An Improved High-performance Liquid Chromatographic Method for the Determination of Soluble Sugars in Root Exudates of Greenhouse Cucumber Grown under CO₂ Enrichment

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ABSTRACT. This study described a simple and quick method to detect trace quantities of a non-reducing sugar (viz. sucrose) in the root exudates of cucumber (*Cucumis sativus*) under CO₂ enrichment. Sucrose was determined by analyzing fructose and glucose before and after invertase digestion using high-performance liquid chromatography. Using this technique, the optimal hydrolysis condition was 5.00 µg·mL⁻¹ invertase for 10 minutes. The detection limit of ultraviolet-visible detector by post-column derivatization with tetrazolium was 0.25, 0.43, 0.48, and 1.95 µg·mL⁻¹ for fructose, glucose, sucrose, and maltose, respectively, and sensitive enough for determination of sugars in root exudates. The dry weight of cucumber at the seedling stage (19 days after transplant) increased by 58.4% when the CO₂ level was elevated from 380 to 1200 µmol·mol⁻¹, whereas the differences were not significant at the initial fruiting stage (63 days after transplant). The photosynthesis rate in 1200 µmol·mol⁻¹ CO₂ was 58.0% higher than that in 380 µmol·mol⁻¹ CO₂ at the seedling stage and 74.2% higher at the initial fruiting stage. Total amount of sugars in cucumber root exudates was significantly increased with increasing CO₂ concentration. The total sugars in root exudates increased by 130.4% and 102.3% in 1200 µmol·mol⁻¹ CO₂ compared with that in 380 µmol·mol⁻¹ CO₂ at seedling and initial fruiting stages, respectively. Elevated CO₂ altered sugar composition in root exudates. Sugars in root exudates released per plant were significantly higher at the initial fruiting stage than that at the seedling stage, whereas the differences in sugars released per gram of root tissue between these two growth stages were not significant. Our results suggest that sugars were increased only in as much as root mass increased. This study provides a simple and quick method to detect 1 to 500 µg·mL⁻¹ sugars in root exudates, and the results illustrate the variation in the sugar composition in cucumber root exudates among the CO₂ levels and growth stages.

In China, greenhouses are often not ventilated during cold winter months until midday when greenhouse temperature reaches the optimal value for plant growth. Therefore, at midday, the CO₂ concentration in greenhouses will drop to very low levels, which may result in inhibition of plants photosynthesis (Kläring et al., 2007). For this reason, CO₂ concentration inside the greenhouse usually is increased by adding CO₂ gas (Mortensen, 1987). CO₂ enrichment not only has a dramatic effect on above-ground plant growth and physiology by changing the photosynthetic assimilation rates, but also affects below-ground processes by altering the quantity and composition of root exudates (Berntson and Bazzaz, 1996; Cardon, 1996). Root exudates, which comprise 15% to 25% of

below-ground allocated carbon, include sugars, organic acids, amino acids, secondary metabolites, peptides, proteins, and lipids (Kuzaykov, 2002). Among them, sugars are the most abundant and serve as a direct energy source for most of the microorganisms in the rhizosphere (Curl and Truelove, 1986; Schönwitz and Ziegler, 1982). Consequently, it is essential to understand the effects of CO₂ enrichment on sugars in root exudates, which are released to the rhizosphere.

Because identification and quantification of individual and trace amounts of sugars pose several analytic problems, most research only determined the total content of sugars in the root exudates by using anthrone's method (Heim et al., 2000; Kato et al., 1997). Owing to the low volatility of sugars, some tedious derivatization steps such as silylation (Sweeley et al., 1963), acetylation (Albersheim et al., 1968), and methylation (Hanisch, 1994) are usually involved before gas chromatography–mass spectrometry analysis. As a result of the high specificity of each enzyme, the enzymatic assays developed recently can be only used for a few common sugars such as sucrose, glucose, and fructose

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(Spackman and Cobb, 2002). Therefore, high-performance liquid chromatography (HPLC) is considered as a powerful and widely used technique for quantifying various types of sugar mixtures (Montero et al., 2004).

