Biosci. Biotechnol. Biochem., **65** (9), 2037–2043, 2001

Capability of Wild *Rosa rugosa* and Its Varieties and Hybrids to Produce Sesquiterpene Components in Leaf Glandular Trichomes

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Received March 8, 2001; Accepted April 19, 2001

The sesquiterpene contents in leaves of wild *Rosa rugosa* and of sixty-one hybrid rugosas were quantitatively measured by a GC analysis. In this group of samples, the greater the number of glandular trichomes the hybrid rugosas possessed on their leaves, the larger the amount of sesquiterpenes they accumulated. In contrast, those having no leaf glandular hairs contained only a trace amount of sesquiterpene components. The concentrations of bisaborosaol A (1) and carota-1,4-dienaldehyde (2) as representative sesquiterpenes of *R. rugosa* were positively correlated with the density of the glandular trichomes. Furthermore, an approximately regular correlation was observed between the concentrations of 1 and 2 in most of the sesquiterpene-producing hybrid rugosas, regardless of their productivity. This suggests that a major part of these hybrid rugosas have inherited from *R. rugosa* the ability to produce two skeletally different sesquiterpenes in parallel with a phenotype to develop leaf glandular trichomes. This investigation also led to discovering 1-dominant (e.g., Amelie Gravereaux and Purple Pavement), 2-dominant (e.g., David Thompson), and other-dominant (e.g., Martin Frobisher) types of sesquiterpene-producing hybrid rugosas.

Key words: *Rosa rugosa*; hybrid rugosa; glandular trichome; bisabolane sesquiterpene; carotane sesquiterpene

Among Rosaceae that is known to be a tanniferous family, very few species are able to produce mono- or sesqui-terpeneoids.[1] *Rosa rugosa* Thunb. is one of the unique sesquiterpene-producing Rosaceae plants that possesses mushroom-shaped glandular trichomes located on the lower surface of the running leaf veins. *R. rugosa* exudes a large amount of syrup-like droplets (10–20 mg/g fresh leaves) from multicellular tips of the leaf glandular trichomes,[2] in which carotane and bisabolane sesquiterpenoids are contained as predominant constituents.[3] In these exudates, carotane sesquiterpenes are mainly accumulated as epidioxy derivatives (rugosal A and rugosic acid A) that are the oxidized form of carota-1,4-dienaldehyde (**2**),[4] while the bisabolane-class sesquiterpene, bisaborosaol A (**1**), is another major constituent.[5] In fact, compounds **1** and **2** were detected as two major peaks in a gas-chromatographic analysis of the leaf volatile components of *R. rugosa* (Fig. 1).

According to their parentage, garden roses are grouped into ten different forms of lineage (e.g., Polyantha from *R. multiflora* × *R. chinensis* var. *minima*, and Hybrid Tea from Hybrid Perpetual × (*R. gallica* × *R. moschata*)).[6,7] Varieties and hybrids emerging from *R. rugosa* originally imported from Japan are categorized in one group and called hybrid rugosas. A large part of these hybrid rugosas have inherited the morphological feature from their mother of possessing glandular trichomes, while others possess only sparse or some completely lack glandular trichomes on the phylloplane. These facts led to an idea that some hybrid rugosas may be better samples than others to study sesquiterpene biosynthesis and also that hybrid rugosa is a better group to look for a correlation between sesquiterpenes and the population of glandular trichomes. We hence analyzed the volatile components from leaves of 61 hybrid rugosas and their mother species, *R. rugosa* during three different seasons to investigate the correlation between density of glandular trichomes and the concentration of produced sesquiterpenes. We describe here a positive correlation between population of leaf glandular trichomes and the concentrations of two representative sesquiterpenes, **1** and **2**, of hybrid rugosas. We also discuss the genotypes of some hybrid rugosas that show their unique sesquiterpene composition.

