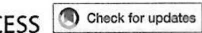
ISSN 1349-1008
Volume 26, Issues 1–4, 2023

Plant Production Science

PLANT PRODUCTION SCIENCE
2023, VOL. 26, NO. 2, 174–186
https://doi.org/10.1080/1343943X.2023.2210766



Rapid evaluation of leaf photosynthesis using a closed-chamber system in a C_4 plant, sugarcane

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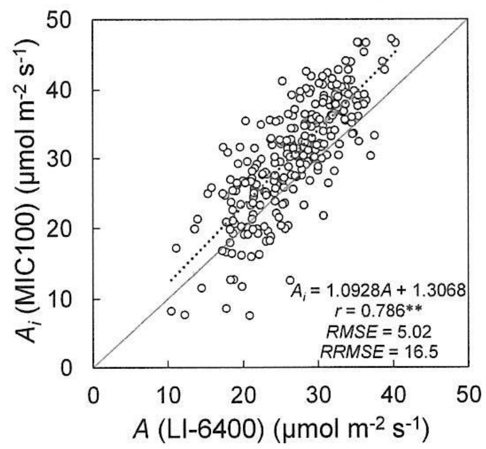
ABSTRACT

To accelerate research on improving sugarcane biomass production, a rapid phenotyping method for individual leaf photosynthetic rates is required. Recently, a closed-type measurement system, which is faster, lighter, and less expensive than conventional open-type systems, has been developed and utilized for C_3 crops. For future utilization of the system in phenotyping photosynthetic rates in sugarcane, which exhibits higher photosynthetic rate than C_3 crops, diurnal changes and genotypic differences were measured simultaneously using an open-type and a closed-type system to verify the accuracy of the measurements in assessing environmental responses and genetic variation. As the relative root-mean-square error, a regression accuracy between the measurements with two systems, was <20% when evaluating diurnal changes and genotypic differences, closed system accurately evaluated photosynthetic rates in multiple samples. Overall, the measured values with the closed system tended to be higher than those with the open system, especially in high values above 30 μmol m^{-2} s^{-1}. The reason for this was presumably not leaf morphology, such as stomatal distribution, but a fundamental difference in the measurement systems (steady-state values for the open system and instantaneous values for the closed system). The open system required 5–7 min to measure a single record, whereas the closed system could measure at <40 s per record. Although it would be desirable to develop a regression equation using measurements involving the open system for each cultivar to examine physiological response in detail, we conclude that the closed system has greater potential for use in phenotyping sugarcane photosynthesis.

ARTICLE HISTORY
Received 10 November 2022
Revised 10 February 2023
Accepted 24 April 2023

KEYWORDS
Closed-type gas exchange system; diurnal change; *Erianthus arundinaceus*; genotypic variation; open-type gas exchange system; phenotyping



Introduction

Sugarcane (*Saccharum* spp.) is a C_4 plant that exhibits a photosynthetic response different from that of C_3 plants such as rice, and is a tropical crop that is highly tolerant to high temperatures, intense radiation, and water stress (Sage et al., 2014). However, similar to other crops, adaptation to climate change, such as unstable rainfall, has become an important challenge

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/1343943X.2023.2210766

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in sugarcane production (Inman-Bamber et al., 2011; Park, 2008; Zhao & Li, 2015). Sugarcane is produced on approximately 26 million hectares in over 90 tropical and subtropical regions and countries (FAOSTAT, 2022), and is an important cash crop for global sugar and bioethanol production. In addition, the by-products of sugar and ethanol production, such as bagasse after squeezing sucrose, are utilized comprehensively (Autrey & Tonta, 2005). Therefore, improving not only yields of the target product but also total biomass production is important from the perspective of energy balance (Hattori & Morita, 2010; Inman-Bamber et al., 2011). In particular, sugarcane grown as a biomass energy feedstock, also known as energy cane, has a higher fiber yield and greater energy production than sugar-producing varieties (Leal, 2007; Matsuoka et al., 2014). Sugarcane is known to have a higher biomass production capacity than other crops (Hattori & Morita, 2010; Murata, 1981), and is estimated to fix carbon dioxide (CO_2) equivalent to >60% of the millable stalk yield (Kawamitsu et al., 1999; Ueno et al., 2008).

Photosynthesis in individual leaves is the most basic process of plant growth and biomass production. In sugarcane, photosynthetic rate is a growth indicator used to assess environmental responses and stress tolerance (Du et al., 1996; etc.) and is an important element for determining resource use efficiency, such as photosynthetic water use efficiency and photosynthetic nitrogen use efficiency (Dinh et al., 2017; Jackson et al., 2016). These measurements can then be scaled-up by inputting canopy photosynthesis and crop growth models to understand crop production and carbon cycling (Hoffman et al., 2018; Inman-Bamber et al., 2016). However, de Souza et al. (2014) pointed out that delays in studies on plant physiology, such as photosynthesis and sink–source relationships, have hindered improvements in sugarcane biomass productivity.

Historically, the journey of sugarcane photosynthesis research is very important (Hatch, 1999). In the 1960s, a closed-type method, ^{14}C labelling, was primarily used to assess photosynthetic rates and related metabolites of sugarcane (Irvine, 1967). These methods and biochemical approaches, including measurements of enzyme activity, have led to the discovery of C_4 photosynthesis (Hatch & Slack, 1966; Kortschak et al., 1965). Photosynthesis measured in closed-type systems, such as ^{14}C labelling, oxygen electrodes, and closed assimilation chambers, is characterized by time-integrated rather than steady-state values. Its disadvantage is that the environment in the system cannot be kept constant because CO_2 concentration decreases, water vapor concentration increases, and air temperature changes within the system (Hunt, 2003; Long & Hällgren, 1993). Therefore, an open-type system was developed for continuous measurements in a constant environment with environmental controls (Sestak et al., 1971). Furthermore, the simultaneous measurement of photosynthesis and transpiration has led to the development of gas exchange models based on various theoretical assumptions (Farquhar et al., 1980), making the open-type system a more robust measurement method (Long et al., 1996). In the initial phase, open-type assimilation chambers were handmade in each laboratory, and the flow rate and temperature/humidity control were important factors in chamber creation (Agata et al., 1986; Kawamitsu et al., 1993; Long et al., 1996). The use of the open-type system for photosynthesis measurements in sugarcane has been practiced since the 1960s (Bull, 1969; Heskech & Moss, 1963), while the system has dominated photosynthesis research since the 1970s (Du et al., 1996; Irvine, 1975; Nose et al., 1994). Although such custom-built assimilation chambers can accurately observe environmental responses to photosynthesis by a large target leaf area (Long & Bernacchi, 2003), they have certain disadvantages, such as a prolonged time to stabilize measurements and difficulty in measuring the large size of sugarcane plants, especially during the late growth stage. During the 1990s and 2000s, compact and portable measurement systems (e.g. closed-type LI-6200, open-type LI-6400, etc.) were introduced, which facilitated in-field and multi-point measurements and promoted research regarding physiological photosynthetic phenomena and stress tolerance in many crops (Long et al., 1996), including sugarcane (Du et al., 1999; McCormick et al., 2006). Further, conventional portable open-type systems (LI-6400, LI-6800, etc.) remain subject to the problems of measurement speed, cost, and labor saving; therefore, they are not suitable for rapid multi-point measurements, that is, high-throughput phenotyping, in the field.

