Details of each sampling site are shown in Table 1.

Sampling was conducted in August 2004 (flowering season). Five to 8 flowering individuals were randomly selected from each population. All samples were weighted and 0.3 g of the total weight extracted in 8 ml of MeOH for quantitative analysis.

UV-B exclusion experiment

The experiment was designed to reveal the effects of UV-B exclusion. In September 2003, 7 inland individuals and 17 coastal individuals were collected from sampling sites mentioned previously, and were transplanted in pots and kept in the greenhouse in Tsukuba Botanical Garden. The relative UV-A and UV-B intensities in the greenhouse (GH) were 32% and 59% regarding UV intensities under all-sky as 100%. Water was automatically supplied once a week. Sampling was conducted in June 2004. Sampling procedures were the same as described above except that all the individuals sampled in the coastal area was regarded as one population.

Chemical analysis of UV absorbing compounds

(1) Qualitative analysis

Fresh leaves from coastal and inland individuals (15 g fresh weight each) were cut into pieces, extracted

Table 1. Description of sampled plant populations and their habitats

Sampled population	Horizontal distance from the shoreline (km)	Relative openness (%)	Relative UV intensity (%)		Altitude (m)	Slope direction
			UV-A	UV-B		
S3	0.17	89	125	116	13	W
S2	0.13	89	80	79	10	W
S1	0.05	60	38	43	4	E

Relative openness was calculated by using free software CANOPON ver.2. (Takenaka 2003). Relative intensity of UV, measured in June 2004, was calculated regarding UV under all-sky is 100%.