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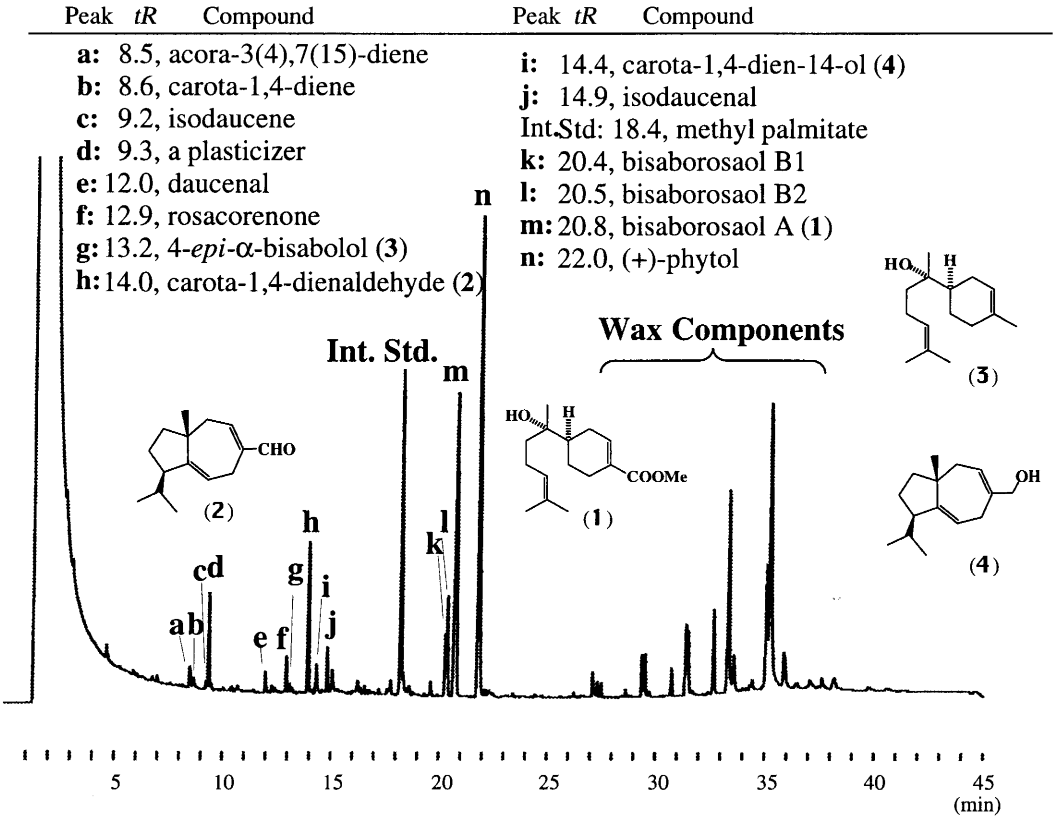

Fig. 1. Gas-chromatogram of the Leaf Volatile Components of Wild-type *Rosa rugosa*.

GC peaks of the major and/or significant volatile sesquiterpenes are listed with alphabetic characters on the chromatogram to show the retention time and compound name. All of the sesquiterpenes were detected in front of the peak of (+)-phytol at *tR* 22.0 min. Other peaks appearing in the range from *tR* 27 to 33 min were *n*-alkanes with odd numbers of carbons (C_{25}, C_{27}, C_{29}, C_{31} and C_{33}), *n*-alk(en)yl alcohols with even numbers of carbons (C_{22}, C_{24}, C_{26} and C_{28}) and steroids (β-stigmasterol and an ursen-3-ol).

