Photosynthetic and respiratory activities of spinach in an unheated greenhouse during winter in Sapporo, Japan

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Abstract

Leafy vegetables cultivated in greenhouses during the winter are sometimes exposed to cold air from outside the greenhouse to enhance sugar and nutrient content. To analyze the possible involvement of photosynthetic and respiratory activities in this process, we evaluated the gas-exchange activity of spinach (\textit{Spinacia oleracea} L.) plants cultivated in an unheated greenhouse in mid-winter in Sapporo, where the daily mean air and soil temperatures are approximately -5 and 0 °C, respectively. Shoot fresh weight showed little increase, whereas the net leaf photosynthetic rate ($P_{\text{n}}$) attained 20 $\mu$mol m$^{-2}$ s$^{-1}$ and the CO$_2$ concentration in the greenhouse ([CO$_2$]) was sometimes lower than 200 $\mu$mol mol$^{-1}$, which was suggestive of active photosynthetic CO$_2$ uptake. After its peak in the morning, $P_{\text{n}}$ decreased in the afternoon, presumably owing to ‘midday depression’ caused by suppressed water uptake in the root zone. Observed diurnal [CO$_2$] change was consistent with a significant CO$_2$ uptake during the daytime. The change also suggested that respiration was active immediately after sunset and suppressed at night. In addition, we calculated the whole-greenhouse CO$_2$ emission rate ($R$) as a measure of night respiration in the plants, taking into account the air ventilation of the greenhouse. The $R$ value was positive under sub-zero air temperatures in the greenhouse and was positively correlated with the nighttime air and soil temperatures. These experimental data suggest active photosynthesis and respiration of winter-sweetened spinach in the greenhouse, despite the low air and soil temperatures and growth retardation, and implies their involvement in the sweetening process.

Key words: BRIX, Environmental control, Spinach, Value-added vegetable, Ventilation

1. Introduction

Although horticultural facility environments are usually controlled with the aim of achieving high yields, attempts have been made to cultivate plants under suboptimal environments to produce value-added products. Low-temperature stress has been utilized effectively by commercial farmers to produce value-added leafy vegetables in northern Japan (e.g. Kato \textit{et al.}, 1994, 1995, 1996; Aoki \textit{et al.}, 1997) and the USA (Orde \textit{et al.}, 2018). In this process, leafy vegetables are raised in greenhouses in late fall and subsequently maintained during winter to produce ‘sweetened’ vegetables with high sugar and ascorbic acid content (e.g. Kato \textit{et al.}, 1995). To attain an intense sweetening effect, vents of the greenhouse are sometimes opened to expose the plants to the cold outside air. The sweetening treatment also contributes to higher content of antioxidants and free amino acids, without increasing nitrate and oxalate content (Watanabe and Ayugase, 2015; Yoon \textit{et al.}, 2017). In addition, local farmers are able to adjust the shipping schedule because plant growth is delayed or halted by the treatment.

The mechanism of cold-induced sweetening is as follows: 1) low soil temperature limits water uptake in the root zone (Kramer, 1983), 2) the leaf transpiration rate exceeds the water uptake rate, leading to leaf dehydration (Kato \textit{et al.}, 1996), and 3) solutes in the cells are concentrated owing to the osmotic adjustment to increase freezing tolerance. While leaf dehydration results in an increase in the sugar content per fresh weight, it also causes water stress on the plant and triggers the sugar accumulation associated with cold acclimation (Alberdi and Corcuera, 1991). In addition to these effects (leaf dehydration and cold acclimation), since accumulated carbohydrates are derived from photosynthates and consumed by respiration, these gas-exchange processes are likely involved in the induction and maintenance of sweetening. However, given that the plant ceases growth during the sweetening treatment, it is suggested that photosynthesis and respiration may balance over this period. In winter greenhouses in cold regions of Japan, such as Hokkaido, vents are often closed to prevent frost damage, and ventilation is limited. If leaf photosynthesis is active during the sweetening treatment, the CO$_2$ concentration inside the greenhouse may be depleted. A low temperature may mitigate respiratory CO$_2$ emissions and accelerate CO$_2$ depletion. Therefore, the low CO$_2$ concentration in a closed greenhouse may limit photosynthesis and act as an obstacle to further sweetening.

