Aroma Evolution during Flower Opening in *Rosa damascena* Mill.

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The changes of aroma ingredients during the process of flower opening from Bulgarian rose were monitored by head space method and solvent extraction.We also analyzed contents of glycosidic alcoholic aroma together with activities of the hydrolytic enzymes throughout the flower development and the opening.

At flower petal opening time, the total amount of aromas in the head space gas reached the highest level. The concentration of citronellol was abundant in the head space gas at this stage, whereas the concentration of 2-phenylethanol became higher than that of citronellol 4 hr after the opening stage. In the volatile extracts, higher accumulation was observed in 2-phenylethanol than those of monoterpenoids at this stage, and the content of the former still increased after flower opening. Glycosidic citronellol, geraniol, and other monoterpenes started their accumulation just before flower opening stage and then reached the maximum level. The amount of these glycosidic compounds were less than those in the volatile extracts. In contrast to the monoterpenes, 2-phenylethyl glycosides accumulated in a higher level than in the volatile extracts starting at least 12 hr before the opening stage. The amount of the glycosidic precursors of 2-phenylethanol detected in the rose petals before flower opening always was higher than the amount of 2-phenylethanol which was released later. The decline of glycosidic 2-phenylethanol at flower opening stage may be due to partial enzymatic hydrolysis. Thereafter a drastic decline was observed, indicating that rapid enzymatic hydrolysis occurred during these stages.

Introduction

Several alcoholic aromas of flowers were reported to be formed from the glycosidic precursors which have no floral scents and were biochemically changed to alcoholic compounds (Francis *et al.*, 1970). The main glycoconjugated aroma isolated from rose flowers were β-D-glucopyranosides of 2-phenylethanol and geraniol (Francis *et al.*, 1969; Berikashvili *et al.*, 1981; Guseva *et al.*, 1975; Banthorpe *et al.*, 1972). Since mevalonate was incorporated to monoterpenols and their β-D-glucopyranoside derivatives (Francis *et al.*, 1969), it was tried to elucidate the aroma formation of flowers by focusing on the glucoconjugates (Ackermann *et al.*, 1988).

In contrast to the results mentioned above, the main glycoconjugates of monoterpene alcohols are disaccharide glycosides in the flowers of *Rosa damascena* var. *bulgaria*, Bulgarian rose (Oka *et al.*, 1997; 1998), which is the famous variety for the production of essential oil and has an excellent rosy note.Besides these disaccharide glycosides, various mono- and diglycosides of alcoholic aroma were isolated from the flowers together with 2-phenylethyl β-D-glucopyranoside (Watanabe *et al.*, 1997; 1998; Straubinger *et al.*, 1997; 1998).We also reported the important role of glycosidase as well as glycoconjugates of alcoholic aroma in scent evolution from the flowers of *Gardenia jasminoides* E (Watanabe, *et al.*, 1993; Watanabe *et al.*, 1994; Watanabe, 1997). Our experimental results so far obtained encourage us to push forward to further investigation on scent evolution in rose flowers.We reexamined the process of aroma release and the accumulation of glycosidic alcoholic

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aroma during the development and the opening of the flowers of *R. damascena.* The change in the activities of hydrolytic enzymes were also reported.

Materials and Methods

Reagents

The enzymes (β-glucosidase: from almonds; activity 4.6 units / mg protein, and naringinase: from *Penicillium decumbens*; activity 365 units / mg solid), and all *p*-nitrophenyl glycosides were purchased from Sigma Chemical Co.*p*-Nitrophenyl β-primeveroside was synthesized in our laboratory (Murata *et al.*, in press.). All other chemicals were of analytical quality and purchased from Wako, Tokyo, Japan.

