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A simple and rapid extraction method to determine osmolar concentration of soluble carbohydrates from rose petals

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Abstract

To establish a simple and rapid extraction method for soluble carbohydrate for determination of osmolar concentration in petals by HPLC analysis, a method using a centrifugal filter device with microwave heating was developed. Rose 'Sonia' petals were placed in a centrifugal filter device and heated in a microwave oven to boiling. The centrifugal filter device was centrifuged with the petals at 12,000 g for 10 min. The resulting leached solution was subjected to HPLC analysis. No significant difference in soluble carbohydrate composition was observed between the solution obtained from this method and that obtained from a conventional extraction method in which tissues are homogenized using hot ethanol solution. Changes in soluble carbohydrate concentration with flower opening in 'Rote Rose' roses were investigated using the new method. The osmolar concentrations of glucose and fructose in the petals increased during flower opening. This increase was roughly comparable to the increase in osmotic pressure in the petals. The results suggest that the method using the centrifugal filter device with microwave heating is a simple and rapid way to determine osmolar concentration of soluble carbohydrates of rose petals.

Key words: Centrifugal filter device, extraction method, microwave heating, osmotic pressure, petal, rose, soluble carbohydrates.

Introduction

Soluble carbohydrates have an osmotic role and also provide substrates for respiration and cell wall synthesis. Since cut flowers are usually placed under conditions below the light compensation point for photosynthesis, they can assimilate little carbon by photosynthesis, and thereby lack reserve carbohydrates. Application of soluble carbohydrates, such as sucrose and glucose, extends the vase life of many cut flowers (Halevy and Mayak, 1979; Pun and Ichimura, 2004). For flower opening, large amounts of soluble carbohydrates are required and application of sugars promotes flower opening and pigmentation of petals (Halevy and Mayak, 1979; Ichimura, 1998).

In rose petals and flowers, the starch level decreased whereas glucose and fructose levels increased with flower opening (Evans and Reid, 1988; van Doorn *et al.*, 1991). Similarly, in *Gladiolus* flowers, glucose and fructose concentrations increasd with flower opening (Yamane *et al.*, 1991). In daylily flowers, fructan concentration decreased, which is accompanied with increased in glucose and fructose concentrations with flower opening (Bieleski, 1993). The increase in monosaccharide concentrations is associated with an increase in osmotic pressure, which may lead to cell expansion of petal cells. To clarify to what degree soluble carbohydrates contribute for maintaining osmotic pressure, osmolar concentrations of carbohydrate should be determined.

High performance liquid chromatography (HPLC) is often used for determination of soluble carbohydrate content since it is easy to perform and yield reproducible results. To extract soluble carbohydrates, petals have been traditionally immersed in hot ethanol solution, and then homogenized. The homogenate is centrifuged and the resulting supernatant is concentrated to remove ethanol, then used for HPLC analysis (Koyama *et al.*, 1995; Ichimura and Hisamatsu, 1999; Ogiwara *et al.*, 1999; Doi *et al.*, 2000). These procedures are laborious and time-consuming. Furthermore, the value obtained in this method represents carbohydrate amount per fresh weight including insoluble materials. Since osmolar concentration of soluble carbohydrates is expressed as moles of carbohydrates per volume of cell solution, osmolar concentrations of carbohydrates cannot be determined correctly by this method.

Previously, we developed a simple and rapid extraction method to determine concentration of soluble carbohydrate in petals using a centrifugal filter device (Norikoshi *et al.*, 2006). In this method, tissues are frozen in liquid nitrogen, after which they are centrifuged. The resulting leached fluid was used as a soluble carbohydrate sample for HPLC analysis. However, sucrose concentration decreased over time whereas glucose and fructose concentrations increased even when the samples were kept at low temperature. This change may have been due to invertase activity. To inactivate invertase activity, microwave heating prior to carbohydrate extraction is effective in various plants including persimmon, strawberry and grape berry fruits (Zheng and Sugiura, 1990; Ogiwara *et al.*, 1999; Fujiwara *et al.*, 1999ab).

In the present study, we developed a simple and rapid extraction method to determine soluble carbohydrate concentration from rose petals. We further investigated changes in osmolar concentration of soluble carbohydrate and osmotic pressure in rose petals during flower opening.

Materials and methods

Plant material: Cut 'Sonia' rose flowers were obtained from a grower of Namekata city, Ibaraki prefecture, whereas 'Rote Rose' rose flowers were from a grower of Ishioka city, Ibaraki prefecture. For investigation of flower opening, 'Rote Rose' flowers at the following 3 stages were used in experiments. Stage 1, petals did not reflex; stage 2, outer petals started to reflex; stage 3, outer petals almost completely reflexed.