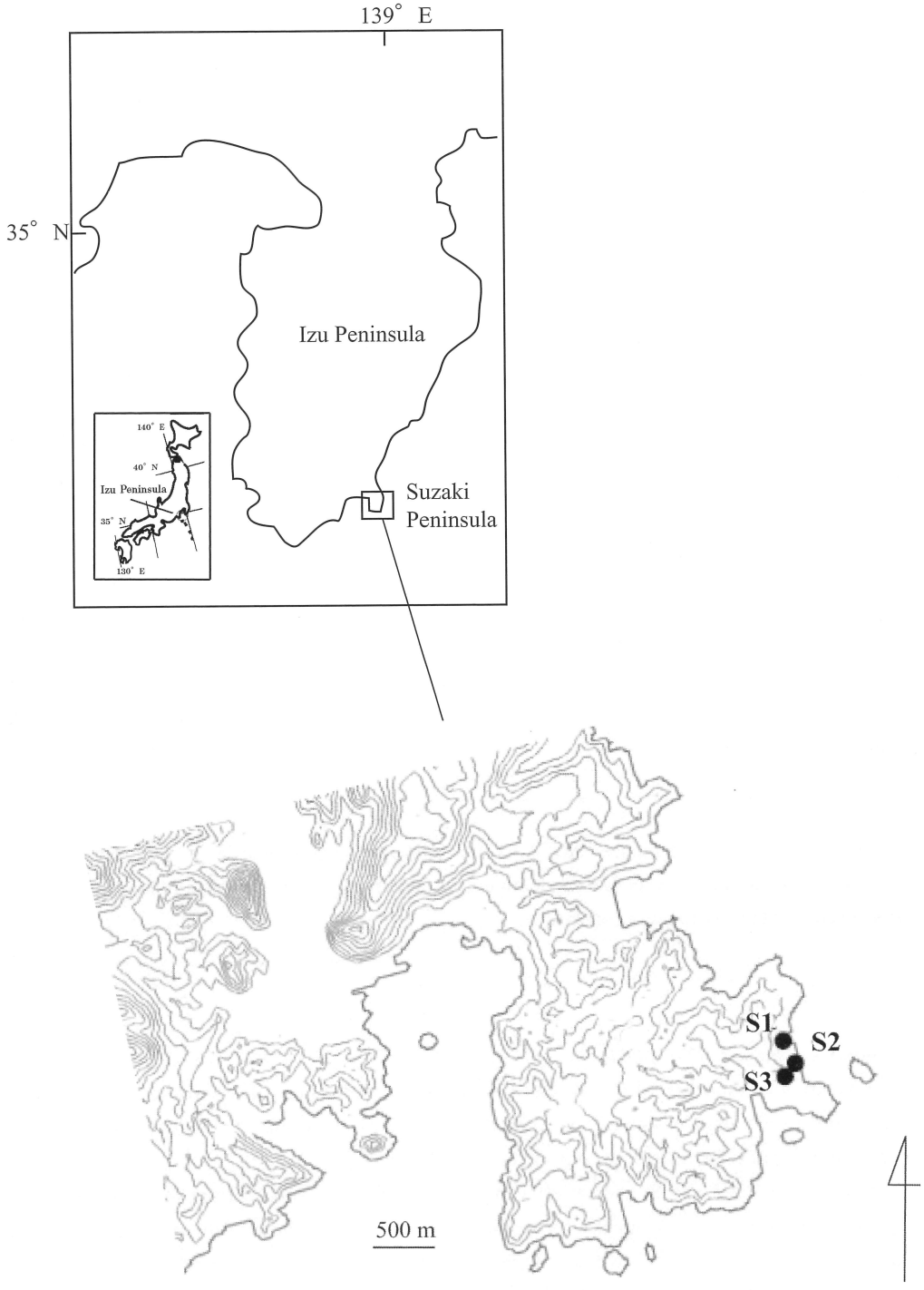

Fig.1. Suzaki Peninsula, the study area located on the southeastern edge of Izu Peninsula, Central Japan. The contours on Suzaki Peninsula are drawn at 20 m intervals.

with MeOH at room temperature and analyzed by two-dimensional paper chromatography (2D-PC) using solvent systems: BAW (*n*-BuOH:HOAc:H_2O = 4:1:5, upper phase) and 15%HOAc. After comparing the results of the 2D-PC between the two ecotypes, only the coastal one was used for further analyses. The flavonoids were isolated by preparative paper chromatography (PPC) using solvent systems: BAW, 15%HOAc and then BEW (*n*-BuOH:EtOH:H_2O = 4:1:2.2), and were finally purified by Sephadex LH-20 column chromatography using solvent system: 70%MeOH.

Procedure of structure identification followed by acid hydrolysis, UV spectral survey was as described in Mabry *et al.* (1970) and Markham (1982), and direct PC (BAW, BEW and 15%HOAc) and HPLC comparisons with authentic specimens.

(2) Quantitative analysis by high-performance liquid chromatography (HPLC)

The concentration of flavonoids was measured from crude MeOH extracts (0.3 g fresh weight in 8 ml MeOH) by using a Shimadzu HPLC system with a Shimpack CLC-ODS [I.D. 6.0 mm×150 mm (Shimadzu)] column at a flow-rate of 1 ml min^{-1}. The analysis was carried out for 20 min with the eluent H_2O-MeCN-H_3PO_4 (78:22:0.2) [note that another eluent, H_2O-MeCN-H_3PO_4 (65:35:0.2), was applied for the identification of aglycones by HPLC]. For each sample, 10 μl of extracts were injected into HPLC after filtration with a Maishori-disk H-13-5 (Tosoh).

Comparisons were made in terms of major UV absorbing compounds which appeared as were major peaks in the HPLC profiles. Peaks that had similar retention times and UV spectra from the wavelengths of 190-700 nm were recognized as the same compounds. Not only the flavonoids but also phenolic acids were compared. The amount of each UV-absorbing compound was expressed as the absorbance at 350 nm (mAU · 10^5). Flavonoid composition of each individual displayed in the HPLC profiles was recorded for comparison of the 4 populations.

Statistical analysis

One-way analysis of variance (ANOVA) was utilized to test whether there were differences in the amount of compounds among UV-B treatments. Quantitative differences among populations were determined by Mann-Whitney's *U*-test.

Results

Qualitative analysis of flavonoids

Two-dimensional (2D) PC flavonoid patterns and HPLC profiles showed that the flavonoid compositions were almost identical among all the populations in the study area (Table 2). Figure 2 is the representative HPLC profiles for coastal and inland individuals.