Plants

Bulgarian rose (*Rosa damascena* var. *bulgaria*) plants were cultured for 3 years from grafting at the field in Fukuroi-city, Shizuoka-prefecture, Japan. For monitoring of aroma release, we used intact plants which would open the next day in May 1996. Each stage of buds (Fig. 1) during its maturing and its flower opening was also harvested in

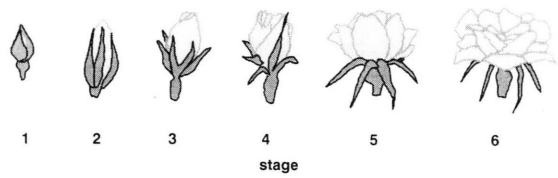

Fig. 1. Stages of buds of Bulgarian rose (*Rosa damascena* var. *bulgaria*) during maturation and flower opening.

May 1996. Flower buds at stage 1 were harvest at least 2 days before the flower opening. The flower buds at stages 2 to 3 were harvested during afternoon to evening. Flowers at the opening stage is defined as the late stage 4 (2 to 4 am). The flowers at initial and the middle stages 4 were observed from 10 pm to 2 am. At stage 5 (4 to 7 am) flowers opened and reached to the full bloom stage 6 at 6 am, the sunrise time. The flowers at the late stage 4 and the initial stage 5 could not be separated completely. All the materials were stored at −20 °C and used for the preparation of enzymes, glycosidic fractions, and volatile fractions.

The collection of dynamic head space gas from the Bulgarian rose and the analysis by GC and GC-MS

From 11 pm to 10 am in the next morning, matured buds of Bulgarian rose which would open in the morning were wrapped with the Tedler bag (21 cm × 30 cm) and sealed at their scapes completely. The pressured clean air was flown at a rate of 500 ml / min into the Tedler bag and sucked through the tube of TENAX-TA resin glass tube (2.5 mm i.d. × 16 cm) by pumping. Every hour, the TENAX tube was replaced by a new one, and so the changes of aroma ingredients during the flower opening of Bulgarian rose were determined by GC-MS with a Thermal Desorption Cold Trap injector (TCT). A Hewlett Packard GCD system G1800A with Chrompack TCT injector and MS as detector with a DB-Wax column (0.25 mm i.d. × 30 m), J&W Scientific (Fursome, California) was used. TCT conditions were as follows: Step I: oven temp. room temp., trap temp. −100 °C, 3 min; Step II: oven temp. 210 °C, trap temp. −100 °C, 5 min; Step III: oven temp. 210 °C, trap temp. 210 °C, 3 min. In a total time of 60 min a linear temperature gradient from 50 °C to 210 °C was performed.

Extraction of volatile compounds from flowers by microwave oven and the analysis by GC, and GC-MS

Two of each frozen flowers or flower buds were cut by scissors and immersed in pentane (50 ml) in a 100 ml flasks (Ganzler *et al.*, 1986; Jean *et al.*, 1992). The flasks with samples were covered with watch glasses and extracted for 90 sec in a microwave oven (TOSHIBA ER-245, 500 W, 2450 MHz), taking care for bumping. The extracts were dried over Na_2SO_4 and concentrated by N_2 stream after adding 2 μl of an internal standard solution, 5 mg of ethyl decanoate in 1 ml of EtOAc. The concentrates were analyzed by GC and GC-MS. For GC analysis a Hewlett Packard 5890 system with flame ionization detection (H_2, 240 °C) was used. The conditions were as follows, helium as carrier gas and a DB-Wax column (0.25 mm i.d. × 30 m), J&W Scientific. Column temp.: 70 °C (2 min) up to 240 °C with linearly increasing gradient of 4 °C / min, injector temp.: 240 °C. The extraction efficacy of *p*-nitrophenol was 91% by this method, although the glycosidic derivatives were not extracted at all.