To clarify photosynthetic activity of winter spinach during growth retardation, in this study we measured diurnal changes in net leaf photosynthetic rates of plants cultivated in a greenhouse in Sapporo, where the daily mean air temperature...
is usually lower than 0 °C in winter. We also measured the CO₂ concentration and its diurnal changes inside the greenhouse, which reflect the balance between photosynthesis and respiration of the plants. To evaluate the determinants of photosynthesis and respiration, we analyzed the relationships between the greenhouse environment and these activities.

2. Materials and methods

2.1 Plant materials and greenhouse operation

Seeds of spinach (Spinacia oleracea L.) were sown in an unheated Quonset greenhouse located in Sapporo (43.01° N, 141.42° E), Hokkaido, Japan on September 30, 2019. The inner volume of the greenhouse was approximately 250 m³ (5.4 m in width, 20 m in length, and 3.6 m in height). The ventilation windows on both sides of the greenhouse opened automatically when the air temperature in the greenhouse exceeded 30 °C. Two cultivars, ‘Houou’ (HO; Tohoku Seed Co. Ltd, Utsunomiya, Tochigi, Japan) and ‘High-Sunpia’ (HS; Kaneko Seeds Co., Ltd, Maebashi, Gunma, Japan), were used in the experiment; HO shows a typical rosette growth form and ceases growth at low temperatures, whereas HS shows considerable leaf elongation at low temperatures (Fig. 1). Seeds of HS and HO were sown at a density of 20 seeds m⁻² to cover approximately 50 m² and 8 m², respectively. The seedlings were raised to a commercially sellable weight (greater than 30–35 g per plant) and subsequently maintained in the greenhouse without irrigation. The plants were subjected to sampling and photosynthetic activity measurements, as detailed in the following sections, between November 2019 and March 2020.

2.2 Environmental monitoring

Air and soil temperatures in the greenhouse (Tₘ and Tₛoil, respectively) were measured above and beneath the plants at 10-min intervals using thermometers (TR-5106 and RTR-502; T&D Corporation, Matsumoto, Nagano, Japan). A cylinder equipped with a ventilation fan was installed at a height of 30 cm to measure the air temperature. The soil temperature was measured at depths of 5 and 10 cm. The CO₂ concentration in the middle of the greenhouse ([CO₂]) was measured at 30 s intervals using a silicon-based non-dispersive infrared sensor (GMP343; Vaisala Corporation, Vantaa, Helsinki, Finland) installed at a height of 110 cm and connected to a data logger (FTJR-2CH, M.C.S Corp., Sapporo, Hokkaido, Japan). The time courses of air temperature (Tₘ) and photosynthetic photon flux density (PPFD) outside the greenhouse were measured at a meteorological observation station located approximately 700 m from the greenhouse.

2.3 Gas-exchange measurement

The net photosynthetic rate of intact leaves (Pₑ) was measured using a portable gas-exchange measurement instrument (MIC-100-S1, Masa International Co., Ltd., Simogyo, Kyoto, Japan) with a closed chamber system (Field et al., 2000). The instrument was equipped with a transparent top chamber and incident sunlight was used as the actinic light for the measurement. The air temperature in the chamber was not controlled. We selected an individual plant and measured the Pₑ of the largest fully expanded leaf that was not shaded by neighboring individuals. This procedure was repeated throughout the day. The Pₑ value was calculated from the time course of the decrease in chamber CO₂ concentration by 15 µmol mol⁻¹ from the ambient value ([CO₂]₀). Note that [CO₂]₀ was higher when the Pₑ measurements were taken because the researcher stayed in the greenhouse and emitted CO₂ during the measurements. Although the CO₂ concentration in the chamber is displayed, the instrument does not calculate negative photosynthetic rates (i.e., respiration rates) when the CO₂ concentration rises. The CO₂ rise was monitored to detect the occurrence of negative Pₑ.

Hourly rates of increase in [CO₂] in the greenhouse (E) were calculated from the change in [CO₂] within 10 min using linear regression. This value is a crude measure of the balance between photosynthetic CO₂ uptake and respiratory CO₂ emission of the plants. The effect of soil organisms is considered to be marginal, as [CO₂] did not increase at night in a nearby greenhouse without plants (data not shown). Artificial changes in [CO₂] caused by

![Fig. 1. Spinach plants during the winter sweetening treatment (left: ‘Houou’; right: ‘High-Sunpia’). Both cultivars were sown at the same planting density (20 plants m⁻²).](image-url)
window opening and human respiration were removed from the calculation. The sweetening treatment period included 23 days without these artificial disturbances.