Six flavones (**1–5** and **8**) and two flavonols (**6** and **7**) were isolated from the leaves of coastal *A. triphylla* var. *japonica*. UV spectral properties of major **1** and **3** showed the presence of free 5,3′,4′-trihydroxyl and a substituted 7-hydroxyl groups, and free 5,3′-dihydroxyl and substituted 7,4′-dihydroxyl groups, respectively (Table 3). Both of the glycosides liberated luteolin and glucose by acid hydrolysis. Flavonoid **1** was identified as luteolin 7-*O*-glucoside by direct PC and HPLC comparison with authentic specimen. Similarly, flavonoid **3** was characterized as luteolin 7,4′-di-*O*-glucoside, but PC and HPLC properties of the compound did not agree with those of authentic specimen. Since retention time of **3** was longer than that of luteolin 7,4′-di-*O*-glucoside (**4**), it was presumed that the glycoside was acylated by

aliphatic acid.

Of minor flavonoids, **5**, **6** and **8** were identified as luteolin, quercetin 3-*O*-glucoside and vicenin-2 (apigenin 6,8-di-*C*-glucoside) by UV spectral properties and direct PC and HPLC comparisons with authentic samples (Tables 2 and 3). Other flavonoids, **2** and **7** were characterized as luteolin 7-*O*-diglucoside and quercetin 3-*O*-diglucoside by acid hydrolysis and UV spectral data. Minor flavonoids were as small amount as not to be visualized on HPLC profiles (Fig. 2).

Quantitative analysis of flavonoids

As was mentioned in the method section, the major UV absorbing compounds that appeared as major peaks in the HPLC profiles were compared. Two large peaks (**1** and **3**) in the HPLC profiles (Fig. 2) were confirmed to be luteolin 7-*O*-glucoside and luteolin 7,4′-di-*O*-glucoside (acylated?), and the total amount of these flavones was considered as the flavonoid quantity (mAU·10^5). Unknown phenolic acids (**Phe 1** and **Phe 2**) detected at the retention times from 3.15 to 3.58 were also used for quantitative comparisons.

In the greenhouse (GH) where UV-B intensity was decreased by 40%, amounts of flavonoids and phenolic acids, and relative amounts of flavonoids (fl ratio) were higher in the inland individuals than in the coastal individuals (Mann-Whitney's *U-test*, $p>0.05$; Fig. 3). Similar result was found in field-grown plants. The amount of flavonoids was significantly higher in the inland population than the coastal populations (Mann-Whitney's *U-test*, $p = 0.0004$; Fig. 3). The relative amounts of flavonoids (fl ratio) was higher in the inland population than in the coastal populations.

Discussion

In spite of differences in habitats and in morphological characteristics, the results of this study suggest that flavonoid composition was the same between coastal and inland populations.

Differences were found in the amounts of major flavonoids, although the results appeared to be the

Table 2. Chromatographic properties of the flavonoids in leaves of *Adenophora triphylla* var. *japonica*

Compounds	Rf value (×100)			Spot color under UV-A		HPLC Rt (min)
	BAW	BEW	15%HOAc	Without NH_3	With NH_3	
luteolin 7-*O*-glucoside (**1**)	27	34	6	d	y	5.91
luteolin 7-*O*-diglucoside (**2**)	32	37	11	d	y	5.39
luteolin 7,4'-*O*-glucoside (acylated) (**3**)	48	50	8	d	d	7.75
luteolin 7,4'-di-*O*-glucoside (**4**)	17	20	14	d	d	3.82
luteolin (**5**)	75	76	0	d	y	-
quercetin 3-*O*-glucoside (**6**)	46	51	19	d	y	5.49
quercetin 3-*O*-diglucoside (**7**)	42	41	4	d	y	4.95
vicenin-2 (**8**)	18	22	31	d	dy	3.52

Rf value of each substance when developed in several solvents (BAW, BEW, 15 %HOAc) are shown.
BAW = *n*-BuOH/HOAc/H_2O (4:1:5, v/v, upper phase), BEW = *n*-BuOH/EtOH/H_2O (4:1:2.2, v/v), 15%HOAc = HOAc/H_2O (15:85, v/v). Ultraviolet-A light at 356 nm was used for the detection of spot and for the observation of colors with and without NH_3. d = dark, y = yellow, dy = dark yellow, dor = dark orange.
Rt = retention time in HPLC analysis.