Extraction of glycosidic aroma and enzymatic hydrolysis

Mature buds and blooming flowers were extracted twice with 50 ml EtOAc by microwave oven as mentioned above (Bureau, 1997). The extracts were combined and concentrated in vacuo. The concentrate was suspended in 100 ml of water by the aid an ultra sonicator and subjected to a column of DIAION HP-20 (20 mm i.d. × 30 cm) equilibrated with water, which was washed with 10% MeCN (100 ml). The glycosidic aromas were eluted with 50% MeCN (100 ml). The eluate was dried in vacuo and dissolved in 2 ml citrate buffer (0.1 M, pH 5.0). The solution was washed with an azeotropic mixture of pentane : dichloromethane = 2 : 1, v/v (b.p. 37 °C) four times and evaporated under N_2 to give the glycosidic sample for enzymatic hydrolysis. To 400 µl of the glycosidic solution were added the powders of 400 µg naringinase and β-glucosidase at a time and 1200 µl of the citrate buffer and incubated at 40 °C for 16 hours. A zero control experiment, with buffer instead of enzyme solution was performed. After incubation the aromas liberated were extracted twice with above mentioned azeotropic mixture. After adding 2 µl internal standard, ethyl decanoate with a concentration of 5 mg / ml in EtOAc, these extracted aromas were concentrated again with N_2 gas. GC was performed on a Hewlett Packard 5890 system with flame ionization detection (H_2, 240 °C), helium as carrier gas and a DB-Wax column(0.25 mm i.d. × 30 m), J&W Scientific. Column temp.: 70 °C (2 min) up to 240 °C with linearly increasing gradient of 4 °C / min, injector temp.: 240 °C. The contents of alcoholic aroma were calculated based on the peak area of each alcoholic aroma, being liberated by the action of the enzymes. Subsequently, These values were corrected by the data obtained from an enzyme-free control experiment. The extraction efficacies of *p*-nitrophenol, *p*-nitrophenyl β-D-glucopyranoside, and *p*-nitrophenyl β-primeveroside were 98, 99, and 93%, respectively.

Preparation of crude enzyme extract from acetone powder

Mature buds and blooming flowers were homogenized by a homogenizer in cold acetone at – 20 °C. After filtration, the residues of the samples were washed with acetone until the floral aromas of the residues were lost completely. Next, the residue were dried in a desiccator *in vacuo* to yield a powdery solid, acetone powder.

Each acetone powder (0.5 g), 0.5 g of quartz sand, 2.5 g of Polyclar SB-100, 20 ml of 0.1 M citrate buffer (pH 4.5; containing 1% Triton and 2 mM dithiothreithol (DTT) and 12 mg of ascorbic acid were mixed and homogenized in a motor for 5 minutes under ice cooling condition. After centrifugation (17,000 *g* × 10 min, 4 °C) the homogenate, the supernatant was used as a crude enzyme extract.

To 450 µl of 100 mM citrate buffer (pH 4.5) were added 100 µl of the enzyme sample solution and 50 µl of 10 mM substrate solution. The reaction was started by adding the protein sample at 37 °C and stopped by addition of 500 µl of 1 M Na_2CO_3. The liberated *p*-nitrophenol was spectrophotometrically quantified at 405 nm. One unit was defined as the amount of enzyme liberating 1 µmol of *p*-nitrophenol / min under the assay conditions.

Results and Discussion

The changes of aroma ingredients during the process of flower opening from Bulgarian rose were monitored by the head space method (Fig. 2). At the flower petal opening time, the late stage 4 to stage 5, the total amount of aromas in the head space gas reached the highest level. A significant decline down to one seventh of late stage 5 could be observed throughout the stage 6 (from 7 to 10 am). The concentration of citronellol was abundant in the head space gas at late stage 4 and the initial stage 5, whereas the concentration of 2-phenylethanol became higher than that of citronellol at the initial stage 6. Besides this observation, geraniol was quantitatively released throughout the flower opening. The other minor hydrocarbon monoterpenes, such as limonene, myrcene, and β-pinene decreased by 50% from late stage 5 to the initial stage 6. These results may suggest that there is a different mechanism for scent evolution from the flowers for each series of aromas, 2-phenylethanol, monoterpene alcohols and hydrocarbon monoterpenes.