Recently, there has been a growing interest in high-throughput phenotyping, as molecular biological studies such as genotyping have progressed and there has been an increased demand for rapid and accurate measurement of target traits in a large number of genotypes, such as genetic populations (Cobb et al., 2013). Against this background, a rapid, inexpensive, and labor-saving closed-type photosynthesis measuring system, MIC-100 (Masa International, Inc.), was developed (Tanaka et al., 2021) which is now being used for efficient photosynthetic phenotyping of C_3-type crops, such as rice and soybean, in temperate zones (Honda et al., 2021; Shamim et al., 2022;

Kondo & Morizono, 2022; Yamatani et al., 2021; Murakami et al., 2021; Nishida & Kondo, 2021). The importance of breeding selection based on physiological understanding has also been highlighted in sugarcane (Jackson et al., 1996). If photosynthetic phenotyping of sugarcane can be easily performed using the newly developed system, it would not only be useful for efficient trait improvement through breeding (Li et al., 2017), but also for cultivation management such as fertilization and irrigation based on growth diagnosis (Watanabe et al., 2022). However, there has been no application of this closed-type rapid measuring system to sugarcane, which exhibits higher photosynthetic rate than C_3-type crops and grows in the tropical and subtropical humid climates. Higher photosynthetic rate could potentially influence the rapidity and precision of measurements using the closed-type system because the in-chamber CO_2 concentration reduces more rapidly. Therefore, it is necessary to verify whether such a system is useful for assessing environmental responses and genetic variation in terms of photosynthesis in this species.

In this study, the accuracy of photosynthesis measurement in sugarcane was verified by simultaneously measuring the same leaf using an open-type system (LI-6400, LI-COR) and a closed-type system (MIC-100). First, diurnal changes in photosynthesis were measured to verify measurement accuracy when the plant responded to environmental changes (Experiment 1). Second, variations among Japanese cultivars were measured to verify the measurement accuracy of genetic variation (Experiment 2). Regression analysis of the obtained results was performed to evaluate the applicability of the closed-type rapid measuring system for sugarcane phenotyping.

Materials and methods

Exp. 1. Evaluation of photosynthetic responses to environmental change under pot conditions

A pot trial was performed in a temperature- and humidity-controlled glasshouses (30/27°C, relative humidity 70%) at the Tropical Agriculture Research Front, Japan International Research Center for Agricultural Sciences (24°22′43″N, 124°11′4″″ E). Single-bud setts of sugarcane cultivar 'NiF8' and *Erianthus arundinaceus* accession 'JW630' were raised for 3 months and transplanted into 1/5000a Wagener pots ($n = 3$) filled with local red soil (Ultisol, pH 5.0) on 11 December 2020. *E. arundinaceus* is a wild relative used for crossbreeding with sugarcane (D'Hont et al., 1995; Pachakkil et al., 2019) and also performs C_4-type photosynthesis (Sage et al., 2014). A slow-release fertilizer (N: P: K = 24:7:5) was applied at 2.0 g/pot and irrigation was carried out twice a day. Photosynthesis measurements were performed simultaneously on the same leaf using an open-type system LI-6400 (LI-COR BioSciences, Lincoln, Nebraska, USA) and a closed-type system MIC-100 (Masa International Corporation, Kyoto, Japan). MIC-100 is a closed system that is lightweight because it specializes in photosynthesis measurements and has improved responsiveness owing to the shortened distance between the sensor and the measurement unit (Tanaka et al., 2021). Measurements were conducted every 2 h from 08:00 to 16:00 on four days from March 2 to 5, 2021. The

Figure 1. The outline of photosynthesis measurements using open system LI-6400 at the basal side of leaf blade and closed system MIC-100 at the tip side under field condition in Exp. 2.

youngest fully expanded leaf exposed to adequate sunlight was selected, and its photosynthetic rate was simultaneously measured at the tip and basal sides of the middle part of the leaf blade using the MIC-100 and LI-6400 instruments, respectively, where the distance between chambers was <10 cm (Figure 1). In a preliminary investigation, the leaves of several varieties were measured in two different chambers by switching the clamping portion, and it was confirmed that there was little effect of the measurement portion (Supplemental Figure. S1). The in-chamber conditions for the MIC-100 were as follows: the light intensity of the LED light source was 1,200 μmol m^{-2} s^{-1}, the stabilization time to start the measurement was set to 20 s, and the CO_2 concentration to start the measurement was 500 ppm considering higher CO_2 values under greenhouse conditions. The photosynthetic rate was calculated from the time required for the CO_2 concentration to decrease by 20 ppm, i.e. 480 ppm. The in-chamber conditions of LI-6400 were set to a flow rate of 400 μmol s^{-1} and a reference CO_2 concentration of 500 ppm. The leaf temperature was 33.1 ± 0.6°C by controlling block temperatures attached to the chamber. Vaper pressure deficit (VPD) was controlled at 2.1 ± 0.2 kPa. Light intensity was set to ambient light intensity in the growing environment, followed by 1,200 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) using an LED light source (6400-02B, LI-COR) equivalent to the MIC100. Under the light intensity 1,200 μmol m^{-2} s^{-1}, two species exhibited almost saturated photosynthetic rate (Suplementary Figure S2).

Stomatal replicas of the adaxial and abaxial surfaces of photosynthesis-measured leaves were collected according to a modified Suzuki's Universal Micro-Printing (SUMP) method with nail polish (Tominaga et al., 2018) and observed under an optical microscope (Eclipse E800, Nikon, Tokyo, Japan) and image analysis software (NIS-elements, Nikon) to examine stomatal density.