Many chemical derivatization methods have been developed for detection of sugar mixtures. However, most chromogenic or fluorescent agents like tetrazolium blue (D'Amboise et al., 1980) and benzamidine (Kai et al., 1985) have weak responses to non-reducing sugars, which are abundant in root exudates. So, in many previous studies, only the reducing sugars in root exudates were reported (Kravchenko et al., 2003; Tawaraya et al., 1994), or sucrose was measured by another method (Lugtenberg et al., 1999). Although arginine (Mikami and Ishida, 1983) and periodic acid with guanidine hydrochloride (Masuda et al., 1996) have been reported for the derivatization of sucrose, much lower activity (10% activity of reducing sugars), long separation time (more than 1 h), high reaction temperature (near 150 °C), and ultra-long tubing system (as long as 24 m) limit their use for root exudate analysis. Therefore, a simple, quick method to detect trace quantities of sucrose with high sensitivity and selectivity is urgently needed.

Invertase is an enzyme that hydrolyzes sucrose into fructose and glucose with high efficiency in mild conditions (Nelson and Schubert, 1928). So, it is reasonable to determine sucrose by analyzing fructose and glucose before and after invertase digestion by a regular HPLC process. The objectives of this study were 1) to find the optimal reaction conditions for hydrolysis of sucrose by invertase; 2) to identify the soluble sugars in root exudates by HPLC; and 3) to investigate the amounts and components of soluble sugar in cucumber root exudates under different CO₂ concentrations at different growth stages.

Materials and Methods

HYDROLYSIS OF SUCROSE BY INVERTASE. Time-dependent experiments were carried out to trace the hydrolysis process of sucrose. Invertase (invertase from baker's yeast, Grade VII, 300 U·mg⁻¹ or greater solid; Sigma-Aldrich St. Louis, MO) solution (1000 µg·mL⁻¹) was prepared with ultrapure water. Sucrose solutions (10.0 mL, 500 µg·mL⁻¹) were kept at 50 °C in a water bath, and enzymatic hydrolysis was started by adding a specific volume of invertase solution to obtain final invertase concentrations of 5.00, 1.00, 0.10, and 0.01 µg·mL⁻¹. After 5, 10, 30, and 60 min, the reaction was stopped by immersing the mixed solution in a boiling water bath for 10 min. Fructose and glucose in the final solutions were analyzed by HPLC.

GROWING CONDITIONS. The experiment was carried out in the glasshouse at Institute of Soil Science, Chinese Academy of Sciences, Nanjing, P.R. China, from Nov. 2012 to Jan. 2013. Seeds of cucumber (cv. Jinlv 3) were germinated on moist filter paper in a growth chamber at a temperature of 28 °C and a relative humidity of 70%. Two days later, the seeds with radicles were sown into trays containing a peat-vermiculite mixture (2:1, v/v). When two true leaves were fully expanded, seedlings were transplanted to elaborate polyvinyl chloride polymer pots (5 L), which contain 4 L of Yamazaki nutrient solution for cucumber (Yamazaki, 1982) with two plants per pot on 16 Nov. 2012. The nutrient solution was composed of 826 mg·L⁻¹ Ca(NO₃)₂·4 H₂O, 115 mg·L⁻¹ NH₄H₂PO₄, 607 mg·L⁻¹ KNO₃, 493 mg·L⁻¹ MgSO₄·7 H₂O, 29.27 mg·L⁻¹

Na₂Fe-EDTA, 2.86 mg·L⁻¹ H₃BO₃, 2.03 mg·L⁻¹ MnSO₄·4 H₂O, 0.22 mg·L⁻¹ ZnSO₄·7 H₂O, 0.08 mg·L⁻¹ CuSO₄·5 H₂O, and 0.02 mg·L⁻¹ (NH₄)₆Mo₇O₂₄·4 H₂O. The pH was adjusted to 6.5 using diluted KOH and H₂SO₄. The solution was aerated intermittently for 30 min every hour and renewed completely every 4 d. When the plant had five to six true leaves, one plant in each pot was harvested for analysis on 5 Dec. 2012. When small fruit of 5 to 8 cm long formed, the plant left in each pot was harvested on 18 Jan. 2013. Six pots of replications (12 plants of replications at seedling stage and six replications at initial fruiting stage) were grown within open-top chambers [OTC (2.3 m length × 0.8 m width × 1.4 m height)] under natural light. The CO₂ concentration in three identical OTCs was set as 380, 625, and 1200 µmol·mol⁻¹, respectively, and was continuously monitored and controlled through an infrared gas analyzer (Ultramat 6; Siemens, Munich, Germany) from 0800 to 1700 HR everyday. The temperature and relative humidity within the OTCs were recorded by a L95-83 recorder (Hangzhou Loggertech Co., Hangzhou, China) every 10 min. The temperature and relative humidity during the experiment range from 12 to 30 °C and 30% to 80%, respectively.