Fig. 3 shows the change in the contents of aroma extracted by pentane throughout the development and opening of the flowers. Geraniol, citronellol,

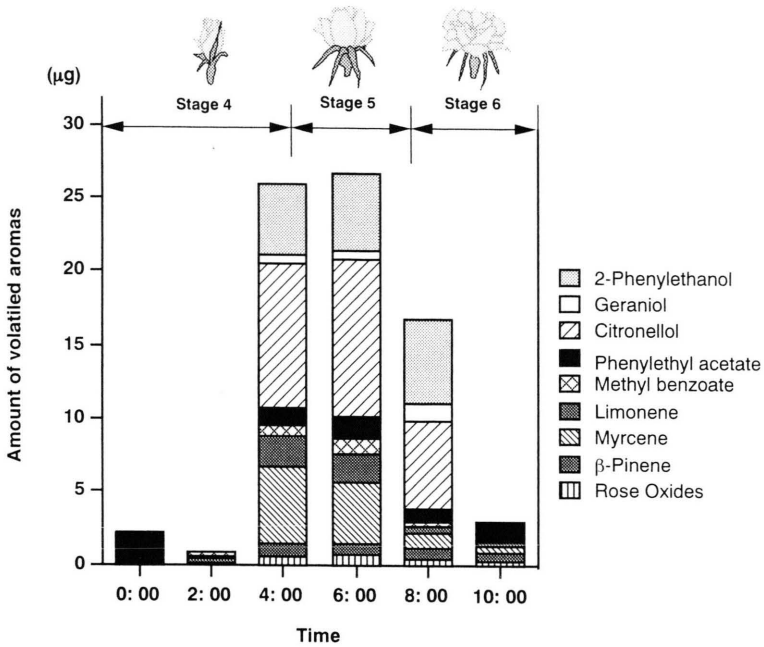

Fig. 2. Change in the amounts of aromas detected by the head space method during the flower opening of *Rosa damascena* var. *bulgaria*.

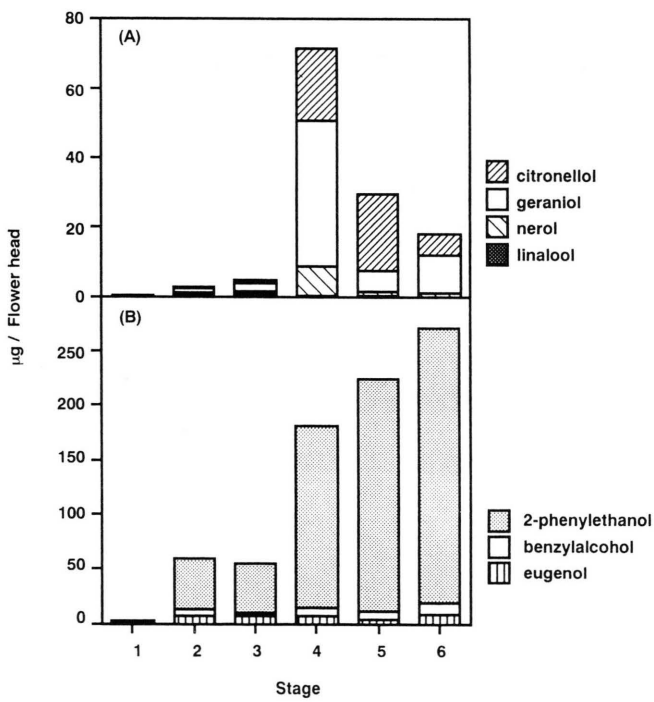

Fig. 3. Change in the contents of monoterpene alcohols (A) and aromatic alcohols (B), extracted with pentane from *Rosa damascena* var.-*bulgaria* flowers.

and the minor monoterpenes were detected in the highest level (79 μg / flower head) at stage 4. The ratio of citronellol to geraniol was not coincided to that in the head space gas. Geraniol, produced from geranyl pyrophosphate, might be transformed into citronellol and other monoterpene derivatives, whereas citronellol thus produced might be simply released from the flowers without modi-

fications. The higher accumulation was observed in 2-phenylethanol (166 μg / head) than those of monoterpenoids at stage 4. The contents of 2-phenylethanol still increased after the opening stage, stages 5 and 6, whereas the amount released was only a few percent of that accumulated in flowers. Here the scent formation and the evolution should be separately discussed in each aroma.