Results and Discussions

Initially, we analyzed the leaf extract of *R. rugosa* by a gas-chromatograph (GC) connected with an OV-1 glass capillary column (TC-1, GL Science, 30 m × 0.32 mm i.d.) and detected nearly thirty peaks as shown in Fig. 1. Most peaks ranging from 8 to 20 min in their retention times were those of sesquiterpene-like components. Among these peaks, eleven sesquiterpenes were identified by GC-MS and by direct comparison with the chromatograms of authentic compounds. Since both bisaborosaol A (**1**) and carota-1,4-dienaldehyde (**2**) gave relatively large peaks on the gas-chromatographic profile of *R. rugosa*, we used compounds **1** and **2** as representative sesquiterpenes of the bisabolane and carotane classes, respectively. Accordingly, we made standard curves of **1** and **2** for the GC analysis, using methyl palmitate as the internal standard, and quantified **1** and **2** in fresh leaves of each hybrid rugosa.

Leaves from a total of 146 samples, including 61 different hybrid rugosas and the mother species, were collected during three different seasons (spring, summer and autumn), and their volatile sesquiterpenes were analyzed by GC. In parallel with the quantification, the population of glandular trichomes in a determined area was also counted on the sample leaves. GC analysis of the volatile sesquiterpenes indicated that most of the hybrid rugosas accumulated significant amount of these representative sesquiterpenoids, similar to their mother species, but some others accumulated none of them. Likewise, many hybrid rugosas possessed dense glandular trichomes, while some were sessile.

According to the population of glandular trichomes (number per 1.25 mm^2 of leaf surface), the hybrid rugosas examined in this study were classed into five groups as follows: group 1, none (0 as countable glandular hairs per 1.25 mm^2); group 2, rare (1–5); group 3, sparse (6–20); group 4, medium (21–40); and group 5, dense (over 41). Each group consisted of the following hybrid rugosas: group 1 (none), Montelene, R. Xalocarpa, Alexander Mackenzie, R. Nitida, Procnbent, Robusta, and *Rosa pallo*; group 2 (rare), Vanguard, Roselina, Tall Poppy, Dr. Eckener, Fimbriata, Monte Rosa, Monte Cassino, Yellow Dagmar Hastrup, Rose a Parfum de l'Hay, Topaz Jewel, Snow Pavement, Corylus, Mrs. Anthony Waterer, Peter Beales, and Rote Max Graf; group 3 (sparse), Conrad Ferdinand Meyer, Sarah Van Fleet, Nova Zembla, Pink Grootendorst, Mme. Georges Bruant, The Hunter, Sir Thomas Lipton,

Table 1. Significant or Unique Hybrid Rugosas, and Their Glandular Trichome Density and Sesquiterpene Concentration in the Leaves
Some of the hybrid rugosas, which have been renamed from their old names described in Verrier's Rosa Rugosa,[7] are listed with the current names. The parentage, average number of counted glandular trichomes, concentrations of bisaborosaol A (**1**) and carota-1,4-dienaldehyde (**2**), and quantitative data on phytochemically significant sesquiterpenes (**3** and **4**) are shown in this table. The value for glandular trichomes is given as an average number of trichomes per 1.25 mm^2 of leaf.