Exp. 2. Evaluation of genotypic differences in photosynthesis under field conditions

A total of 43 sugarcane cultivars was planted on 14 September 2020, in a field at the same site as in Exp. 1 with two plots where a plot was designed at a 3-m row length × 1.5-m inter-row distance (9 plants per m^2 in each plot). A slow-release fertilizer was applied at N: P: K = 24:7:10 kg per 10a, and irrigation was conducted the day before measurements if there was no rainfall during the two days before measurements. Photosynthesis measurements were performed from 12:00 to 15:30 on six days on 19 March, 26 March, 30 March, 1 April, 6 April, and 19 April 2021 (n = 3 per plot) to account for the effects of morning dew and evening decline. As in Exp. 1, the youngest fully expanded leaves exposed to adequate sunlight were selected, and different adjacent portions within the same leaf were measured simultaneously using the MIC-100 and LI-6400. The in-chamber conditions of the MIC-100 were as follows: the light intensity of the LED light source was 1,200 μmol m^{-2} s^{-1} of PPFD, the stabilization time to start the measurement was set to 20 s, and the CO_2 concentration to start the measurement was 400 ppm. The photosynthetic rate was calculated from the time required for the CO_2 concentration to decrease by 20 ppm. The in-chamber conditions for the LI-6400 were set as follows: flow rate of 400 μmol s^{-1}, reference CO_2 concentration of 450 ppm, and light intensity of 1,200 μmol m^{-2} s^{-1} of PPFD. Reference CO_2 concentration was set considering high photosynthetic rate, i.e. high ΔCO_2 in the chamber, under field condition, where ambient CO_2 concentration in the chamber was around 400 ppm. The leaf temperature was 31.1 ±1.1°C by controlling block temperatures attached to the chamber. VPD was controlled at 1.4 ± 0.3 kPa.

The stomatal density of the measured leaves was observed using the same methods as in Exp.1. After stomatal sampling, leaf blades were collected from the basal part, SPAD and leaf area were measured using a leaf area meter (CI-203 and CI-203CA, CID Bio-Science Inc., WA, US) and SPAD meter (SPAD-502plus; Konica Minolta Inc., Tokyo, Japan), respectively, and dry matter weights were determined.

Climatic conditions

Climate data for the two experimental periods were acquired from the database of the Japan Meteorological Agency (JMA, 2022). The dataset for Ishigaki station was extracted from this database because it was the closest to the experimental field. Climate conditions were consistent with typical weather conditions without severe typhoons and drought events during the experimental periods (Figure 2).

Statistical analysis

Data analysis was performed using statistical analysis software (Bell Curve for Excel, Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences among the mean values of the examined parameters for each genotype in Exp. 1 were determined using Student's *t*-test, with statistical significance assumed

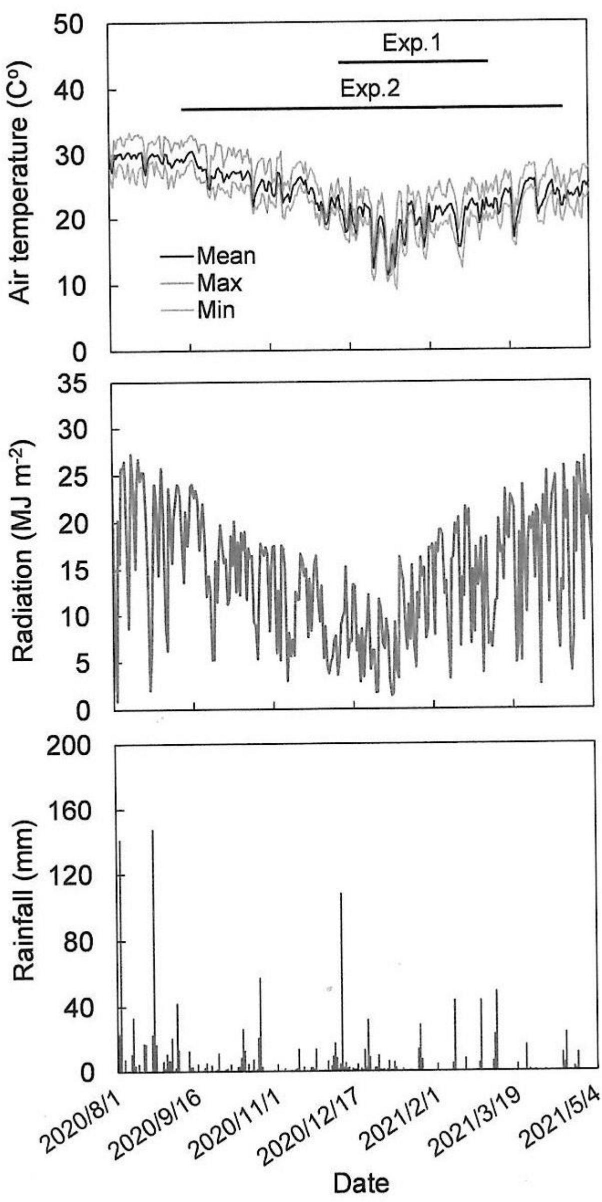

Figure 2. Climatic conditions during the experimental period in Ishigaki. Note: All daily data were acquired at Ishigaki station of the Japan Meteorological Agency (JMA, 2022).

at $P < 0.05$ and 0.01 (n = 3). Correlation analysis was performed between measurement values using the MIC-100 and LI-6400 instruments in Exp.1 and 2, followed by linear regression analysis with the measurement values of LI-6400 as explanatory variables. In addition, the root-mean-square error (*RMSE*) and relative *RMSE* (*RRMSE*) for each relationship were calculated as linear regression accuracy according to the following equations:

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=0}^{n-1}\left(A_i - \widehat{A_i}\right)^2}$$

$$RRMSE\ (\%) = (RMSE\ /\ \text{mean}\ A_i) \times 100$$

where A_i and $\widehat{A_i}$ are the values observed using the MIC-100 and the predicted value, respectively. For Exp. 2, correlation analysis was performed between photosynthetic parameters and leaf characteristics.

Results

Exp. 1. Photosynthetic responses to diurnal changes measured using closed system

Diurnal changes in the photosynthetic rates of sugarcane NiF8 and *Erianthus* JW630 grown in a temperature- and humidity-controlled glasshouse are shown in Figure 3. The trend of diurnal changes in photosynthetic rates under ambient light conditions measured using the open-system LI-6400 (A_{amb}) was consistent with the changes in light intensity under growing conditions (ambient PPFD) in both genotypes. Diurnal changes in photosynthetic rates under strong light conditions (1,200 μmol m^{-2} s^{-1}) measured by LI-6400 (A_{1200}) exhibited a trend toward higher photosynthesis during the daytime when ambient light was greater for both genotypes, whereas genotypic differences were observed in the morning between 08:00 and 10:00: JW630 exhibited greater A_{1200} in the morning (08:00) when ambient light was weak and the plant was not yet active and on cloudy days (2 and 5 March, 2021) (Figure 3). Diurnal changes in photosynthesis measured by MIC-100 (A_i) showed a similar trend to A_{1200}, with the exception of 5 March 2021, the genotypic differences were greater with A_i: significant differences between genotypes were observed even after 10:00. Significant positive correlations ($P < 0.01$) were found between A_i and A_{1200} in sugarcane NiF8 ($r = 0.951$) and *Erianthus* JW630 ($r = 0.724$) (Figure 4). Plotting measurements of the two genotypes together in the same scatterplot yielded significant correlations ($r = 0.904$, $P < 0.01$). RMSE, an accuracy indicator of the linear regression equation with A_i as the objective variable and A_{1200} as the explanatory variable, was 2.0, 2.9, and 2.6 for sugarcane NiF8, *Erianthus* JW630, and two genotypes, respectively. The RRMSE values for sugarcane NiF8, *Erianthus* JW630, and two genotypes were 10.8%, 12.4%, and 12.1%, respectively. The photosynthetic rate of *Erianthus* JW630 was generally higher than that of sugarcane NiF8, with no leaves having a photosynthetic rate<15 μmol m^{-2} s^{-1}. In both genotypes, when A_{1200} exceeded 25 μmol m^{-2} s^{-1}, A_i tended to be higher. In particular, the slope of the obtained regression equation was greater for