COLLECTION AND FRACTIONATION OF ROOT EXUDATES. The root exudates of cucumber were collected at seedling (4 Dec. 2012) and initial fruiting stages (17 Jan. 2013) by a modified continuous trapping system similar to that designed by Tang and Young (1982) (Fig. 1). The nutrient solution in each pot was simultaneously driven and continuously recycled at the rate of 25 mL·min⁻¹ by a peristaltic pump (BT100-1L; Baoding Longer Precision Pump Co., Baoding, China) from 0900 to 1500 HR on the day after the nutrient solution was renewed. The trapping column was packed with 20 cm³ of resin (Amberlite XAD-4; Alfa Aesar, Ward Hill, MA) and filled with cotton on both sides. All the tubes and the column were covered with aluminum foil completely to avoid algal growth. After collection, the column was taken off to drain off excess nutrient solution, and then the resin was washed with 100 mL of ethanol. The ethanol was removed using a vacuum rotary evaporator at 40 °C and the residue was redissolved in 10 mL ultrapure water. Root exudates in the aqueous solution were separated by ion exchange resins (Gransee and Wittenmayer, 2000). The aqueous solution was first passed through a cation exchange column

Fig. 1. Schematic illustration of the continuous trapping system for collecting the root exudates of cucumber. The nutrient solution in the polyvinyl chloride polymer pot was driven by a peristaltic pump and flow-through sieve, Millipore membrane (Merck Millipore, Billerica, MA) and XAD-4 resin (Alfa Aesar, Ward Hill, MA) and then back to the pot.

(Amberlite IR-120, H⁺ form, column bed 20 cm³; Alfa Aesar), and the effluent was then passed through an anion exchange column (Amberlite IRA-410, formate form, column bed 20 cm³; Alfa Aesar). The final effluent was lyophilized and the residue was redissolved in 4 mL ultrapure water, 2 mL of which was hydrolyzed by 5 µg·mL⁻¹ invertase for 10 min. Sugars in the root exudates solution before and after hydrolysis were determined by HPLC.

CHROMATOGRAPHIC CONDITIONS. HPLC analysis was performed on a LC-10AVP HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-10ATVP pump, SPD-10AVP ultraviolet-visible detector, CTO-10AVP column oven, CRB-6A chemical reaction box, SIL-10AVP autosampler, and Shimadzu LC solution software (Class-VP Version 6.1). Samples were filtered through 0.20-µm Millipore membrane (Merck Millipore, Billerica, MA) and 10 µL filtrate was injected for analysis. Separation of sugars was achieved on a Shim-pack CLC-NH₂(M) column (250 mm length × 4.6 mm diameter; Shimadzu) maintained at 25 °C by elution with acetonitrile–water (75:25 v/v, HPLC-grade acetonitrile; Sigma-Aldrich) at a flow rate of 1.0 mL·min⁻¹ (Lugtenberg et al., 1999). After separation, the eluate was mixed through a T-joint with tetrazolium reagent. The reagent was composed of 2 g·L⁻¹ blue tetrazolium chloride (Alfa Aesar) in 7.2 g·L⁻¹ NaOH and pumped at 0.2 mL·min⁻¹. The derivatization was conducted at 85 °C through a stainless steel tube (3 m length × 0.25 mm i.d.) in the chemical reaction box. Derivatized mixtures were cooled by passing through a stainless steel tube (1 m length × 0.25 mm i.d.) in an ice-water bath; the sugars were detected at 487 nm using the ultraviolet-visible detector. Sugars were identified by comparing the retention times of sample peaks with standards.

DRY WEIGHT AND PHOTOSYNTHETIC RATE ANALYSIS. Plants were separated into roots, stems, and leaves after the whole plants were removed from the pots. Dry weight (DW) of each organ was determined by drying the fresh tissues at 105 °C for 30 min and then further drying at 80 °C for 24 h. Photosynthetic rate (Pn) was measured on clear days (3 Dec. 2012 at the seedling stage and 10 Jan. 2013 at the initial fruiting stage) from 0900 to 1500 HR using a portable photosynthetic apparatus (LI-6400; LI-COR, Lincoln, NE) equipped with a standard leaf chamber. Twelve leaves (the fourth leaf from the apex on each plant at the seedling stage; the fourth and fifth leaves on each plant at the initial fruiting stages) from each treatment were measured. The photosynthetic photon flux density was set as 1500 µmol·m⁻²·s⁻¹, which was provided by an attached LI-6400-02B blue–red light-emitting diode light. Temperature

of the air in the leaf chamber was set to 25 °C and the CO₂ concentration in the leaf chamber was set the same as the CO₂ environment where the plants were grown.