Variety/Cultivar/Hybrid (parentage)	Glandular Trichomes	Season	Compound (μg/g f.w.)		
			1	**2**	others
R. rugosa alba plena (natural sport)	42 (dense)	July	1858	262	
Fru Dagmar Hastrup (*R. rugosa* seedling)	42 (dense)	May	560	116	**3**:27
Hansa (parentage unknown)	42 (dense)	July	1008	48	
David Thompson ((Schneezwerg × F. D. Hastrup) × unknown seedling)	41 (dense)	May	334	152	
		July	609	211	
Henry Hudson (Schneezwerg seedling)	41 (dense)	May	1389	146	**3**:116
Belle Poitevine (parentage unknown)	40 (medium)	May	1480	192	
Charles Albanel (Sonvenir de Philemon Cochet × unknown)	39 (medium)	July	1705	64	
		May	909	112	
R. rugosa (mother species)	37 (medium)	May	488	85	**3**:4, **4**:29
Magnifica (Lucy Ashton seedling)	36 (medium)	July	217	205	
Martin Frobisher (Schneezwerg × unknown)	33 (medium)	May	0	0	**3**:466
		October	0	0	**3**:157
Moje Hammarberg (parentage unknown)	33 (medium)	July	1796	235	**4**:153
Purple Pavement (parentage unknown)	29 (medium)	July	2534	295	
Jens Munk (Schneezwerg × F. D. Hastrup)	28 (medium)	May	706	155	
R. rugosa alba (natural sport)	26 (medium)	May	1037	127	
Roseraie de L'Hay (sport of a hybrid rugosa)	26 (medium)	July	1339	79	
Flamingo (*R. rugosa* × a Hybrid Tea)	23 (medium)	July	261	36	
Amelie Gravereaux ((a Hybrid Perpetual × a Noisette) × C. F. Meyer)	22 (medium)	July	2891	206	
Carmen (*R. rugosa* × a Hybrid Perpetual)	12 (sparse)	July	207	20	**3**:45, **4**:106
Nova Zembla (sport of Conrad F. Meyer)	12 (sparse)	July	94	3	
Flanc Double de Coubert (*R. rugosa* × a Climbing Tea)	9 (sparse)	May	656	74	**3**:32
Schneezwerg (*R. rugosa* seedling)	9 (sparse)	May	182	49	
Conrad Ferdinand Meyer ((a Climbing Tea × a Centifolia) × Germanica)	9 (sparse)	May	80	11	**3**:17
White Grootendorst (*R. rugosa rubra* × a Polyantha)	9 (sparse)	July	543	99	**4**:98
The Hunter (*R. rugosa rubra* × a Floribunda)	6 (sparse)	July	113	7	
Peter Beales (parentage unknown)	1 (rare)	May	0	0	**3**:23
Vanguard ((*R. wichuraiana* × *R. rugosa alba*) × a Hybrid Tea)	1 (rare)	May	0	0	**3**:69

Mary Manners, Carmen, White Grootendorst, F. J. Grootendorst, Playtime, Max Graf, Schneelicht, Blane Double de Coubert, Lady Curzon, *Rosa rugotida*, and Schneezwerg; group 4 (medium), *R. rugosa*, Martin Frobisher, Belle Poitevine, Jens Munk, Scabrosa, Magnifica, Amelie Gravereaux, *R. rugosa alba, R. rugosa rubra*, Purple Pavement, David Thompson, Moje Hammarberg, Charles Albanel, Roseraie de l'Hay, and Flamingo; group 5 (dense), Henry Hudson, *R. rugosa alba plena, Rosa rugosa plena*, Pierette, Hansa, and Frau Dagmar Hastrup. Phytochemically significant and/or unique hybrid rugosas are listed in Table 1 with their parentage and concentrations of some major sesquiterpenes.

To demonstrate the correlation between the density of glandular hairs and the concentration of sesquiterpenes, correlation plots among the hybrid rugosas were made between these two elements in each season. Both compounds **1** and **2** in the hybrid rugosas showed a correlation with the population of glandular trichomes on their leaves (Figs. 2 and 3). These findings support our previous speculation that the sesquiterpene production of *R. rugosa* was dependent on its leaf glandular trichomes. On the other hand, a high regular correlation between them with a correlation factor $r = 0.87$ was apparent (Fig. 4) in two-dimensional plots for the concentrations of compounds **1** and **2** (μg/g of fresh leaves) throughout the sesquiterpene-producing hybrid rugosas in spring and summer. Glandular trichomes of the hybrid rugosas probably conserved the ability to produce carotane and bisabolane sesquiterpenes in a certain constant ratio, irrespective of their population and capability to produce sesquiterpenes.

Some of the hybrid rugosas in this study were found to produce remarkable amounts of sesquiterpenes. Both Amelie Gravereaux and Purple Pavement were capable of accumulating large amounts of compounds **1** (2891 and 2534 μg/g, respectively) and **2** (206 and 295 μg/g, respectively). In contrast, Charles Albanel was a typical bisabolane-type hybrid rugosa predomonantly accumulating **1**, while David Thompson produced a relatively large amount of **2** (Fig. 4 and Table 1).