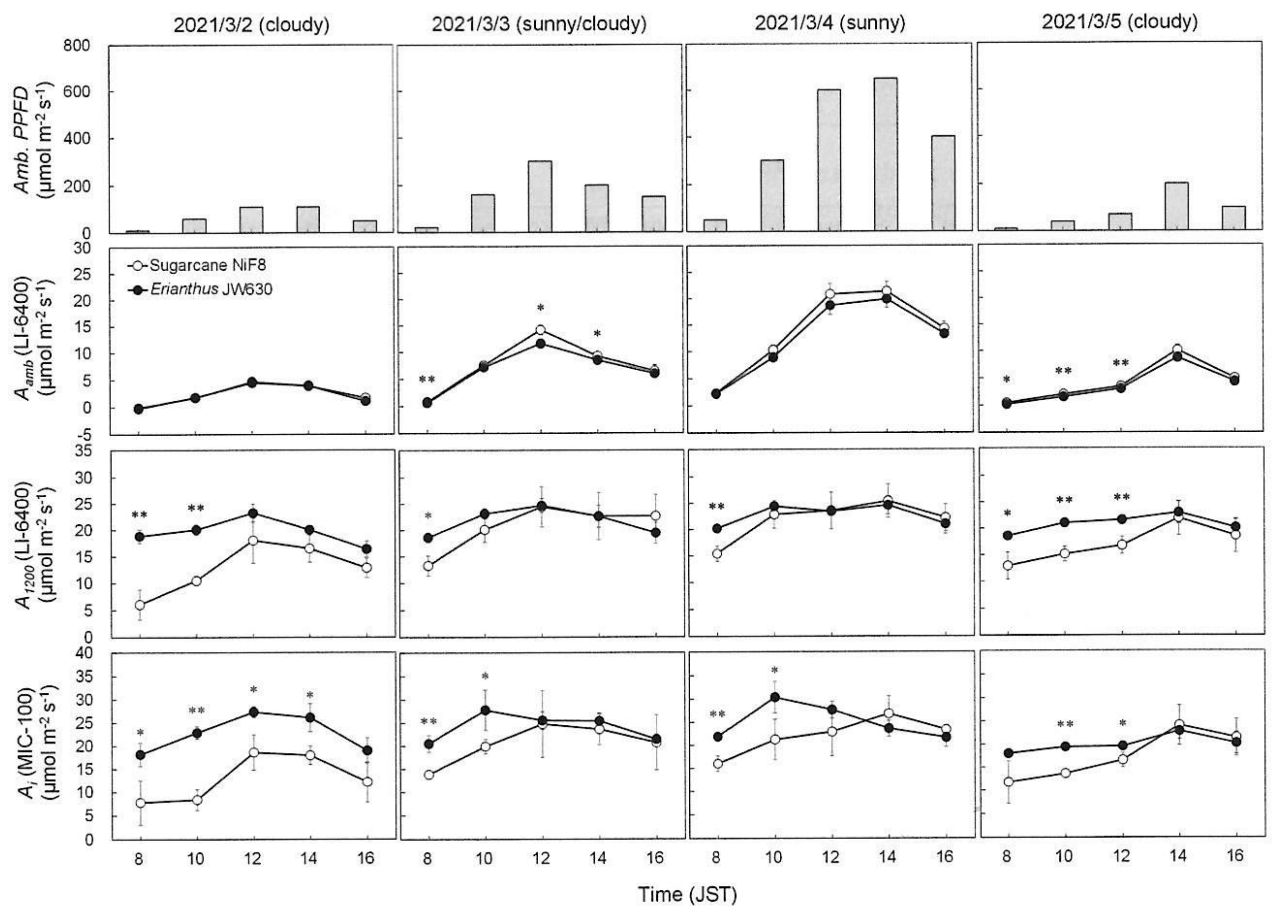

Figure 3. Diurnal changes in photosynthetic rates measured using open system LI-6400 under ambient (A_{amb}) and 1200 μmol $m^{-2}s^{-1}$ of PPFD (A_{1200}) light conditions and using closed system MIC-100 (A_i). Note: Open squares indicate ambient PPFD values measured and set using the LI-6400. The open and closed circles represent the average values of sugarcane NiF8 and *Erianthus* JW630, respectively (n = 3). Bars indicate the standard deviations. * and ** represent significant differences among genotypes at $P < 0.05$ and 0.01, respectively (*t* – test, n = 3).

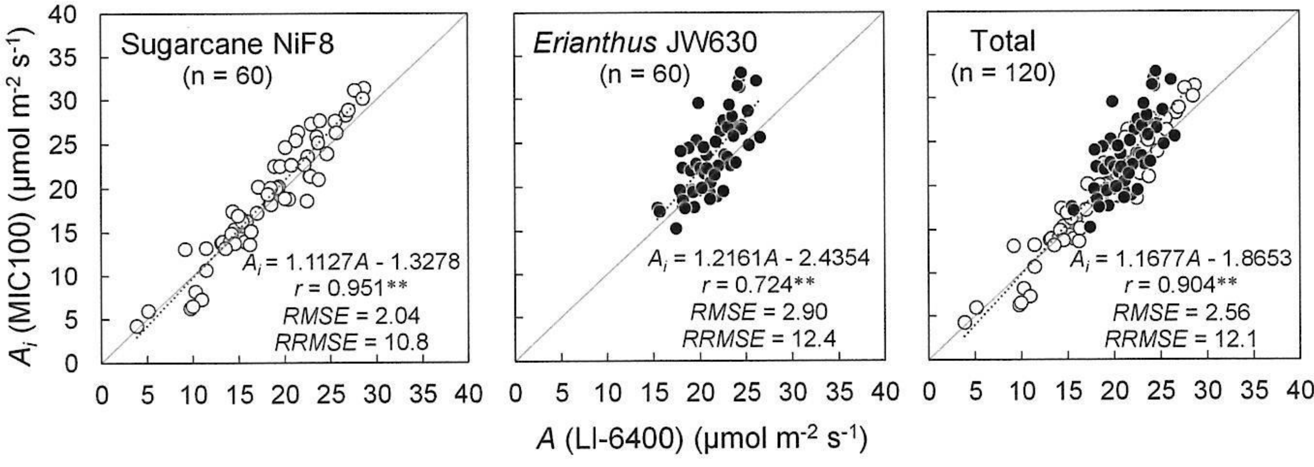

Figure 4. Correlations among photosynthetic rates measured using closed system MIC-100 (instantaneous; A_i) and open system LI-6400 (steady-state; A) in Exp.1. Note: Open and closed circles represent the average values for sugarcane NiF8 (n = 60) and *Erianthus* JW630 (n = 60), respectively. The red line indicates a linear equation of y = x. The blue dashed lines represent linear regression equations using A as explanatory variables. ** represent significant correlation at $P < 0.01$.