To quantify the respiration rate of the plants, considering ventilation between the inside and outside air, we calculated night-time CO$_2$ emission rate ($R$) from the courses of [CO$_2$] in the greenhouse using the following equation (Yoshida et al., 1997):

$$R = nV(C_t - C_a) = (R - nV(C_t - C_a)) e^{-nt},$$

where $n$ is the exchange ratio of the closed greenhouse (h$^{-1}$), $V$ is the volume of the greenhouse (250 m$^3$), $C_t$ is the [CO$_2$] in the greenhouse at time $t$ (µmol mol$^{-1}$), and $C_a$ is the [CO$_2$] of the outside air (400 µmol mol$^{-1}$). Using a nonlinear least square regression function (nls function) implemented in R statistical software (R Core Team, 2019), $n$ and $R$ values were estimated from the change in [CO$_2$] during 20:00–24:00.

### 2.4 Sampling

Plants were harvested at approximately 1-month intervals. Five to ten individuals were sampled at or near 10:30 and leaf number ($> 2.5$ cm), lamina length, and shoot fresh weight were measured. The SPAD value of the largest fully expanded leaf was measured using a chlorophyll meter (SPAD-502 Plus, Konica Minolta Inc., Chiyoda, Tokyo, Japan). To assess the BRIX index as a measure of sweetening, the lamina and petiole of the largest leaf were frozen together. The BRIX index of the juice extracted from the frozen sample was measured using a digital refractometer (PR-101α; ATAGO Co., Ltd., Minato, Tokyo, Japan).

### 2.5 Statistical analysis

We used R statistical software (R Core Team, 2019) for statistical analyses. The one-sample $t$ test was used to test the null hypotheses that $E$ and $R$ values were zero using t.test function. Statistical differences in the shoot fresh weight, lamina length, leaf number, SPAD value, and BRIX index among the sampling dates were tested by the Tukey-Kramer’s test using a TukeyHSD function. The correlation between the $R$ value and night-time $T_a$ and $T_{soil}$ (mean values of the temperatures during 20:00–24:00) were evaluated by the Pearson’s product-moment correlation and their statistical significances were tested using cor.test.

### 3. Results

#### 3.1 Greenhouse environment

During mid-winter, the daily mean values of $T_a$ and $T_{soil}$ were higher than $T_{out}$ by approximately 4 and 9 °C, respectively (Fig. 2). The value of $T_{soil}$ between December 26, 2019 and February 21, 2020 was predominantly lower than 5 °C, a conventional threshold required to obtain highly sweetened spinach (Hamasaki et al., 2007). In the present study, this period is referred to as the sweetening treatment period.

When the greenhouse was not sufficiently warm to trigger window opening, [CO$_2$] decreased steeply in the morning (Fig. 3), presumably owing to photosynthesis. At times, the value of [CO$_2$] decreased to lower than 200 µmol mol$^{-1}$, but changed little during the middle of the day (10:00–15:00). The increase in [CO$_2$] began at approximately 16:00, rose steeply in the evening and then gradually during the night. We evaluated diurnal
changes in [CO₂] in an empty greenhouse of the same geometry and confirmed that the concentration was higher at midday than at night (data not shown).

3.2 Plant growth and BRIX index

Increases in shoot fresh weight, lamina length, and number of leaves were suppressed during the sweetening treatment (Fig. 4A–C). Shoot fresh weight and leaf number increased slightly in both cultivars later in the treatment period, despite low Tₛ and T_soil. The lamina length changed little during the treatment in both cultivars. While shoot fresh weight and lamina length of HS plants increased after treatment, the resumption of growth in HO plants after sweetening treatment was indistinct. The SPAD value changed little in both cultivars during the sweetening treatment but exhibited a sharp increase after the treatment (Fig. 4D).

The BRIX index increased in January to a value higher than the conventional threshold of 8.0 of sweetened spinach (Fig. 5). While the increase in HO was significant, the increase in HS was less pronounced. Note that a broad variation in the BRIX indices of leaves sampled on the same day was observed, particularly when the BRIX was high. The BRIX index decreased in both cultivars after sweetening treatment, presumably in response to the increases in Tₛ and T_soil (Fig. 2).