As shown in Table 1, some of the hybrid rugosas showed a qualitatively unique sesquiterpene composition, one of the most remarkable hybrids being Martin Frobisher. We have previously reported that

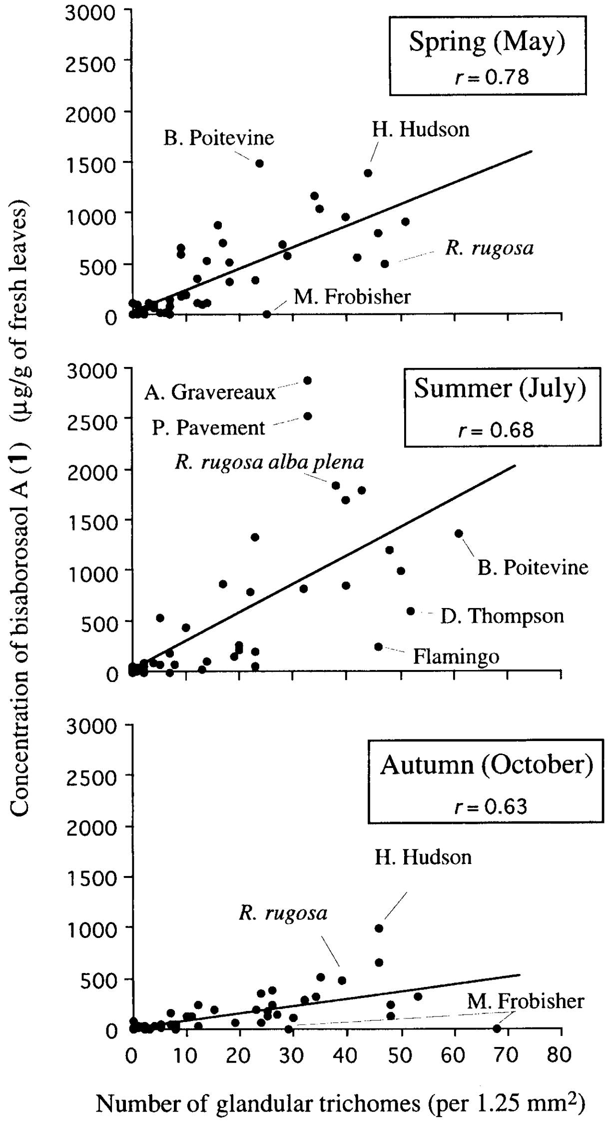

Fig. 2. Correlation between the Density of the Glandular Trichomes on the Leaves and the Concentration of Bisaborosaol A (**1**).

Hybrid rugosas were respectively investigated for the correlation between the concentration of compound **1** and the density of glandular trichomes on the leaves. Respective coefficients of correlation (r) are shown in each plotted figure. Some points obviously dislocated from each correlation standard line were those of phytochemically unique hybrids in producing a bisabolane-type sesquiterpene.