Table 1. Comparison of stomatal density between genotypes grown under glasshouse condition in Exp. 1.

Genotype	Stomatal density (no. mm^{-2})			Adaxial /Abaxial
	Adaxial	Abaxial	Total	
Sugarcane NiF8	101.2	191.7	292.9	0.53
Erianthus JW630	93.9	134.5	228.3	0.70
t - test	NS	**	**	**

Note: The *t* – test was performed to detect genotypic difference (n = 3). ** and NS represent significant difference among genotypes at $P < 0.01$ and no significant difference, respectively.

Table 2. Photosynthesis values measured using MIC-100 (instantaneous; A_i) and LI-6400 (steady-state; A) and parameters of their linear regression in each cultivar grown under field condition in Exp.2.

Genotype	Photosynthesis (μmol m^{-2} s^{-1}) A_i (MIC-100)	A (LI-6400)	A_i/A	A_i vs A regression parameter Slope	*y*-intercept	R^2	*P* value
Yomitanzan	30.6	28.4	1.078	1.098	−0.541	0.803	0.016
POJ2725	19.1	20.7	0.903	1.273	−7.306	0.921	0.002
NCo310	35.1	30.4	1.161	0.942	6.438	0.869	0.007
F161	33.8	29.3	1.117	2.105	−27.996	0.843	0.010
Ni1	17.3	24.1	0.707	1.031	−7.506	0.506	0.113
NiN2	23.1	22.5	0.963	1.339	−7.021	0.944	0.001
NiF3	33.8	26.4	1.289	1.126	4.079	0.826	0.012
NiF4	32.8	26.3	1.274	0.623	16.335	0.566	0.084
NiF5	35.0	29.2	1.243	0.397	23.384	0.165	0.425
Ni6	27.0	26.2	1.027	1.179	−3.853	0.822	0.013
NiN7	34.3	26.1	1.316	1.300	0.393	0.947	0.001
NiF8	31.1	24.1	1.328	1.030	6.321	0.853	0.009
Ni9	32.7	27.9	1.193	0.781	10.896	0.495	0.119
NiTn10	27.6	25.5	1.059	1.733	−16.542	0.787	0.018
Ni11	28.1	26.1	1.045	1.632	−14.561	0.944	0.001
Ni12	29.2	25.7	1.131	1.252	−3.021	0.939	0.001
Ni13	26.9	25.4	1.050	1.727	−16.869	0.543	0.095
Ni14	27.4	27.1	1.027	0.775	6.455	0.445	0.148
Ni15	29.7	25.3	1.186	0.817	9.097	0.702	0.037
Ni16	29.5	24.7	1.203	0.786	9.997	0.817	0.013
Ni17	37.8	29.8	1.273	1.169	2.992	0.892	0.005
NiTn18	32.5	30.7	1.060	1.048	0.363	0.943	0.001
NiTn19	27.3	27.7	0.988	0.821	4.546	0.548	0.092
NiTn20	34.1	29.1	1.167	2.051	−25.567	0.455	0.142
Ni21	25.7	23.9	1.094	1.001	1.803	0.741	0.028
Ni22	28.3	23.7	1.227	0.826	8.728	0.752	0.025
Ni23	28.6	26.0	1.101	1.058	1.038	0.774	0.021
NiN24	27.4	26.0	1.065	0.349	18.324	0.072	0.608
NiH25	30.2	26.8	1.128	1.124	0.110	0.868	0.007
Ni26	34.6	30.8	1.108	1.896	−23.902	0.753	0.025
Ni27	36.3	26.4	1.397	0.743	16.645	0.607	0.068
Ni28	28.6	25.8	1.107	1.185	−1.965	0.362	0.207
Ni29	34.3	27.2	1.256	2.245	−26.875	0.369	0.201
NiN30	32.6	27.5	1.196	−0.917	57.835	0.241	0.323
Ni31	29.6	24.1	1.229	1.259	−0.732	0.560	0.087
NiTn32	31.9	23.2	1.353	3.101	−40.158	0.869	0.007
RK97–14	30.0	26.2	1.166	0.720	11.158	0.615	0.065
Haruno-ogi	30.3	26.0	1.196	0.713	11.829	0.773	0.021
Kurokaido	31.3	25.9	1.234	0.674	13.860	0.547	0.093
KRFo93–1	32.3	27.8	1.214	0.857	8.430	0.925	0.002
KY01–2044	35.4	28.4	1.223	1.637	−11.093	0.994	0.000
Shimanoushie	32.1	28.9	1.106	1.245	−3.883	0.877	0.006
Yaenoushie	34.1	33.3	1.025	1.121	−3.184	0.687	0.041

Note: Photosynthesis values and the A_i/A ratio are mean values (n = 6). Linear regression analysis was perfomed with A_i and A as the objective and explanatory variables, respectively.

Erianthus JW630, indicating a tendency to overestimate the photosynthetic rate when using MIC-100. No significant genotypic differences in stomatal density were found on the adaxial surface of the measured leaves, whereas the stomatal density of *Erianthus* JW630 was significantly lower on the abaxial surface (Table 1). Therefore, the total stomatal density of the abaxial and adaxial surfaces was

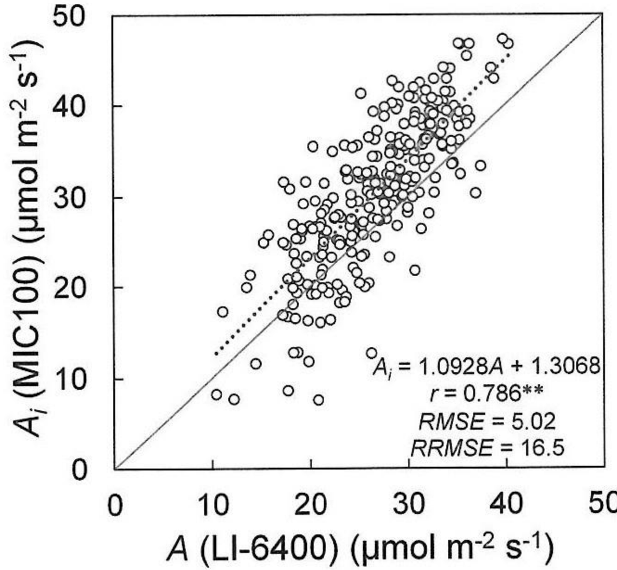

Figure 5. Correlations among photosynthetic rates measured using closed system MIC-100 (instantaneous; A_i) and open system LI-6400 (steady-state; A) in Exp.2. Note: Open circles represent all photosynthesis records (n = 258). The red line indicates the linear equation of y = x, and the blue dashed line represents the linear regression equation using A as an explanatory variable. ** represent significant correlation at $P < 0.01$.

significantly lower and the stomatal distribution ratio of the adaxial to abaxial surfaces was significantly higher in *Erianthus* JW630.