3.3 Photosynthetic and respiratory activities

Both cultivars exhibited similar diurnal variations in Pₐ (Fig. 6). Although the instrument was not equipped with a temperature control system and the air temperature in the gas chamber could rise during the measurement, this should not markedly affect Pₐ because the actual measurement time was not long enough to change the activation states of the photosynthetic enzymes. During the sweetening treatment period (Fig. 6A–C), Pₐ increased after sunrise and peaked at around 09:00. Because the Pₐ decreased after the peak despite the increase in the outside PPFD, other rate-limiting factors are likely involved. We observed some wilted leaves in the afternoon with remarkably low Pₐ values, particularly on HS plants. After the second peak at approximately 14:00, Pₐ started to decrease in response to the decrease in the outside PPFD. After the sweetening treatment (Fig. 6D, E), the changes in Pₐ were found to be proportional to the changes in the outside PPFD. The Pₐ peaked under a high PPFD at approximately 12:00, which suggested that PPFD was the major rate-limiting factor of photosynthesis.

A negative peak in E was observed in the morning at around 09:00 (Fig. 7). Thereafter, E increased and was positive from 13:00, whereas the leaf Pₐ was positive in the afternoon. This gap between E and Pₐ measurements may reflect the difference in greenhouse environment during the respective evaluations; the leaf-level measurements of Pₐ were conducted on sunny days, whereas E was evaluated on cloudy days to avoid disturbance in [CO₂] by window opening. A positive peak in E was attained at around 17:00 immediately after sunset and approached zero. The E value before the dawn was significantly greater than zero. The sum of hourly E values for one day was negative on the majority of days without artificial disturbances (data not shown), which suggested a net uptake of CO₂.

The estimated exchange rate originated from the ventilation between the inside of the closed greenhouse and the outside air during the night ranged from 0.03 to 0.59 h⁻¹. The estimated R value ranged from 4 to 12 × 10⁻³ m³ h⁻¹. This range corresponded to 0.7–2.1 µmol m⁻² s⁻¹ on the basis of cultivation area. The R value was greater than zero (P < 0.001), and the 95% confidence interval was 6.6 to 7.7 × 10⁻³ m³ h⁻¹.

![Fig. 3. Diurnal time course of CO₂ concentration inside the greenhouse. The time courses of cloudy days without side window opening are shown in gray and their mean is shown in black (Jan 18–Feb 21; N = 23).](image-url)
3.4 Relationship between greenhouse environment and gas-exchange activities

A positive correlation between $P_n$ and PPFD was detected (Fig. 8), and $[\text{CO}_2]$ clearly modulated the response of $P_n$ to PPFD. The $P_n$ increased steeply in response to an increase in PPFD under high $[\text{CO}_2]$, whereas the slope was small under low $[\text{CO}_2]$. This result suggested that low $[\text{CO}_2]$ limited photosynthetic $\text{CO}_2$ uptake in the barely ventilated greenhouse.

Fig. 4. Shoot fresh weight (A), lamina length (B), number of leaves (C), and SPAD index (D) of spinach ‘Houou’ (HO) and ‘High-Sunpia’ (HS) plants. Values of samples (small symbols) and their means (large symbols) ± standard deviations are shown ($N = 5–10$). Different letters represent significant differences among sampling dates ($P < 0.05$, Tukey-Kramer’s test; large and small letters are for HO and HS, respectively). Shading indicates the period of sweetening treatment.

Fig. 5. BRIX index of spinach ‘Houou’ (HO) and ‘High-Sunpia’ (HS) plants. Refer to Fig. 3 for the details of the annotations and legends.
No statistically significant correlation was observed between \( P_n \) and \( T_s \), and a negative correlation between \( P_n \) and \( T_{	ext{soil}} \) was observed (data not shown). This result might indicate that low air and soil temperatures did not limit photosynthetic activity. Note that the observed negative correlation does not imply a causal relationship where low \( T_{	ext{soil}} \) increased \( P_n \), given that \( T_{	ext{soil}} \) tracked...