The change in the contents of glycosidic aroma was shown in Fig. 4. Glycosidic geraniol and other monoterpenes started their accumulation at stage 2 and reached the maximum level at stage 4. The amount of these glycosidic compounds was less than those in the volatile extracts. If the process of the scent evolution would be via the glycosidic compounds, higher accumulation should be detected in the level of the glycosidic monoterpene alcohols. The results being obtained strongly suggested that monoterpenes, such as geraniol and citronellol, were directly synthesized from geranyl pyrophosphate (Pichersky *et al.*, 1994; 1995; Dudareva *et al.*, 1996) and the rest after the scent emission was stored in some of the part of flower cells in the glycosidic forms. In contrast to the monoterpenes, 2-phenylethyl glycosides, mainly 2-phenylethyl β-D-glucopyranoside, was accumulated in a higher level than in the volatile extracts starting from stage 2. The accumulation of glycosidic 2-phenylethanol was detected prior to the flower petal opening stages, in the similar amount (270 μg / flower head) sufficient to be released after their hydrolysis to that (250 mg / head) in volatile extracts. The decline (30%) of glycosidic 2-phenylethanol at stage 4 from stage 3 may be ascribed by the partial enzymatic hydrolysis. The drastic decline was also observed between stages 4 and 5, indicating that enzymatic hydrolysis was occurred in high speed during these stages. The rebound in the accumulation of glycosidic 2-phenylethanol at stage 6 may be caused by the post glycosilation of the free 2-phenylethanol, liberated from the glycosidic compounds.

As shown in Fig. 5, the activities of glycosidases elevated dramatically during the flower opening, between stages 4 and 5. β-Glucosidase was the most remarkable and the highest among the glycosidase activities tested in this study, which activity jumped up by 5 times during the flower opening stage. Although β-galactosidase was relatively high throughout the flower development and the opening, its activity did not show a drastic increment (only 2 times during the flower opening). β-Xylosidase and α-arabinosidase showed lower activity (one 8th of β-glucosidase), but the profiles

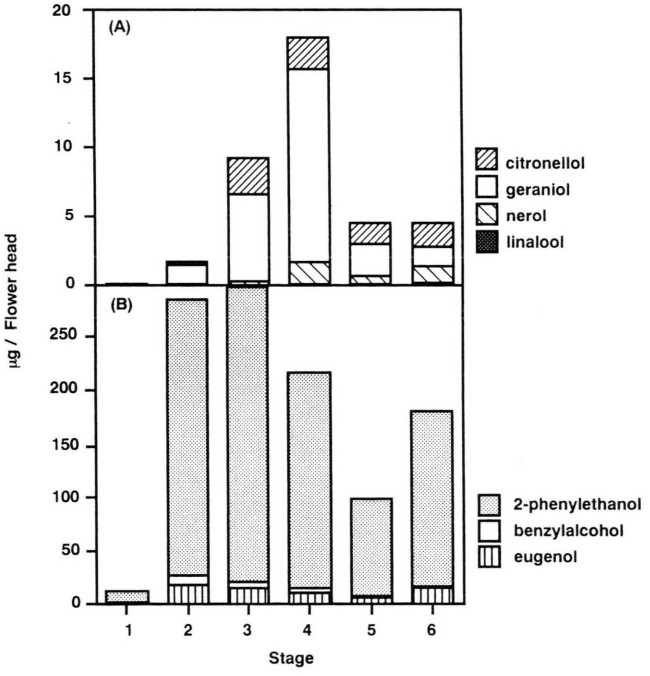

Fig. 4. Change in the contents of monoterpene alcohols (A) and aromatic alcohols (B), liberated by the action of hydrolytic enzymes from the precursor solutions prepared from each stage of flower heads.