Exp. 2. Photosynthetic variation among Japanese cultivars measured using closed system

The average photosynthetic rates measured by MIC-100 (A_i) and LI-6400 (A) in 43 cultivars grown in the field, the slopes, y-intercepts, R^2 values, and P values of the linear regression equation with A_i as objective variables and A as explanatory variables are shown in Table 2. Similar to the results of Exp. 1, a significant positive correlation ($r = 0.786$, $P < 0.01$) was found between A_i and A for all measurements, with *RMSE* and *RRMSE* of 5.0 and 16.5% respectively for the linear regression equation (n = 258; Figure 5). The cultivar-average A_i ranged from 17.3 to 37.8 μmol m^{-2} s^{-1}, whereas the A measurements ranged from 20.7 to 33.3 μmol m^{-2} s^{-1}. The cultivar-average ratio of A_i to A was>1.0, except four cultivars (POJ2725, Ni1, NiN2, and NiTn19); A_i tended to be higher than A. Significant positive correlations between A_i and A were observed for many cultivars (n = 6; Figure 6). When classified by significance level, 15, 12, and 7 cultivars

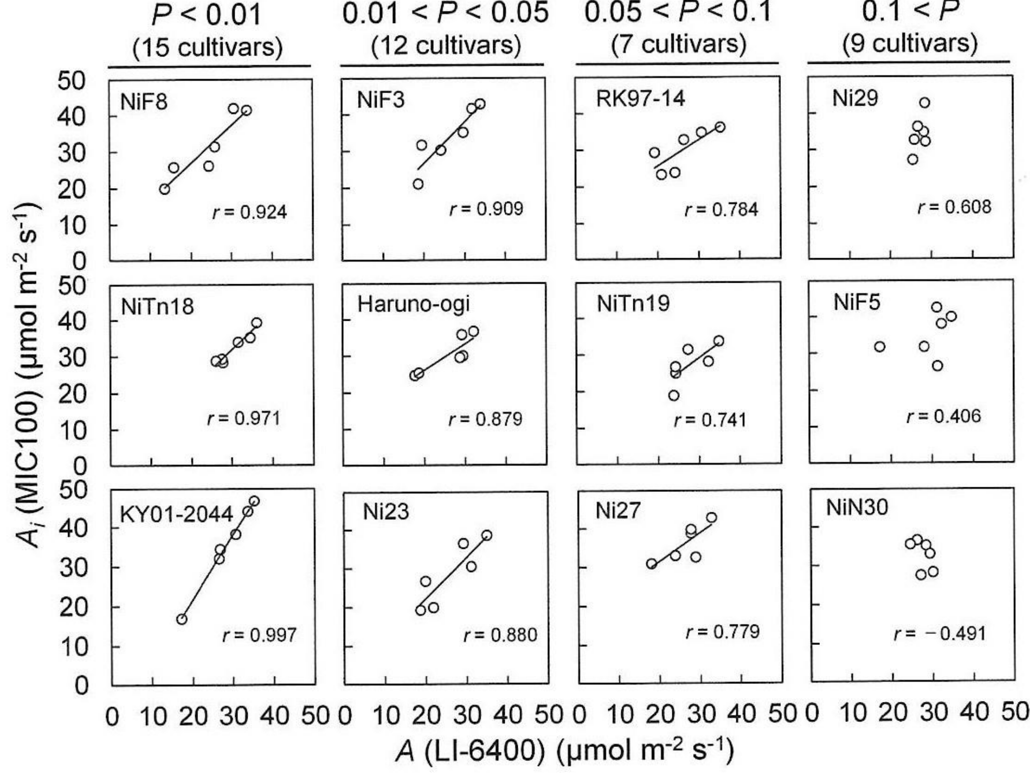

Figure 6. Correlations among photosynthetic rates measured using closed system MIC-100 (instantaneous; A_i) and open system LI-6400 (steady-state; A) of representative sugarcane cultivars in Exp.2. Note: Open circles represent all photosynthetic records for each cultivar (n = 6). P values at the top of the figures indicate ranges of significant levels of the correlation. Numbers in parentheses represent the number of cultivars that showed P values within each significant range.

Table 3. Correlation between photosynthesis and leaf traits of 43 sugarcane cultivars grown under field condition in Exp. 2.

	Leaf area	Leaf dry weight	SLA	SPAD	Stomatal density			Adaxial /Abaxial
					Abaxial	Adaxial	Total	
A (LI-6400)	−0.142	−0.166	0.209	0.133	−0.057	−0.220	−0.136	−0.168
A_i (MIC-100)	0.220	0.164	0.091	0.077	−0.065	−0.306*	−0.178	−0.252
Mean A_i/A	0.440*	0.376*	−0.029	0.019	−0.006	−0.205	−0.091	−0.204
Slope of A_i vs A regression	0.044	0.127	−0.209	0.125	0.126	−0.081	0.062	−0.174

The values in the column represent correlation coefficients between mean values of each trait in each cultivar (n= 43). SLA is the specific leaf area. Asterisks represent significance at $P < 0.05$.

Table 4. Average time required for a photosynthesis measurement using closed (MIC-100) and open (LI-6400) systems.

Experimental condition	Required time		
	MIC-100 (seconds record^{-1})	LI-6400 (seconds record^{-1})	Significance (t - test)
Glasshouse (Exp.1, n = 4 days)	37.8	404.0	$P < 0.001$
Field (Exp.2, n = 6 days)	36.9	315.2	$P < 0.001$

Note: The t – test was performed using daily mean values of time required for photosynthesis measurement to detect difference between measurement systems (n = 4 and 6 days for Exps. 1 and 2, respectively).

revealed significant correlations at $P < 0.01$, $0.01 < P < 0.05$, and $0.05 < P < 0.1$, respectively. The slopes of the regression equations for these varieties varied from 0.7 to 3.1 (Table 2). There were many cultivars for which the slope of the regression equation and the A_i /A ratio between simple measurements exceeded 1.0, and these varieties tended to have higher values for A_i than for A (Table 2). Correlation analysis using cultivar-mean photosynthesis measurements and leaf traits revealed weak correlations between A_i/A ratio and leaf area and weight, and between A_i and adaxial stomatal density, but no clear relationships were found (Table 3).