STATISTICAL ANALYSIS. Time-dependent experiments of sucrose hydrolysis were replicated five times. DW of each plant and sugar composition of root exudates were analyzed as a three × two factorial (CO₂ concentration × growth stage) using a completely randomized block design with six replications. Pn was measured on 12 leaves from 12 individual plants at the seedling stage and from six individual plants at the initial fruiting stage for each treatment. Ten readings were recorded from each leaf and averaged as one observation. Pn data presented are means of 12 observations. Statistical analysis was performed using SPSS software (Version 18.0; IBM Corp., Armonk, NY). Differences between means under the same CO₂ concentration at two different growth stages were evaluated by independent samples *t* test. Differences among means under different CO₂ concentrations at the same growth stage were separated using Duncan tests of one-way analyses of variance at *P* ≤ 0.05. Error was reported as the SEM. Figures and linear fitting procedures were generated by OriginPro (Version 8.0; OriginLab Corp., Northampton, MA).

Results

SEPARATION OF STANDARD SUGAR MIXTURE. A typical chromatogram of a mixture of three standard sugars (each was 250 µg·mL⁻¹) separated by HPLC was shown in Figure 2, in which fructose, glucose, and maltose were eluted at 9.404, 10.598, and 17.049 min, respectively. All the peaks were symmetrical and the total separation time was no more than 20 min. A linear response was obtained with fructose, glucose, and maltose up to 500 µg·mL⁻¹, and the detection limit was 0.25, 0.43, and 1.95 µg·mL⁻¹ (signal/noise = 3). *R*² values of the corresponding fitted lines were 0.996, 0.999, and 0.997, respectively. Sucrose cannot form chromatic derivatives directly with blue tetrazolium, whereas fructose and glucose, which were the hydrolysates of sucrose, can be colored. An equal amount of fructose and glucose was detected in the sucrose solutions after hydrolysis by invertase. Because the sensitivity of fructose was higher than that of glucose, the fructose contribution to sucrose was used to calculate the initial concentration of sucrose. The linear response of sucrose was up to 1000 µg·mL⁻¹, and the detection limit was 0.48 µg·mL⁻¹.

CONDITION OF SUCROSE HYDROLYSIS. Hydrolysis of sucrose by invertase was traced by time-dependent experiments (Fig. 3).

Fig. 2. Separation of a standard sugar mixture (250 µg·mL⁻¹) by high-performance liquid chromatography under isocratic conditions (acetonitrile:water = 75:25 v/v) at a flow rate of 1.0 mL·min⁻¹ at 25 °C (inset = calibration plot).

It can be seen that the rate of sucrose hydrolysis increased with increasing invertase concentration. Completed hydrolysis was achieved at 5, 10, and 30 min when 5.00, 1.00, and 0.10 $\mu\text{g}\cdot\text{mL}^{-1}$ invertase was used, respectively. When 0.01 $\mu\text{g}\cdot\text{mL}^{-1}$ invertase was used, only 20% to 30% sucrose was hydrolyzed when the reaction time lasted 60 min. To ensure the completed hydrolysis of sucrose, all the samples in sucrose detection were hydrolyzed in 5 $\mu\text{g}\cdot\text{mL}^{-1}$ invertase for 10 min.

EFFECT OF THE ATMOSPHERIC CO_2 CONCENTRATION ON PLANT GROWTH AND PHOTOSYNTHETIC RATE. The DW of roots, stems, and leaves of cucumber was altered by CO_2 concentrations (Table 1). At the seedling stage (19 d after transplant), DW of roots, stems, and leaves of the plant grown under 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 was significantly greater than that under 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 . At the initial fruiting stage (63 d after transplant), the greatest root and stem DW were also obtained under 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 , but the differences of leaf and whole plant DW among treatments were not significant. DWs of plant tissues and the whole plant under 380 and 625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 were similar at seedling and initial fruiting stages, except stem at the seedling stage. The root/shoot was not significantly affected by CO_2 concentration or growth stage.

Fig. 3. Extent of standard sucrose ($500 \mu\text{g}\cdot\text{mL}^{-1}$) hydrolysis as a function of time with different invertase concentrations. Data are means $\pm$ SE ($n = 5$).

Table 1. Dry weight of roots, stems, leaves, and the whole plants of cucumber under different CO_2 concentrations at seedling and initial fruiting stages ($n = 6$).