New method: Petals (200mg FW) were cut into pieces of about 5 mm² and placed in a centrifugal filter device (Ultrafree MC, 0.45 μ m, Millipore, Medford, MA, USA). The filter device with sample was transferred into a microwave oven (ER-VS12, Toshiba, Tokyo, Japan) and heated at 500 W for about 45 sec to boiling. Then, the filter device was centrifuged at 12,000 × g. Unless otherwise stated, centrifugation time was 10 min. The leached fluid was used for HPLC analysis and measurement of osmotic pressure.

Conventional extraction method: The conventional method was conducted as previously described (Ichimura and Hisamatsu, 1999). The petals (200 mg FW) were immersed in 5 mL of 80% ethanol at 75°C for 30 min. The sample was then homogenized and centrifuged at $3,000 \times \text{g}$ for 10 min. The pellet was twice reextracted with 2.5 mL of 80% ethanol and the three supernatants were combined and dried *in vacuo* below 50°C. The residue was dissolved in 1 ml of distilled water and used for HPLC analysis.

Determination of soluble carbohydrate: Carbohydrates were separated using an HPLC system (Jasco, Tokyo, Japan) equipped with a refractive index detector on a Shodex SUGAR SP0810 column (Showa denko, Tokyo, Japan). The column was kept at 80°C and eluted with water at flow rate of 0.8 mL min⁻¹. The identity of each peak was confirmed using authentic carbohydrates. Peak area was determined by integration and the amount of each carbohydrate in the sample was calculated.

Effects of storing time: To evaluate stability of carbohydrate concentration of the sample, the extracted solution was stored at 4° C for 3 h or at -30°C for 1 or 7 days.

Measurement of osmotic pressure: The osmotic pressure of resulting solution was measured with a vapor pressure osmometer (VAPRO5520, WESCO, Logan, UT, USA) according to the instruction manual. Osmotic pressure due to total soluble carbohydrate was calculated as described by Thorpe *et al.* (1993).

Results

Changes in recovery of cell fluids obtained from rose petals: Fig. 1 shows recovery of cell fluids obtained from rose petals during different durations of centrifugation. About 110 μ L fluid was recovered by centrifugation for 10 min. The amount of recovered fluid was slightly increased after further centrifugation.

Comparison of carbohydrate composition with conventional and new methods: Glucose, fructose, sucrose, methyl glucoside and *myo*-inositol were detected in samples obtained from the new method. To confirm the validity of this method, we compared carbohydrate composition obtained by the new method with that obtained by a conventional method because the units obtained from the conventional method are mg per fresh weight, which differ from the units obtained from the new method. No significant difference in amount of constitutive carbohydrates was observed between the two methods (Table 1).



Fig. 1. Recovery of cell fluids obtained from rose petals during different durations of centrifugation

Sample stability: To clarify to what degree the extracted samples are stable, rose samples were stored at 4°C for 3 h. The carbohydrate concentration did not change significantly during this period (Table 2). When the samples were stored at -30°C for 1 and 7 days, the carbohydrate concentration also did not change significantly except for xylose stored for 1 day (Table 3).

Changes in soluble carbohydrate concentrations and osmotic pressure during flower opening in roses: To evaluate the contribution of soluble carbohydrate to osmotic pressure, changes in soluble carbohydrate concentrations and osmotic pressure

Table 1. Carbohydrate compositions obtained by conventional and new method from 'Sonia' rose petals

Method	Carbohydrate composition (%) ²					
	Glucose	Fructose	Sucrose	Methyl	myo-	Xylose
				glucoside	Inositol	
Conventional	34.2	45.9	11.0	3.5	3.2	2.3
New	33.2	44.1	12.4	3.9	3.4	3.0
Significancey	NS	NS	NS	NS	NS	NS

^z Mean of 4 independent experiments.

^yNS indicates non-significant at P<0.05 by *t*-test. Statistical analysis was performed on data after arcsine transformation.

Table 2. Effects of storage periods on soluble carbohydrate contents of 'Sonia' rose flowers

Storage time	Carbohydrate concentration (mM) ^z					
(h)	Glucose	Fructose	Sucrose	Methyl	myo-	Xylose
				glucoside	Inositol	
0	55.3	129.3	9.8	7.6	11.9	9.4
3	53.2	133.0	9.6	7.1	10.8	9.3
Significancey	NS	NS	NS	NS	NS	NS

^z Mean of 4 independent experiments.

^yNS indicates non-significant at *P*<0.05 by *t*-test.