Comparison of time requirement for photosynthesis evaluation (Exps. 1 & 2)

A comparison of the time required for the measurements is presented in Table 4. In Exp. 1, the LI-6400 required an average of 404.0 s (6.7 min) to obtain a single measurement record for each leaf, including both A_{amb} and A_{1200} measurements, while the MIC-100 required an average of 37.8 s for a single record (n = 4 days). In Exp. 2, the LI-6400 required an average of 315.2 s (5.7 min), while the MIC-100 required an average of 36.9 s to obtain a single record (n = 6 days).

Discussion

Plant photosynthetic rates respond to diurnal changes in environmental conditions, such as air temperature, humidity, and solar radiation (Adachi et al., 2019; Al-Saidi et al., 2009; Tominaga et al., 2014). The present study investigated diurnal changes in photosynthesis in a temperature- and humidity-controlled glasshouse using a closed-type rapid-measurement system. The *RMSE* values, the accuracy of the measurement, for sugarcane, *Erianthus*, and both genotypes were 2.04, 2.90, and 2.58, respectively (Figure 4), which were favorable values comparable to those of soybean (2.71) and rice (3.18) reported in a previous study (Tanaka et al., 2021). In contrast, the *RMSE* was higher (5.0) for cultivars growing in a field with inconsistent temperature and humidity (Figure 5), which was higher than the value obtained for the C_3 plants reported previously. Photosynthetic rates are generally higher in sugarcane, a C_4 plant, than in C_3 plants; 0–30 µmol m^{-2} s^{-1} in rice and soybean (Tanaka et al., 2021) compared to 10–50 µmol m^{-2} s^{-1} in field-grown sugarcane (Figure 5). Therefore, in the present study, the *RMSE* values may have been high because of the wide distribution range and mean of the data. *RRMSE* is an index for comparing the accuracy of data with different distribution ranges. It is also rated as 'Excellent' for regression accuracy <10%, 'Good' for 10–20%, 'Fair' for 20–30%, and 'Poor' for ≥30% (Jamieson et al., 1991). The present study showed very good regression accuracies corresponding to *RRMSE* of 10.8–16.5% for sugarcane and 12.4% for *Erianthus* (Figures 4 and 5), which are equivalent to 'Good' rating. In conclusion, it was possible to evaluate diurnal and genetic variations in sugarcane photosynthesis with practical accuracy by using a closed-type rapid measurement system, although the accuracy in the field environment was somewhat lower than that under environmentally controlled conditions.

While the availability of a closed-type rapid measuring system was demonstrated, the ratio between measurements with open- and closed-type systems confirmed the overestimation tendency of the latter (Figure 5; Table 2). This overestimation tendency in the present results became greater at higher photosynthetic rate above 30 μmol m^{-2} s^{-1}, where data distribution was almost absent for C_3 plants like rice and soybean (Tanaka et al., 2021). The MIC-100 system is designed to allow gas to flow which is detected by sensors only on the abaxial side when the chamber is completely covered with leaves (Tanaka et al., 2021). In contrast, LI-6400 is a system in which gases in both leaf axial surfaces are mixed and detected by sensors (COR Biosciences, 2008; Márquez et al., 2021). Tanaka et al. (2021) suggested that MIC-100 measurements and their relationship with LI-6400 measurements are affected by whether the leaves completely cover the chamber and whether the stomatal distribution differs on each surface. Accordingly, a correlation analysis was conducted between leaf traits, including stomatal distribution, and photosynthesis values to investigate the factors contributing to the overestimation tendency in the MIC-100, but no clear relationship was found (Table 3). Such discrepancies between measurements have been observed even between the same open-type measurement systems (LI-6400 vs. LI-6800; Saathoff, unknown year; Garen et al., 2022). Therefore, by the process of elimination, it was suggested that the fundamental system differences among the measurement systems were the main cause of the overestimation tendency in the MIC-100.

The photosynthetic capacity of sugarcane varies among cultivars and genetic resources (Irvine, 1967; Jackson et al., 2016; Dinh et al., 2019), and the selection of promising genotypes from genetic populations through screening is important for efficient trait improvement. As the influence of diurnal changes (Figure 3; Adachi et al., 2019) cannot be ignored, it is necessary to measure multiple genotypes rapidly during a short time period (e.g. 2 h from 10:00 to 12:00) to reduce errors due to measurement times and replicates. Variability in measurements is likely to be greater in closed-type chambers because of the significantly changing environment (Hunt, 2003; Long & Hällgren, 1993). As an example of multi-point measurement using MIC-100, Honda et al. (2021) found differences in photosynthetic capacity among rice genetic populations with different genetic backgrounds by measuring 246 leaves in 5 h from 08:00 to 13:00 and repeating this for approximately 20 days. The MIC-100 could be used in our experiments for approximately 40 s per record (Table 4), suggesting that screening can be performed well using the same method for sugarcane, although it must first be determined whether the accuracy of measurements of instantaneous photosynthesis are acceptable (*RRMSE* < 20%). Therefore, it was considered necessary to increase or decrease the number of days of measurement or to change the measurement conditions in consideration of variation according to the purpose of the study in order to rapidly evaluate genetic variation.

The measurements for each cultivar in Exp. 2 show that significant positive correlations were obtained between A_i and A for approximately 60% of the measured cultivars (29 cultivars) at a significance level of 5% or less, whereas the slopes of the regression equations were diverse (Figure 6; Table 2). As the dataset for each cultivar in this experiment was small ($n = 6$), it is highly likely that a significant relationship could be obtained by increasing the number of leaf measurements of the cultivars for which no significant relationship was obtained in this experiment. If the closed-type system MIC-100 is to be used for more detailed studies of photosynthetic capacity rather than for screening purposes, it would be desirable to obtain a regression equation using the photosynthesis values measured with an open-type system, such as LI-6400, for each variety.

Conclusions

In the C_4 crop sugarcane, it was found that a closed-type measuring system could rapidly measure the photosynthetic rate at an average of <40 s per leaf, presuming that approximately 100 leaves could be measured per hour. Our results demonstrated that an overestimation tendency in the closed-type system becomes greater at higher photosynthetic rate. The main reason for the differences in the measured values between the measurement systems observed in both experiments was presumably the fundamental difference in the measurement systems (steady-state values for the open system and instantaneous values for the closed system). Although it is desirable to separately prepare a regression equation with measurements acquired using an open-type system such as LI-6400 for each variety when using it for a more detailed study, the closed-type rapid measurement system MIC-100 is applicable for photosynthesis phenotyping of multiple samples if the accuracies (*RRMSE* < 20%) shown in the measurements of environmental response (Exp. 1) and genetic variation (Exp.2) in sugarcane are acceptable.

Acknowledgments

We are most grateful to Masato Shimajiri, Shinji Ogata, Michiru Kyan, and many JIRCAS-TARF staff for their help with experimental management and measurement.

Disclosure statement

No potential conflict of interest was reported by the authors.