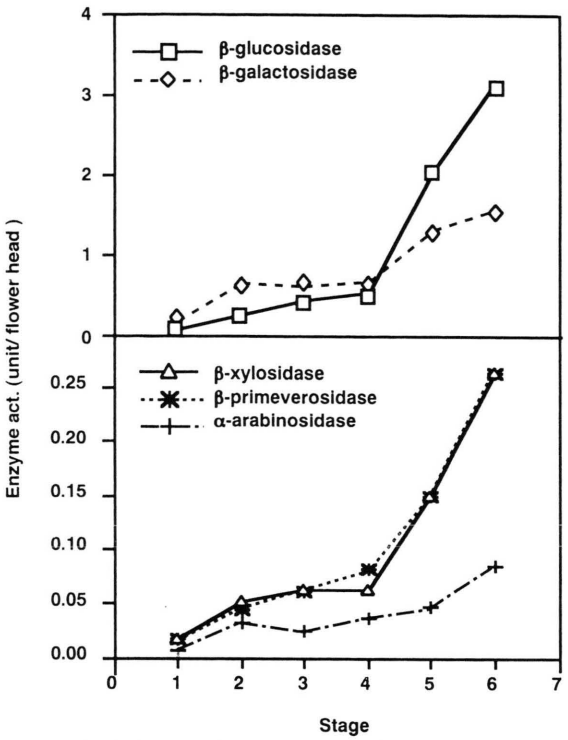

Fig. 5. Change in enzyme activities during development of *Rosa damascena* var. *bulgaria*.

were similar to that of β-glucosidase. As the monoterpene disaccharides have been isolated from the flowers of *R. damascena*, primeverosidase activity was examined as one of the unspecific disaccharidase activities. The activity was found to gradually increase with the flower opening in a low level. These results strongly indicated that enzymes with the activity of β-glucosidase as well as β-xylosidase, and α-arbinosidase, play an important role in the formation of some of the alcoholic aroma, such as 2-phenylethanol and eugenol.

As a conclusion, we can suggest that citronellol and geraniol synthesized *de novo* in flower head at flower opening stage was released from flowers without being converted into glycosidic compounds, whereas 2-phenylethyl glycosides synthesized in the flower buds were accumulated in the buds before flower opening. Then the glycosidic 2-phenylethanol may be hydrolyzed by the action of glycosidase during flower opening to result in the decline of the amount of the glycosides after flower opening. Here the importance of the glycosidic 2-phenylethanol and the hydrolytic enzyme was again confirmed. The low level accumulation of geranyl glycosides may be explainable by an excess synthesis of geraniol during flower opening. The increased synthesis of geraniol might become a signal for the accumulation in a glycosidic form in the cells of flowers, such as in a vacuole.

Previously we reported the isolation of disaccharide glycosides of geraniol and citronellol, and β-glucopyranosides of 2-phenylethanol and benzyl alcohol as main glycosides from Bulgarian rose (Oka *et al.*, 1997; 1998; Watanabe *et al.*, 1997). Based on the results so far obtained, amongst these glycosidic aromas, 2-phenylethyl β-D-glucopyranoside may be regarded as an important aroma precursor, whereas the majority of glycosidic monoterpene alcohols are accumulated as a storage or detoxification forms in the flower head. Thus we conclude that each aroma was formed by a different mechanism.

We are now investigating the accumulation of glycosidic aromas in the vacuoles of rose flowers. It is also an interesting topic to clarify a biogenesis of 2-phenylethyl β-D-glucopyranoside. The data will be reported elsewhere.

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