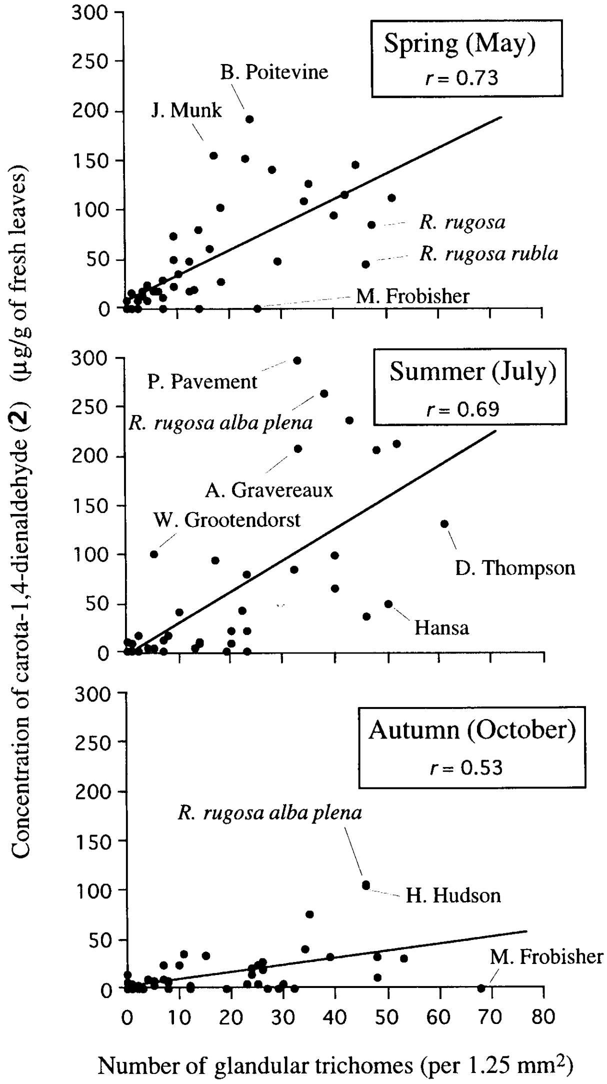

Fig. 3. Correlation between the Density of the Glandular Trichomes on the Leaves and the Concentration of Carota-1,4-dienaldehyde (**2**).

Hybrid rugosas were respectively investigated for the correlation between the concentration of compound **2** and the density of glandular trichomes on the leaves. Respective coefficients of correlation (r) are shown in each plotted figure. Some points obviously dislocated from each correlation standard line were those of phytochemically unique hybrids in producing a carotane-type sesquiterpene. Note the scale bar for carota-1,4-dienaldehyde (**2**) as being one-tenth of the scale for bisaborosaol A (**1**).

Martin Frobisher produced (+)-4-*epi*-α-bisabolol (**3**) as the main and nearly only sesquiterpene component with 466 μg/g of fresh leaves.[8] Vanguard and Peter Beales also accumulated **3** as a single sesquiterpene component (69 and 23 μg/g, respectively). On the other hand, Carmen, which is another hybrid rugosa accumulating **3** as a major bisabolane class of constituent, also produced carota-1,4-dien-14-ol (**4**, at *tR* 14.4 min on the gas chromatogram in Fig. 1)[5] as the single component of carotane-class sesquiterpenes (106 μg/g). These hybrid rugosas probably inherited the capacity of *R. rugosa* to yield bisabolane or bisabolane/carotane skeletons, but not its ability to produce oxygenases in association with further modification of the sesquiterpene structure.

Higher plants possessing glandular trichomes on the leaves are distributed throughout several families, of which Solanaceae[9,10] and Labiatae[11,12] have been well studied in phytochemical, enzymological and plant physiological aspects.[13] In many of them, the chemical components of the hybrids exuding from the trichomes have been investigated and compared with those of wild genotypes. Such a qualitative variation in glandular trichome exudates among hybrids

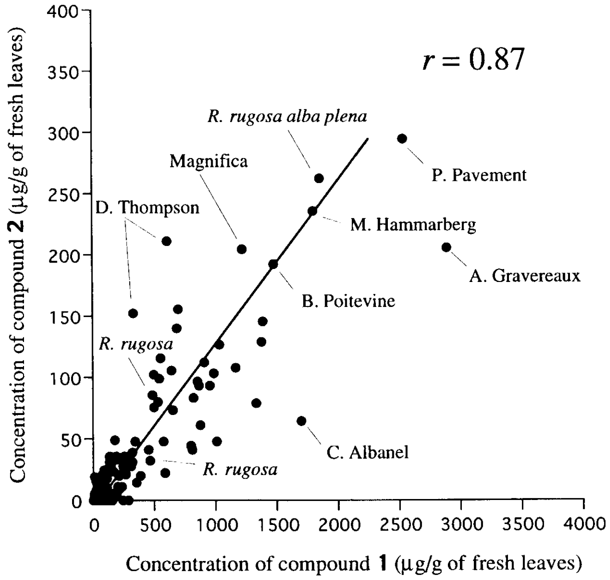

Fig. 4. Correlation between the Concentrations of Bisaborosaol A (**1**) and Carota-1,4-dienaldehyde (**2**) among Sesquiterpene-producing Hybrid Rugosas.