Table 3. UV spectral properties of the flavonoids in leaves of *Adenophora triphylla* var. *japonica*

Compounds No.	λ max (nm)					
	MeOH	NaOMe	$AlCl_3$	$AlCl_3$ /HCl	NaOAc	NaOAc/H_3BO_3
1	255	267	273	265	259	259
	265sh	391 (inc.)	427	272sh	404	373
	349			294		
				360		
				384 sh		
2	255	268	272	262	259	259
	264sh	392 (inc.)	425	272sh	404	374
	350			296		
				360		
				382sh		
3	269	267	258	252	272	269
	335	376 (dec.)	277	280	373	338
			293sh	290sh		
			354	346		
			380sh	380sh		
4	269	267	260sh	255sh	265	269
	334	366 (dec.)	276	278	333	338
			292sh	290sh		
			355	349		
			380sh	380sh		
5	254	271	272	259	269	261
	264sh	407 (inc.)	324	272sh	393	373
	349		422	359		423sh
				382sh		
6	256	273	275	267	272	262
	264sh	329	432	300	325	378
	358	358 (inc.)		361	395	
				395sh		
7	257	273	274	267	273	262
	262sh	325	432	301	326	380
	358	412 (inc.)		362	400	
				395sh		
8	271	282	277	278	282	284
	335	334	304	303	396	321
		401 (inc.)	354	351		360
			381sh	380sh		

Absorption maxima of flavonoids in methanol and several shift reagents in the range of 220~500 nm are shown. Shift reagents used were sodium methoxide (NaOMe), aluminum chloride ($AlCl_3$), hydrochloric acid(HCl) and boric acid (H_3BO_3). inc. = increase in intensity relative to the spectrum of methanolic solution. sh = shoulder.

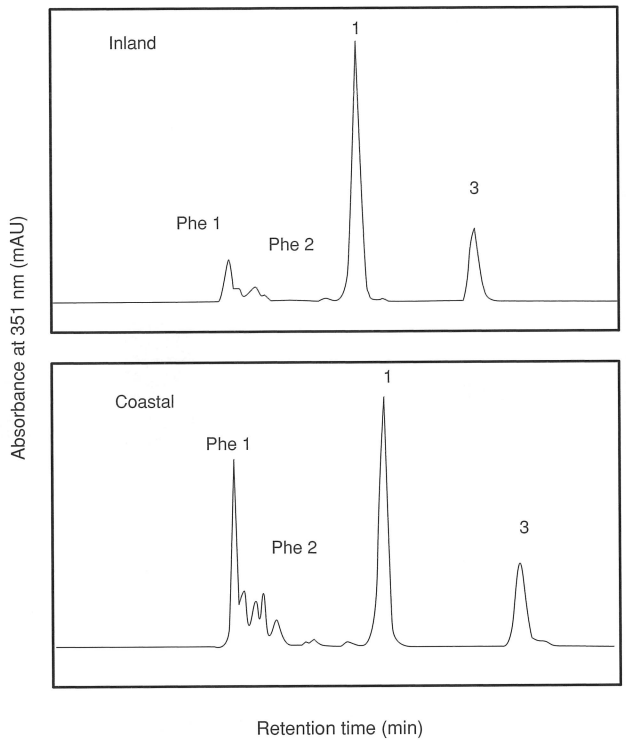

Fig.2. HPLC elution profiles of MeOH extracts from leaves of *Adenophora triphylla* var. *japonica*. Profiles of inland and coastal populations are shown. **1** = luteolin 7-*O*-glucoside, **3** = luteolin 7,4′-*O*-glucoside, **Phe1** and **2** = unknown phenolic acids.

opposite from the expectations. Higher accumulations of flavonoids and phenolic acids were detected in inland population as compared to the coastal populations. One possible assumption for this result is that morphological traits may play more important roles in adapting to the coastal environment than biochemical traits. Another reason may be that quantitative differences in secondary metabolites under various habitat conditions be attributed not only to UV-B intensity but also to many other stresses of both biotic and abiotic, since each flavonoid has multiple functions which contribute to defense against various environmental stresses (Iwashina 2003).