Stage	Plant tissue	Dry wt [mean $\pm$ SE (mg/plant)]		
		380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2	625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2	1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2
Seedling stage	Roots	70.2 $\pm$ 4.8 b ^z	88.2 $\pm$ 5.4 b	121.7 $\pm$ 14.8 a
	Stems	157.7 $\pm$ 10.3 b	227.1 $\pm$ 23.0 a	281.1 $\pm$ 23.6 a
	Leaves	638.8 $\pm$ 27.3 b	748.6 $\pm$ 33.2 b	969.7 $\pm$ 58.4 a
	Total	866.6 $\pm$ 37.1 b	1064.0 $\pm$ 60.0 b	1372.6 $\pm$ 93.6 a
	Root/shoot	0.0890 $\pm$ 0.0074 a	0.0906 $\pm$ 0.0031 a	0.0957 $\pm$ 0.0032 a
Initial fruiting stage	Roots	606.8 $\pm$ 75.6 b	623.0 $\pm$ 86.6 b	923.8 $\pm$ 109.4 a
	Stems	2121.4 $\pm$ 154.1 b	2192.2 $\pm$ 248.1 ab	3092.2 $\pm$ 442.8 a
	Leaves	3863.8 $\pm$ 381.2 a	4235.4 $\pm$ 697.4 a	5485.1 $\pm$ 789.3 a
	Total	6592.0 $\pm$ 589.9 a	7050.6 $\pm$ 1021.3 a	9501.1 $\pm$ 1328.4 a
	Root/shoot	0.1007 $\pm$ 0.0063 a	0.0979 $\pm$ 0.0048 a	0.1098 $\pm$ 0.0030 a

^zMeans within rows not followed by the same letter are significantly different at $P \leq 0.05$.

Pn values under different CO_2 concentrations applied are shown in Figure 4. Pn of the plant grown under 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 was significantly higher than that under 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 treatment at both seedling and initial fruiting stages. During the growth of cucumber, Pn was decreased from the seedling to initial fruiting stage by 31.7% to 43.4% in all three CO_2 treatments.

EFFECT OF THE ATMOSPHERIC CO_2 CONCENTRATION ON SUGAR COMPONENT IN ROOT EXUDATES. The amounts of four common sugars in the root exudates released by one cucumber plant under different CO_2 concentrations are listed in Table 2. Concentrations of sugar from plants growing under 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 were greater than that under 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 . At the seedling stage, fructose and glucose in the root exudates increased when the concentration of CO_2 was elevated to 625 $\mu\text{mol}\cdot\text{mol}^{-1}$, whereas concentrations of other sugars released under 380 and 625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 were similar. At the initial fruiting stage, only glucose concentration under 625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 was greater than that under 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 . Glucose was the major sugar in root exudates and constituted $\approx 40\%$ of the total pool of sugars, and fructose was the second major sugar, which constituted $\approx 25\%$ of the total sugars (Table 3). Two disaccharides, sucrose and maltose, were less than 20% of the total sugars. As plants grew, the total amount of sugars was increased almost 5-fold from seedling to initial fruiting stage.

The CO_2 concentration altered the specific root exudation of sugars per gram DW of root tissue (Fig. 5). Cucumber seedlings growing under 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 released more fructose and glucose in root exudates per gram DW than under 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO_2 . In general, CO_2 concentration had little influence on the specific root exudation of sucrose, maltose, and total sugar. At initial fruiting, only the amount of sucrose released per gram DW was increased as the concentration of CO_2 increased from 380 to 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$. Although the total amount of sugars released per plant was 5-fold higher at the initial fruiting stage than that at the seedling stage (Table 2), it is noteworthy that almost all the sugars released per gram of root tissue were similar between seedling and initial fruiting stages.

Discussion

Early work reported that yeast invertase was a highly effective catalyst for the hydrolysis of sucrose, and the inversion rate is proportional to the concentration of sucrose, water, and invertase at certain temperatures (Nelson and Schubert, 1928). Therefore, when the concentration of sucrose is constant and very low ($500 \mu\text{g}\cdot\text{mL}^{-1}$), the rate of inversion is only determined by the concentration of invertase. In the present study, the rate of sucrose hydrolysis increased with invertase concentration increasing from 0.01 to 5.00 $\mu\text{g}\cdot\text{mL}^{-1}$. To make sure that the hydrolysis of sucrose is finished in 10 min, 5.00 $\mu\text{g}\cdot\text{mL}^{-1}$ invertase is chosen as the optimal dosage.