Table 3. Effects of storage periods on soluble carbohydrate contents of 'Sonia' rose flowers

Storage time	Carbohydrate concentration (mM) ^z					
(day)	Glucose	Fructose	Sucrose	Methyl	myo-	Xylose
				glucoside	Inositol	
0	18.2	30.6	2.2	1.2	1.3	3.7
1	17.4 ^y	30.4	2.3	1.1	1.4	5.0*
7	19.6	33.2	2.5	1.0	1.3	3.5

^z Mean of 4 independent experiments.

^y All values except for xylose stored for 1 day (*) were not significant at $P \le 0.05$ compared with initial values (0 time) by Dunnett's test.



Stage

Fig. 2. Concentration of differnt soluble carbohydrate at different flower opening stages.



Fig. 3. Total osmotic pressure and osmotic pressure due to carbohydrate at different flower opening stages.

during flower opening in roses was investigated using solutions obtained by the new method. Glucose and fructose concentrations in the petals increased whereas other carbohydrate concentrations remained more or less constant during flower opening (Fig. 2). Osmotic pressure in the petals was almost constant from stage 1 to 2 and increased thereafter (Fig. 3). Osmotic pressure due to total soluble carbohydrate increased during flower opening. From stage 2 to 3, total osmotic pressure and osmotic pressure due to soluble carbohydrates increased by 254 and 279 kPa, respectively.

Discussion

Previously, we reported that sucrose concentration in extracts obtained from frozen rose petals by centrifugation decreased with time (Norikoshi *et al.*, 2006). As found in many plants, invertase activity in rose petals is relatively high (Ho and Nichols, 1977). Thus, this decrease may be due to invertase activity. In the present study, petals were heated in a microwave oven to inactivate enzyme activities prior to extraction of soluble carbohydrates, since microwave heating also apparently inhibits invertase activity (Zheng and Sugiura, 1990; Ogiwara *et al.*, 1999; Fujiwara *et al.*, 1999ab). Sugar concentrations did not change during the storage

period (Tables 2 and 3). Thus, invertase activity in the samples in this method may be inactivated by microwave irradiation, resulting in high stability of the carbohydrate samples.

In rose petals, glucose, fructose, sucrose, methyl glucoside, xylose and *myo*-inositol have been reported as the carbohydrate constituents (Ichimura *et al.*, 1997). In the present study, these carbohydrates were detected in samples prepared by the new method. Little difference in the carbohydrate composition was observed between the new method and the conventional method (Table 1). It takes less than 90 min from preparation of plant sample to the start of HPLC. In contrast, it takes more than 1 day by the conventional method. Furthermore, apparatuses, such as centrifugal evaporators and homogenizers, are not used in the new method. Thus, the new method appears to be simple and rapid for extraction of soluble carbohydrates of petals.

In the present study, we measured the osmotic pressure of the cell fluid obtained by centrifugation. For measurement of osmotic pressure, in general, cell solution is obtained from plant materials by degrading their membranes (Nonami, 2001). To degrade membranes, tissues are generally frozen (Evans and Reid, 1988). In our previous paper, rose petals were frozen in liquid nitrogen and cell solution was collected from them by centrifugation. However, the soluble carbohydrate concentrations of the resulting solution changed with time (Norikoshi *et al.*, 2006). This suggests that osmotic pressure of the solution from frozen tissues changes with time. In contrast, carbohydrate concentrations did not change when tissues had been subjected to microwave heating (Table 2 and 3). Thus, we concluded that the solution obtained by the method using microwave heating is suitable for the measurement of osmotic pressure.

We investigated changes in soluble carbohydrate concentration and osmotic pressure during flower opening to confirm whether soluble carbohydrate contributes to increase in osmotic pressure. In our study, glucose and fructose concentrations increased during rose flower opening (Fig. 2). The increase in total osmotic pressure and osmotic pressure due to soluble carbohydrate in the petals from stage 2 to 3 was 254 and 279 kPa, respectively (Fig. 3). Thus, as reported in daylily flowers (Bieleski, 1993), an increase in glucose and fructose concentrations largely contributes to the increase in osmotic pressure, which may lead to expansion of petal cells. Although soluble carbohydrate concentration increased from stage 1 to 2, osmotic pressure did not increase (Fig. 3). Other than soluble carbohydrate, inorganic ions and organic acid act as osmotica (Cram, 1976). These compounds contribute to changes in osmotic pressure of rose petals.

In conclusion, we developed a new method to determine the osmolar concentrations of soluble carbohydrates using rose petals. Carbohydrate composition was almost the same as those determined by the conventional method. This method is simple and rapid and the extracted samples are stable. The solutions appear to be suitable for measurement of osmotic pressure. The method could be used to clarify to what degree soluble carbohydrate contributes to maintaining osmotic pressure in petals of floricultural plants.

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