The present study would provide the first indication for the adaptive traits of coastal plants against UV-B or excessive light by analyzing the MeOH soluble compounds from whole leaves. For further discussion on adaptive traits seen in the coastal plants, however, chemical analyses should be done on each leaf tissue, considering that quantitative and qualitative differences may be present not only among leaf tissues but between types of leaves (e.g., sun and shade) of the same species (Liakoura *et al.* 2003).

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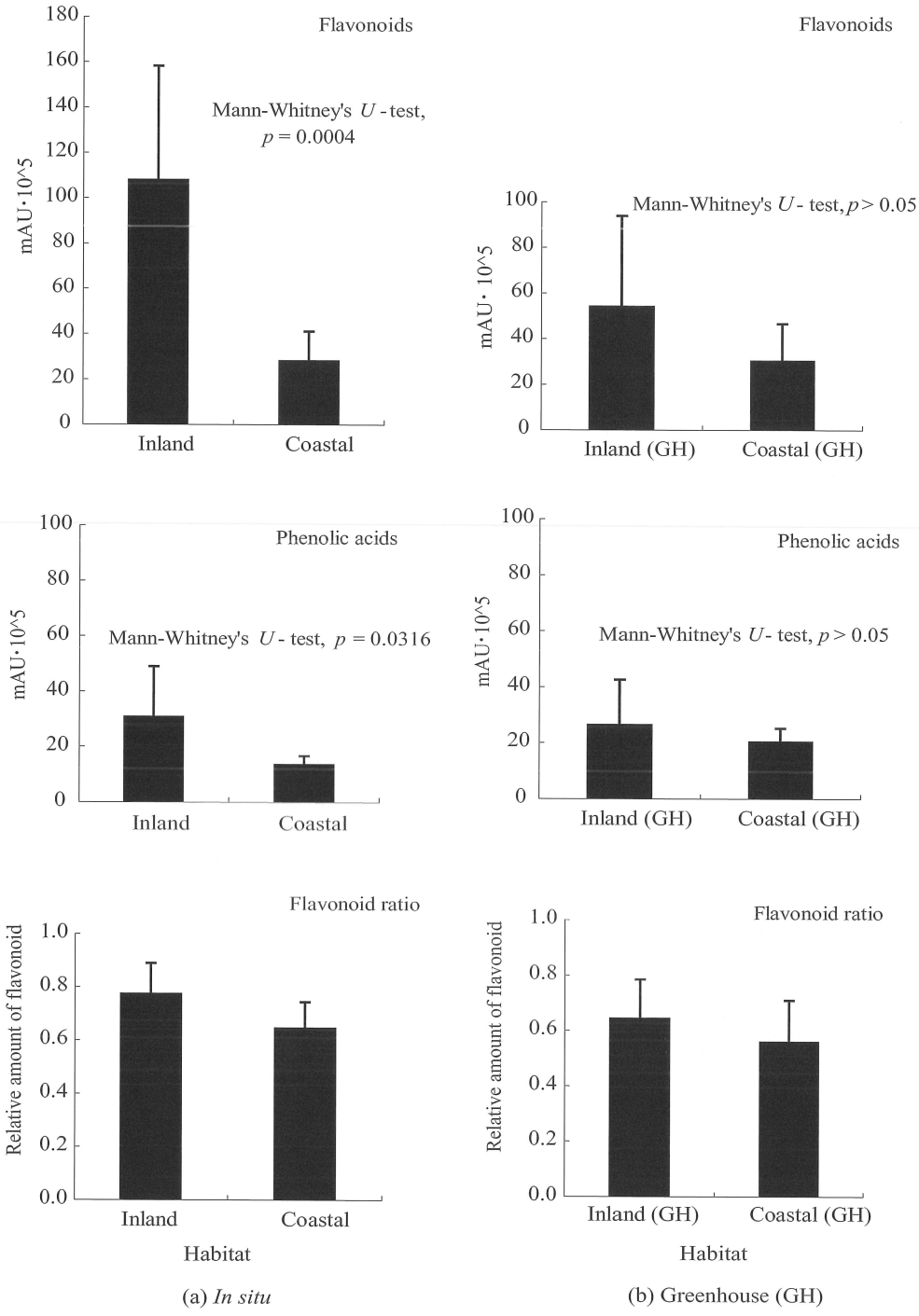