Refractive index detector (RID) is usually used in quantifying sugar mixtures by HPLC, but its detection limit can be only 100 $\mu\text{g}\cdot\text{mL}^{-1}$ for

fructose, glucose, and sucrose (Sharma et al., 2009). In this work, most sugars collected at the seedling stage in the solution for HPLC analysis were less than 100 $\mu\text{g}\cdot\text{mL}^{-1}$, so RID would be unsuitable for detecting the trace quantities of sugars in root exudates. The detection limit of evaporative light-scattering detector (ELSD) has been reported as 44 and 11 $\mu\text{g}\cdot\text{mL}^{-1}$ for glucose and sucrose, respectively (Sharma et al., 2010). However, ELSD is not specific for sugars, and other compounds such as organic acids and amino acids in root exudates could interfere with the detection of sugars. Soga et al. (1992) used pulsed amperometric detector (PAD) for the determination of sugars, and the detection limits for fructose, glucose, sucrose, and maltose were 0.09, 0.07, 0.21, and 0.27 $\mu\text{g}\cdot\text{mL}^{-1}$,

Fig. 4. Effects of CO₂ concentration on net photosynthesis rates of cucumber at seedling (17 d after planting) and initial fruiting stages (55 d after planting). Data are means $\pm$ SE (n = 12). Means within the same stage not followed by the same letter are significantly different at $P \leq 0.05$. *, *** Means between different growth stages within each CO₂ treatment are significantly different at $P \leq 0.05$ or 0.001, respectively.

respectively (assuming injected volume was 10 μL). However, PAD requires a special stationary phase and tubing system designed for high pH conditions. The detection limit of the ultraviolet-visible detector in this work was 0.25, 0.43, 0.48, and 1.95 $\mu\text{g}\cdot\text{mL}^{-1}$ for fructose, glucose, sucrose, and maltose, respectively, which is similar to a previous report (D'Amboise et al., 1980). Therefore, the detection limit of the present method was much better than that of RID and ELSD and was slightly lower than that of PAD. Considering the sugar concentration in root exudates was more than 10 $\mu\text{g}\cdot\text{mL}^{-1}$, the present method provides greater economy and adequate sensitivity alternation for determination of sugars in root exudates.

It is well known that CO₂ enrichment can increase plant yield and enhance photosynthesis (Kimball, 1983; Makino and Mae, 1999; Peet and Willits, 1987). In cucumber, the plants with CO₂ enrichment (700 to 2000 $\mu\text{mol}\cdot\text{mol}^{-1}$) had 14% to 259% more DW and 76% to 175% higher Pn than that without CO₂ enrichment (Agüera et al., 2006; Sánchez-Guerrero et al., 2005; Segura et al., 2001; Wei et al., 2002). In our study, a 58.4% increase in DW was observed at the seedling stage when the CO₂ level was elevated from 380 to 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ (Table 1). Similarly, the Pn in 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ was 58.0% higher than that in 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ at the seedling stage and 74.2% higher at the initial fruiting stage, respectively. The unaltered root/shoot means there was no significant difference in dry matter partitioning as a result of CO₂ treatment (Hodge et al., 1998). These results were consistent with previous work, and the increased DW and Pn were possibly caused by more fixation of CO₂ and accumulation of biomass (Makino and Mae, 1999).

Although the Pn was much larger in plants grown in 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ than in 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂, the difference in DW was not significant at the initial fruiting stage. We found at

Table 2. Sugar composition of cucumber root exudates growing under different CO₂ concentrations at seedling and initial fruiting stages (n = 6).

Stage	Sugar	Sugar [mean $\pm$ SE ($\mu\text{g}/\text{plant}$)] ^z		
		380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO ₂	625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO ₂	1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO ₂
Seedling stage	Fructose	199.3 $\pm$ 55.5 b ^y	388.1 $\pm$ 43.1 a	525.9 $\pm$ 51.8 a
	Glucose	358.6 $\pm$ 74.3 c	640.3 $\pm$ 85.0 b	926.5 $\pm$ 66.9 a
	Sucrose	172.8 $\pm$ 32.2 b	218.0 $\pm$ 26.6 b	333.7 $\pm$ 41.1 a
	Maltose	146.9 $\pm$ 29.1 b	210.0 $\pm$ 26.2 ab	236.0 $\pm$ 18.0 a
	Total	877.6 $\pm$ 184.8 c	1456.4 $\pm$ 173.3 b	2022.1 $\pm$ 160.8 a
Initial fruiting stage	Fructose	1854.4 $\pm$ 360.2 b	1676.0 $\pm$ 317.0 b	2863.6 $\pm$ 238.6 a
	Glucose	2237.7 $\pm$ 253.7 b	3412.0 $\pm$ 381.7 a	4203.1 $\pm$ 226.0 a
	Sucrose	770.3 $\pm$ 108.7 b	1153.9 $\pm$ 159.7 b	2583.3 $\pm$ 392.2 a
	Maltose	775.6 $\pm$ 150.9 b	1186.2 $\pm$ 153.4 ab	1758.0 $\pm$ 299.5 a
	Total	5638.0 $\pm$ 766.4 b**	7428.1 $\pm$ 785.4 b***	11408.0 $\pm$ 991.2 a***