**Fig. 6.** Diurnal changes in net photosynthetic rate of spinach ‘Houou’ (HO) and ‘High-Sunpia’ (HS) leaves. Black lines indicate the diurnal trend of photosynthetic rate calculated using a locally estimated scatterplot smoothing (LOESS) method. Occurrences of negative photosynthetic rates, detected as rises in the CO\(_2\) concentration in the gas chamber, are indicated by triangles. Orange lines indicate photosynthetic photon flux density (PPFD) outside the greenhouse.

**Fig. 7.** Rates of increase in CO\(_2\) concentration inside the greenhouse measured as the balance between photosynthetic and respiratory activity of spinach plants. Hourly median, quartiles, and ranges are shown with outliers shown as dots. The time courses and values on cloudy days without side window opening are shown (Jan 18–Feb 21; \( N = 23 \)). Asterisks indicate that the rates are statistically different from zero (*: \( P < 0.05 \), **: \( P < 0.01 \), ***: \( P < 0.001 \)).
changes in $T_\text{in}$ with a time delay; $T_\text{soil}$ was low in the morning whereas $P_\text{n}$ usually peaked at that time.

We analyzed greenhouse environmental effects on the respiratory activity of the plants based on the $R$ values calculated from the time courses in $[\text{CO}_2]$ during 20:00–24:00. Night-time $R$ values were used because we were not able to separate photosynthetic CO$_2$ uptake and respiratory CO$_2$ emission from the day-time data. It was found that the value of $R$ was positively correlated with mean $T_\text{in}$ ($r = 0.56$, $P < 0.001$) and $T_\text{soil}$ ($r = 0.73$, $P < 0.001$) during 20:00–24:00 (Fig. 9).

Fig. 8. Net photosynthetic rate of spinach leaves plotted against photosynthetic photon flux density (PPFD) outside the greenhouse (left: ‘Houou’; right: ‘High-Sunpia’). Regression lines are calculated for values grouped by the CO$_2$ concentration inside the greenhouse. The data collected on Jan 23, Feb 23, Mar 12, and Mar 25 are shown.

Fig. 9. Relationships between the whole-greenhouse CO$_2$ emission rate ($R$) and air temperature ($A$: $T_\text{in}$) and soil temperature at a depth of 10 cm (B: $T_\text{soil}$). The $R$ values were estimated from changes in the greenhouse CO$_2$ concentration during 20:00–24:00. $T_\text{in}$ and $T_\text{soil}$ are mean values calculated from the values during 20:00–24:00. Data from Jan 18 to Mar 11 are shown ($N = 51$).
4. Discussion

4.1 Active photosynthesis and CO$_2$ depletion in the winter greenhouse

We observed active net photosynthetic CO$_2$ uptake by spinach leaves ($P_n$) during the sweetening treatment despite growth retardation (Figs. 4A–C, 6A–C). A negative peak in $E$ in the morning also indicated active photosynthesis (Fig. 7). The third quartile of the photosynthetic rates was 13.0, which was comparable to the value for major agricultural species. For instance, a net photosynthetic rate of field-grown wheat and rice cultivars ranged from 11.8 to 27.0 µmol m$^{-2}$ s$^{-1}$ (Reynolds et al., 2000) and 7.7 to 26.6 µmol m$^{-2}$ s$^{-1}$ (Sasaki and Ishii, 1992), respectively. The rate of greenhouse-grown tomato cultivars ranged from 11.2 to 23.0 µmol m$^{-2}$ s$^{-1}$ (Zeist et al., 2018). The $P_n$ value in our study was sometimes greater than 20 µmol m$^{-2}$ s$^{-1}$, which is comparable to the light-saturated $P_n$ of spinach leaves cultivated and measured under 5 °C (Boese and Huner, 1990) and under suitably controlled environments with artificial light (e.g., Matsuda et al., 2007). The active photosynthesis at the low daily mean $T_{an}$ (< 0 °C) indicates the remarkable capacity of leaf photosynthesis for temperature acclimation. Similarly, in gas-exchange experiments with spinach plants cultivated under low (15/10 °C) and high (30/25 °C) day/night air temperatures, Yamori et al. (2005) reported a shift in the optimum temperature for $P_n$.