These correlative plots include all of the samples, except hybrids, that contained no **1** or **2**. The correlation coefficient (r) for the 2-dimensional plots was 0.87. The linear regression line is expressed by the function, $y = 7.7x$ (x and y are the concentrations of **2** and **1**, respectively).

is known in tobacco,[14] tomato,[15] geranium,[16] mint[17] and tansy.[18] In geranium, one gene expressed in glandular trichomes to be linked with their aphid-resistant or susceptible nature has been characterized to be a fatty acid desaturase that regulated the unsaturation of anacardic acids in the aliphatic side-chain.[19,20]

The capacity of hybrid rugosas to produce sesquiterpenoids was thus highly linked with the phenotype to develop glandular trichomes. In respects of the qualitative variation of the sesquiterpenes exuded from the glandular trichomes, most of the varieties displayed a regulated turnover of two different skeletons in the sesquiterpene biosynthesis. Similar to geranium,[20] some hybrid rugosas that produce qualitatively unique sesquiterpenoids probably have alternative genes associated with sesquiterpene biosynthesis. Biosynthetic research on the sesquiterpene components in *R. rugosa* will be conducted by using these hybrid rugosas as advanced plant materials.

Materials and Methods

General. Gas-chromatography was conducted with a Hitachi G 5000 instrument and TC-1 glass-capillary column (GL Science, 30 m × 0.32 mm., corresponding to OV-1) equipped with a Hitachi D-2500 Chromato-Integrator. GC-MS analyses were performed by a ThermoQuest GC-Q Plus instrument combined with a ThermoQuest TRACE 2000 GC equipped with the same TC-1 column as that just mentioned.

Plant materials. The leaves (approx. 0.3–2.7 g) of *R. rugosa* and its hybrids were collected from Yurigahara Park in Sapporo (see the address of T. K., one of the authors) in late May (1998), late July and mid October (1999). These hybrid rugosas had been imported from the rose breeding stations, Spring Valley Roses (Spring Valley, USA), Pickering Nurseries (Pickering, Canada), Hortico (Waterdown, Canada) and Peter Beales Roses (Norfolk, England) in 1997–1998, and were planted into a nursery garden to spend the winter season. The parentage of each hybrid is based on Beales' "Roses"[7] and Verrier's "Rosa Rugosa".[21] Wild-type *R. rugosa, R. rugosa alba* and *R. rugosa plena* were also collected in May 1998 from the Botanical Garden of the Faculty of Agriculture at Hokkaido University.

Extraction, clean-up and GC analysis. The leaflets were separately soaked in approx. 15 ml of EtOAc in a 20-ml glass vial, and kept in a freezer for at least a week. The resulting EtOAc extracts were then adjusted to 25 ml in volume in a volumetric flask, and exactly 10 ml of the solution was concentrated and re-dissolved in 1 ml of 10% EtOAc/*n*-hexane to apply to a Sep Pak silica cartridge that had previously been conditioned with the same solvent. The cartridge was washed with 5 ml of the solvents and the resulting eluate was again concentrated and then mixed with 100 μl of an internal standard solution (in EtOAc, containing 167 μg of methyl palmitate). From the sample solution thus prepared, 0.5 μl was subjected to the GC analysis.