^zSugars released by individual plant at the seedling stage were calculated as half the value of the exudates collected from one pot.

^yMeans within rows at the same stage not followed by the same letter are significantly different at $P \leq 0.05$.

, * Means between different growth stages within each CO₂ treatment are significantly different at $P \leq 0.01$, or 0.001, respectively (n = 6).

Table 3. Percentage of each sugar in the total amount of four sugars in cucumber root exudates growing under different CO₂ concentrations at seedling and initial fruiting stages (n = 6).

Stage		Seedling stage			Initial fruiting stage		
CO ₂ concn ($\mu\text{mol}\cdot\text{mol}^{-1}$)		380	625	1200	380	625	1200
Sugar [mean $\pm$ SE (%)]	Fructose	21.5 $\pm$ 1.5 b ^z	26.8 $\pm$ 0.4 a	25.9 $\pm$ 0.9 a	32.1 $\pm$ 3.7 a	22.4 $\pm$ 2.5 b	25.4 $\pm$ 1.9 ab
	Glucose	40.9 $\pm$ 1.2 b	43.5 $\pm$ 0.9 ab	46.0 $\pm$ 1.0 a	40.4 $\pm$ 1.9 ab	46.0 $\pm$ 2.2 a	37.4 $\pm$ 1.5 b
	Sucrose	20.5 $\pm$ 1.5 a	15.2 $\pm$ 0.8 b	16.3 $\pm$ 0.8 b	13.9 $\pm$ 1.2 b	15.6 $\pm$ 1.6 b	22.3 $\pm$ 2.4 a
	Maltose	17.0 $\pm$ 0.7 a	14.5 $\pm$ 0.8 a	11.9 $\pm$ 1.0 b	13.6 $\pm$ 2.0 a	16.0 $\pm$ 1.4 a	14.9 $\pm$ 1.3 a

^zMeans within rows at same stage not followed by the same letter are significantly different at $P \leq 0.05$.

the end of initial fruiting stage (≈ 10 d), the daily average temperature in the OTC was less than 18 °C and the daily minimum temperature was 10 to 12 °C. The effect of CO₂ enrichment on biomass may be limited by the low temperature. On the other hand, there were no significant differences in DW and Pn between the cucumber growing in 380 and 625 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂. Because the effect of CO₂ enrichment is affected by many factors such as plant species, growth age, temperature, light, nutrients, water, and so on, we speculate that the CO₂ concentration that has the largest effect on cucumber is higher than 625 $\mu\text{mol}\cdot\text{mol}^{-1}$ in our OTC experiments. It is also noteworthy that the Pn was lower at the initial fruiting stage than that at the seedling stage. This phenomenon has been reported as photosynthetic acclimation and down-regulation of photosynthesis during long-term CO₂ enrichment (Ayari et al., 2000; Makino and Mae, 1999).

Sugars are one of the most abundant primary metabolites in root exudates. Vančura and Hovadík (1965) have reported that nine identifiable reducing sugars, viz. arabinose, fructose, glucose, maltose, desoxyribose, xylose, galactose, ribose, and rhamnose, were detected in the root exudates of cucumber seedlings. Changes in the amounts of specific sugars in the root exudates of cucumber have been quantified by Kamilova et al. (2006). They found glucose and fructose were two major sugars in the root exudates of cucumber cultivated on glass beads, which contributed 44.2% and 40.8% reducing sugars,

respectively, whereas maltose (11.9%) and xylose (3.1%) were two minor compositions. However, non-reducing sugars were not detected by the method described in their work. We measured similar glucose and fructose concentrations in the root exudates of cucumber as reported by Kamilova et al. (2006). The sugars in the root exudates of cucumber seedlings grown in 380 $\mu\text{mol}\cdot\text{mol}^{-1}$ CO₂ consisted of glucose (40.9%), fructose (21.5%), sucrose (20.5%), and maltose (17.0%). The total amount of sugars released per plant in our study was ≈ 100 times more than that in Kamilova et al. (2006). Differences in sugar concentrations between our study and those reported by Kamilova et al. (2006) may be a result of different growth conditions, collection methods, and collection durations.