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References

Adachi, S., Tanaka, Y., Miyagi, A., Kashima, M., Tezuka, A., Toya, Y., Kobayashi, S., Ohkubo, S., Shimizu, H., Kawai-Yamada, M., Sage, R. F., Nagano, A. J., Yamori, W., & Raines, C. (2019). High-yielding rice Takanari has superior photosynthetic response to a commercial rice Koshihikari under fluctuating light. *Journal of Experimental Botany, 70*(19), 5287–5297. https://doi.org/10.1093/jxb/erz304

Agata, W., Kawamitsu, Y., Hakoyama, S., & Shima, Y. (1986). A system for measuring leaf gas exchange based on regulating vapour pressure difference. *Photosynthesis Research, 9*(3), 345–357. https://doi.org/10.1007/BF00029799

Al-Saidi, A., Fukuzawa, Y., Ueno, M., Baba, S., & Kawamitsu, Y. (2009). Temporal and vertical variations in photosynthetic drivers in mangrove canopies, Okinawa, Japan. *Plant Production Science, 12*(3), 336–340. https://doi.org/10.1626/pps.12.336

Autrey, L. J. C., & Tonta, J. A. (2005). From sugar production to biomass utilisation: The reform process to ensure the viability of the Mauritian sugar cane industry. *International Sugar Journal, 107*(1283), 646–652.

Bull, T. A. (1969). Photosynthetic efficiencies and photorespiration in calvin cycle and C_4 -dicarboxylic acid plants 1. *Crop Science, 9*(6), 726–729. https://doi.org/10.2135/cropsci1969.0011183X000900060015x

Cobb, J. N., DeClerck, G., Greenberg, A., Clark, R., & McCouch, S. (2013). Next-generation phenotyping: Requirements and strategies for enhancing our understanding of genotype–phenotype relationships and its relevance to crop improvement. *Theoretical and Applied Genetics, 126*(4), 867–887. https://doi.org/10.1007/s00122-013-2066-0

de Souza, A. P., Grandis, A., Leite, D. C., & Buckeridge, M. S. (2014). Sugarcane as a bioenergy source: History, performance, and perspectives for second-generation bioethanol. *BioEnergy Research, 7*(1), 24–35. https://doi.org/10.1007/s12155-013-9366-8

D'Hont, A., Rao, P. S., Feldmann, P., Grivet, L., Islam-Faridi, N., Taylor, P., & Glaszmann, J. C. (1995). Identification and characterisation of sugarcane intergeneric hybrids, *Saccharum officinarum* x *Erianthus arundinaceus*, with molecular markers and DNA in situ hybridisation. *Theoretical and Applied Genetics, 91*(2), 320–326. https://doi.org/10.1007/BF00220894

Dinh T. H., Takaragawa H., and Kawamitsu Y. (2019). Nitrogen use efficiency and drought tolerant ability of various sugarcane varieties under drought stress at early growth stage. *Plant Production Science, 22*(2), 250–261. https://doi.org/10.1080/1343943X.2018.1540277

Dinh, T. H., Watanabe, K., Takaragawa, H., Nakabaru, M., & Kawamitsu, Y. (2017). Photosynthetic response and nitrogen use efficiency of sugarcane under drought stress conditions with different nitrogen application levels. *Plant Production Science, 20*(4), 412–422. https://doi.org/10.1080/1343943X.2017.1371570

Du, Y. C., Kawamitsu, Y., Nose, A., Hiyane, S., Murayama, S., Wasano, K., & Uchida, Y. (1996). Effects of water stress on carbon exchange rate and activities of photosynthetic enzymes in leaves of sugarcane (Saccharum sp.). *Functional Plant Biology, 23*(6), 719–726. https://doi.org/10.1071/PP9960719

Du, Y. C., Nose, A., & Wasano, K. (1999). Effects of chilling temperature on photosynthetic rates, photosynthetic enzyme activities and metabolite levels in leaves of three sugarcane species. *Plant, Cell & Environment, 22*(3), 317–324. https://doi.org/10.1046/j.1365-3040.1999.00415.x

FAOSTAT, (2022). Sugarcane area harvested. FAO. Retrieved May 12, 2022, from https://www.fao.org/faostat/en/#data/QCL

Farquhar, G. D., von Caemmerer, S. V., & Berry, J. A. (1980). A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta, 149*(1), 78–90. https://doi.org/10.1007/BF00386231

Garen, J. C., Branch, H. A., Borrego, I., Blonder, B., Stinziano, J. R., & Michaletz, S. T. (2022). Gas exchange analyzers exhibit large measurement error driven by internal thermal gradients. *The New Phytologist, 236*(2), 369–384. https://doi.org/10.1111/nph.18347

Hatch, M. D. (1999). *C_4 photosynthesis: A historical overview.* (Sage RF, Monson RK, Eds.). Academic Press.

Hatch, M. D., & Slack, C. R. (1966). Photosynthesis by sugar-cane leaves: A new carboxylation reaction and the pathway of sugar formation. *The Biochemical Journal, 101*(1), 103–111. https://doi.org/10.1042/bj1010103

Hattori, T., & Morita, S. (2010). Energy crops for sustainable bioethanol production; which, where and how? *Plant Production Science, 13*(3), 221–234. https://doi.org/10.1626/pps.13.221

Heskech, J. D., & Moss, D. N. (1963). Variation in the response of photosynthesis to light 1. *Crop Science, 3*(2), 107–110. https://doi.org/10.2135/cropsci1963.0011183X000300020002x

Hoffman, N., Singels, A., Patton, A., & Ramburan, S. (2018). Predicting genotypic differences in irrigated sugarcane yield using the Canegro model and independent trait parameter estimates. *European Journal of Agronomy, 96*, 13–21. https://doi.org/10.1016/j.eja.2018.01.005

Honda, S., Ohkubo, S., San, N. S., Nakkasame, A., Tomisawa, K., Katsura, K., Ookawa, T., Nagano, A. J., & Adachi, S. (2021). Maintaining higher leaf photosynthesis after heading stage could promote biomass accumulation in rice. *Scientific Reports, 11*(1), 1–11. https://doi.org/10.1038/s41598-021-86983-9

Hunt, S. (2003). Measurements of photosynthesis and respiration in plants. *Physiologia plantarum, 117*(3), 314–325. https://doi.org/10.1034/j.1399-3054.2003.00055.x

Inman-Bamber, N. G., Jackson, P., & Bourgault, M. (2011). Genetic adjustment to changing climates: Sugarcane. In S. Yadav, R. Redden, J. Hatfield, H. Lotze-Campen, & A. Hall (Eds.), *Crop adaptation to climate change* (pp. 439–447). Wiley.

Inman-Bamber, N. G., Jackson, P. A., Stokes, C. J., Verrall, S., Lakshmanan, P., & Basnayake, J. (2016). Sugarcane for water-limited environments: Enhanced capability of the APSIM sugarcane model for assessing traits for transpiration