Counting the glandular trichomes on each leaf of the hybrid rugosa samples. A middle-positioned leaflet was excised from the compound leaves of each hybrid rugosa that had been sampled as the most typical one in the season. The lower surface of the leaflet was observed under a × 40 microscope on a glass plate. Avoiding a photographic view of the midvein, a positive microphotograph (35 × 40 mm^2) was then taken of the selected area for each hybrid and the mother species to count the number of glandular trichomes. The photographs as positive slide film were then screened on a × 2 viewer (70 × 80 mm^2). A glass plate set in a rectangular frame of 40 × 50 mm^2 was put on the screen of the viewer, and the glandular trichomes in a selected area inside the frame were counted and recorded. The area magnified to 40 × 50 mm^2 on the slide viewer is 1.25 mm^2 in actual size. The glandular trichomes on the leaflet were counted for two different views, and the counted numbers were averaged by omitting the decimal. Since the density of the glandular trichomes was somewhat dependent on the condition of the leaves, such as the developing stage, position and season, it was not always constant even in the same variety or specimen. The means of the number of glandular trichomes per 1.25 mm^2 area of the same variety but of different samples were further averaged to give a truer value for the trichome density in each variety.

Standard curve for bisaborosaol A (**1**). A standard curve for bisaborosaol A (**1**) was made for a quantitative analysis. A solution of compound **1** was first prepared by dissolving 11.6 mg of **1** in 100 ml of *n*-hexane. From this stock solution, 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 ml were each taken and poured into a separate test tube. 100 μg of methyl palmitate (as 2.0 ml of a 50-ppm solution in EtOAc) was then added to each test tube, and the solvent evaporated to dryness. Each sample was then re-dissolved in 1.0 ml of EtOAc and analyzed by GC. Sample preparation for each concentration was triplicated. Standard plots of the peak ratios for **1** (*tR* 20.7 min) against the internal standard (*tR* 18.5 min) were on a straight line in the range from 0.08 to 7.6 of peak ratio. It was thus possible to quantify 12 μg to 1.16 mg of the absolute amount of **1** with this system. When the peak ratio was exactly 1.00, the absolute amount of compound **1** in the test tube was calculated to be 145 μg. This standard curve was reliable when the peak intensity of the internal standard was in the range of 2,100 to 8,500. In the practical analysis of **1** in these samples, we adjusted the volume of the internal standard solution to be a maximal 10-fold and minimal 0.16-fold. The volume of the sample solution was always 0.5 μl, and the absolute amount of the internal standard was approximately 0.25 μg, so that compound **1** in a range from 1.9 μg to 5.3 mg was accepted as a reliable value in the quantification by GC.

Standard curve for carota-1,4-dienaldehyde (**2**). A standard curve of carota-1,4-dienaldehyde (**2**) was also constructed, basically in the same manner as that for compound **1**. Pure **2** (19.3 mg) was dissolved in EtOAc (100 ml), and then solutions of 0.5, 1.0, 2.0, 5.0 and 10.0 ml were respectively poured into separate test tubes. After methyl palmitate (100 μg) had been mixed, the solvent in the tubes was again removed. Sample solutions re-dissolved in 0.2 ml of EtOAc were each analyzed by GC. Preparation of the samples at each concentration was duplicated. A plot of the peak ratios of **2** (*tR* 14.2 min) against the internal standard gave a straight line in the range from 0.39 to 8.23 of the peak ratio. When a peak ratio was exactly 1.00, the absolute amount of compound **2** in the mixture was calculated to be 0.94 mg. The sensitivity of compound **2** was higher than that of **1** in the GC analysis, it being possible to quantify 39 μg to 0.77 mg of the absolute amount of **2** with this system. We also adjusted the absolute amount of the internal standard added to the leaf extracts, so that compound **2** in the range from 2.0 μg to 3.9 mg was a reliable value in the quantification by GC. We used the standard curve of **2** for convenience to quantify (+)-4-*epi*-α-bisabolol (**3**).

Acknowledgments

The authors thank Dr. Y. Fujita in the Botanic Garden of Faculty of Agriculture at Hokkaido University for providing the leaves of *R. rugosa* and some of its varieties.

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