Owing to enhanced Pn and increased plant growth, sugars in root exudates may similarly be expected to increase under CO₂ enrichment. Hodge et al. (1998) reported that the free sugars in the root exudates of *Lolium perenne* were increased under elevated CO₂ treatment, whereas the total sugars were decreased. In Haase et al.'s (2007) work, total sugars in the root exudates of *Phaseolus vulgaris* were increased with CO₂ enrichment only in nitrogen (N)-deficiency treatments at 12 d after sowing, whereas they were increased regardless of N supply at 18 d after sowing. In our work, total amount of sugars in cucumber root exudates was increased with an increase in CO₂ concentration from 380 to 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ at both seedling and initial fruiting stages (Table 2).

There is little published research describing changes in sugar compositions in root exudates when plants are grown with elevated CO₂. Our results indicate that at the seedling stage, the percentage of fructose and glucose in root exudates increased with an increase in CO₂ concentration from 380 to 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$. In contrast, at the initial fruiting stage, the percentage of sucrose in root exudates increased with an increase in CO₂ concentration from 380 to 1200 $\mu\text{mol}\cdot\text{mol}^{-1}$ (Table 3). Studies on weeds showed that the percentages of glucose and fructose in root were increased in the growing season from February to July, whereas sucrose was increased after July (Cyr et al., 1990; Wilson et al., 2001). Based on these results, we assume that most photosynthates, which are transported to roots, are present as sucrose and this sucrose hydrolyzed into glucose and fructose for the growth of roots at the seedling stage. As plants mature, sucrose from above ground is accumulated and stored in root. Therefore, it is reasonable that the percentage of glucose in root exudates was directly proportional to CO₂ concentration at the seedling stage, whereas the percentage of sucrose was directly proportional to CO₂ concentration at the initial fruiting stage.

Although sugars in root exudates released per plant were significantly higher at the initial fruiting stage than that at the seedling stage, the differences in sugars released per gram DW between these two growth stages were not significant. These results indicated that sugars were increased only in as much as

Fig. 5. Sugars released by cucumber roots under different CO₂ concentrations at seedling and initial fruiting stages. Data are means $\pm$ SE (n = 6). Means within same stage not followed by the same letter are significantly different at $P \leq 0.05$ (n = 6). *, ** Means between different growth stages within each CO₂ treatment are significantly different at $P \leq 0.05$ or 0.01, respectively.

root mass increased. The decrease of root exudation with an increase in plant age has been observed on alfalfa [*Medicago sativa* (Hamlen et al., 1972)], rice [*Oryza sativa* (Aulakh et al., 2001; Bacilio-Jiménez et al., 2003)], and wheat [*Triticum aestivum* (Přikryl and Vančura, 1980)]. Four possible explanations for decreased root exudation with plant maturation have been hypothesized (Aulakh et al., 2001; Bacilio-Jiménez et al., 2003; Hamlen et al., 1972): 1) accumulation of high levels of organic compounds in the vicinity of the root, which represses the release of more root exudates; 2) reabsorption of the organic compounds by the root; 3) lack of aeration reduces the activity of root; and 4) senescence and death of root over time. Considering the rapid adsorption of root exudates by resin, the concentration of root exudates could not be too high to limit the release of root, and the reabsorption of root exudates was impossible. Additionally, our growing pots were well aerated, so root activity could not be repressed. Therefore, the slight decrease of sugars released per gram DW of root may be caused by the senescence of root from the seedling to initial fruiting stage.

Conclusions

The optimal reaction conditions for determination of sucrose by analyzing fructose and glucose before and after invertase digestion by a regular HPLC process were studied. This method can be used to identify the soluble sugars in root exudates of cucumber at seedling and initial fruiting stages grown in hydroponic conditions under different CO₂ concentrations. Using this method, we determined that glucose was the major sugar in root exudates and constituted ≈40% of the total sugars. The effect of enriched atmospheric CO₂ concentrations on the total amount of sugars in the root exudates of cucumber was mainly the result of the increase of root mass.

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