During the sweetening treatment, $P_n$ peaked in the morning (up to 20 µmol m$^{-2}$ s$^{-1}$) and subsequently decreased (Fig. 6A–C). This decrease was similar to the phenomenon termed midday depression (e.g., Tenhunen et al., 1984). Midday depression may be caused by stomatal closure, a key modulator of the balance between photosynthetic carbon uptake and water loss by transpiration (Farquhar and Sharkey, 1982). Given that low $T_{water}$ limits water uptake in the root zone and imposes water stress on the plants (Kramer, 1983), stomatal closure would be induced during the sweetening treatment, as reported by Okada et al. (2006). The decline in stomatal conductance can cause a decrease in photosynthetic light-use efficiency (i.e., the slope of the PPFD-$P_n$ curve) under low [CO$_2$] conditions (Fig. 8). Alleviation of midday depression after the sweetening treatment (Fig. 6D) may reflect the promotion of root water uptake resulting from the increase in $T_{water}$ (Fig. 2). Another possible cause of midday depression is the sink limitation of photosynthesis. The demand for photosynthate would be suppressed because of the retardation of plant growth (Fig. 4), leading to excessive accumulation of photosynthates in the leaves. As spinach is a starch-accumulating species, the accumulated starch might cause a reduction in leaf photosynthesis by limiting CO$_2$ diffusion in the chloroplasts (e.g., Nakano et al., 2000). The alleviated depression after the sweetening treatment might reflect increased demand for photosynthates in sink organs because of the resumption of plant growth (Fig. 4).

The evaluation of whole-greenhouse gas-exchange implied that night respiration was evident (Figs. 7 and 9). The $R$ value was correlated with $T_{water}$ and $T_{air}$ and was positive under sub-zero air temperatures (Fig. 9). This result suggested that greenhouse night environments may affect the carbohydrate consumption of the plants.

4.2 Mechanism of sweetening: dehydration, cold acclimation and photosynthesis

A cause of higher sugar content might be the limitation of water uptake in the root zone followed by leaf dehydration leading to an increase in sugar content per fresh weight (see also the Introduction). The contribution of this effect is supported by the findings that water uptake is restricted by low temperature in the root zone (Kramer, 1983) and the BRIX index is higher in a dehydrated leaf with a smaller water content (Kato et al., 1996). Decreasing the root-zone temperature without changing the aerial temperature caused an increase in the BRIX index of leafy vegetables (Ito et al., 2013; Okada et al., 2006). While these previous studies suggested that the dehydration effect contributes to the sweetening, the involvement of additional mechanisms in the sweetening process has been proposed. The sucrose content per dry weight increased in response to the winter-sweetening treatment in spinach (Moriyama and Aoki, 2004; Okada et al., 2005). Kato et al. (1996) showed that the increase in sugar content on the basis of fresh weight was much larger than that expected from the decrease of water content. A likely cause of the higher BRIX index was cold acclimation triggered by water stress, which is associated with sugar accumulation (Alberdi and Corcuera, 1991, Guy et al., 1992).

As well as the cold acclimation, the remaining proportion of the BRIX increase might be attributable to leaf photosynthesis activity during the sweetening treatment (Fig. 6). This contribution of photosynthesis to sweetening was supported by experimental data showing that decreases in PPFD using shade cloth and mutual shading suppressed the increase in BRIX index (unpublished data, T. Hamasaki). In an experiment using tomato, Fujimura et al. (2012) also reported that an increase in sugar content in fruits through root chilling treatment was attributable to a change in sink-source balance, or repression of growth rate and sustained photosynthesis. Slight but sustained growth during the sweetening treatment in HS (Fig. 4) might involve photosynthetic consumption by construction respiration, which in turn might mitigate the rise in the BRIX index in HS (Fig. 5).

It has also been hypothesized that carbohydrate consumption by respiration should not be significant at low temperatures during the sweetening treatment. However, the present study revealed a substantial night respiratory rate (Fig. 7). The BRIX index is reported to decline rapidly in response to a rise in temperature after sweetening treatment (Okada et al., 2005). This rapid decrease in the BRIX index might be partly caused by the increase in respiratory demand in response to the rise in air and soil temperatures (Fig. 9), which suggests that respiration may have an important impact on BRIX maintenance.

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Author contribution

K.M. designed the study, performed gas-exchange and meteorological measurements, analyzed the data, and wrote the manuscript. T. Hamasaki designed the study, and performed sampling and meteorological measurements. M.N. performed meteorological measurements and made critical revisions of the manuscript. S. I. and T. Hirota made critical revisions of the manuscript.

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