(a) *In situ* (b) Greenhouse (GH)

Fig. 3. Amount of the major phenolic compounds in leaves of *Adenophora triphylla* var. *japonica* in August 2004. Data of plants growing in the field (a) and in the greenhouse (b) were shown. Vertical axes are the amount of flavonoids, the amount of phenolic acids and flavonoid ratio. All the data of phenolic compounds are shown in Table 2 and Table 3. Error bars indicate the standard deviations. Different letters indicate statistically significant differences at $p < 0.05$ (Mann-Whitney's U - test).

Summary

The flavonoids in leaves of *Adenophora triphylla* var. *japonica* (Campanulaceae) were analyzed qualitatively and quantitatively. Analyses were made with comparison between coastal populations and inland populations, which aims to discuss the adaptive traits of coastal populations to ultraviolet-B radiation (280～315 nm; UV-B).

The flavonoid composition was nearly identical between coastal and inland populations. Six flavones, luteolin 7-*O*-glucoside (**1**), luteolin 7-*O*-diglucoside (**2**), luteolin 7,4′-di-*O*-glucoside (acylated?) (**3**), luteolin 7,4′-di-*O*-glucoside (**4**), luteolin (**5**) and vicenin-2 (**8**), and two flavonols, quercetin 3-*O*-glucoside (**6**) and quercetin 3-*O*-diglucoside (**7**) were identified. Flavones, **1** and **3** were appeared to be major components. In terms of quantitative results, higher accumulations of flavonoids and phenolic acids were seen in the inland populations, both under natural and experimental conditions.

The results of the present study suggest that some other traits such as leaf morphology may play more important role in adaptation to the coastal environment.

摘　要

ツリガネニンジン（*Adenophora triphylla* var. *japonica*, キキョウ科）の葉に含まれるフラボノイドの組成と相対量を，海岸型と内陸型の個体群とで比較した。その結果を踏まえてフラボノイドが海岸における植物の紫外線（280～315 nm; UV-B）防御機構にどのような役割を果たすのかを考察した。

フラボノイド組成は海岸型と内陸型でほぼ一致した。すなわち，６種類のフラボン，luteolin 7-*O*-glucoside (**1**), luteolin 7-*O*-diglucoside (**2**), luteolin 7,4′-di-*O*-glucoside (acylated?) (**3**), luteolin 7,4′-di-*O*-glucoside (**4**), luteolin (**5**) および vicenin-2 (**8**)と２種類のフラボノール，quercetin 3-*O*-glucoside (**6**) および quercetin 3-*O*-diglucoside (**7**)が分離・同定された。高速液体クロマトグラフィーによる比較の結果，luteolin 7-*O*-glucoside とluteolin 7,4′-di-*O*-glucoside (acylated?)の２種類が主要成分であると判明した。

フラボノイド量と有機酸量は，紫外線量が海岸に比べると弱い内陸部で採集した個体群の方が多かった。温室内の同一条件下においてポットで10ヶ月間育てた個体でも同様の結果が得られた。以上のことから，ツリガネニンジンの紫外線防御機構においてフラボノイドではなく，葉の形態など他の形質がより重要な役割を担っていることが示唆された。

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