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Regulation of Photosynthesis during the Light Period in CAM Plants
—Evaluation by a Gas-Phase O_2 Electrode and a Compensating Infrared CO_2 Analysis System—

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The CO_2 dependent O_2 evolution during the light period, at which the exogenous CO_2 uptake was suspended, in 12 CAM plants (including pineapple (*Ananas comosus*)) was evaluated with a gas-phase oxygen electrode. At 5% CO_2, the rate of photosynthetic O_2 evolution in pineapple was saturated at 1 500 μmol m^{-2} s^{-1} PPFD and the maximum rate was 60 μmol m^{-2} s^{-1}, which was 10 times those obtained at ambient CO_2 conditions with the CO_2 exchange system and significantly higher than the other CAM plants. At the saturated PPFD, the O_2 evolution in pineapple substantially increased with increasing CO_2 concentration up to 3% and decreased above 4%. However in the other CAM plants, such increment was small. By the use of a novel compensating CO_2 gas exchange system, the rate of CO_2 exchange in pineapple at very high CO_2 was measured and found the enhancement of CO_2 uptake in both light (Phases III and IV) and dark (Phase I) periods. Based on the results obtained, possibilities of the further increases in CO_2 uptake in CAM plants, especially of pineapple, are discussed in terms of stomatal functions and malate storage capacities.

Keywords: CO_2 dependent O_2 evolution, crassulacean acid metabolism, high CO_2, pineapple, stomatal closure

INTRODUCTION

In pineapple cultivation, at least 2 years is needed from transplanting to harvest under natural conditions. The use of plant hormones such as indoleacetic acid and ethylene accelerates the floral differentiation and can reduce the cultivation period at most 6 months. In general, the growth rate of pineapple is extremely slow due to its crassulacean acid metabolism (CAM), as compared with C_3 and C_4 plants (Nose et al., 1986). Recently, some farmers in Okinawa, Japan, are cultivating pineapple in greenhouses to promote growth rate and simultaneously to improve fruit quality. Although they can improve fruit quality in winter and early spring, they still unsuccessful to shorten the cultivation period. Main reason of this is that most farmers do not understand pineapple as a CAM plant and its tremendous performances in physiological and biochemical aspects are not accounted for the actual

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cultivation. For example, some farmers close the greenhouses in order to raise night temperature, irrespective of the evidence that pineapple plants absorb CO_2 at night through CAM. This makes the pineapple leaf into CO_2 starved state at the end of night (Kawamitsu and Nakayama, 1997).

According to Osmond (1978), the gas exchange and stomatal behaviour of CAM plants were divided into four phases, i.e. Phase I with CO_2 uptake in darkness, Phase II with CO_2 uptake in the early morning, Phase III with stomatal closure during the middle of day and Phase IV with CO_2 uptake again in the later afternoon. During Phase III, when stomata close tightly, malic acid previously formed in the dark is decarboxylated and refixation of CO_2 by ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) takes place. As a result of stomatal closure, the conventional gas exchange apparatus with an infrared gas analyzer can not be used to evaluate the CO_2 fixation by Rubisco in CAM leaf. Fortunately, a gas-phase O_2 electrode system can detect the O_2 evolution from CAM leaves (Thomas and Andre, 1987; Maxwell et al., 1998). Even if the stomata are closed, CO_2 with very high concentrations (1–10%) can penetrate through waxy epidermis or cutting edge, so that CO_2 dependent O_2 evolution operating in the mesophyll cells may be detected with a gas-phase leaf disc O_2 electrode (Delieu and Walker, 1981).

Although the rise in CO_2 is a world wide phenomenon, few studies on the effects of CO_2 enrichment for pineapple plants were undertaken (Zhu et al., 1997, 1999). Elevated CO_2 would be expected to have little effect on dark CO_2 fixation because it is mediated by phosphoenolpyruvate carboxylase (PEPCase), which is assumed to be CO_2 saturated at near-ambient CO_2 levels (Ting, 1994). However, Zhu et al. (1997, 1999) demonstrated that PEPCase in pineapple leaf is not CO_2-saturated at ambient CO_2 levels and 25°C night temperature.

The goal of this research was to establish an ideal technique for the cultivation of pineapple with reducing the growth period at least to 12–13 months. We expect that an increase in leaf CO_2 exchange rate may be an important factor to increase the carbon accumulation in pineapple, a CAM plant. In this paper, we examined the responses of O_2 evolution to photosynthetic photon flux density (PPFD) and CO_2 concentration during light period in which exogenous CO_2 uptake was suspended (Kluge and Ting, 1978). In addition, to ensure the effects of very high CO_2 concentration on the CO_2 uptake during light period, we conducted the measurements of CO_2 exchange by a new, compensating gas-exchange system with an infrared gas analyzer (Graan and Boyer, 1990; Kawamitsu and Boyer, 1999).

MATERIALS AND METHODS

Plant materials. We examined 12 CAM species: *Ananas comosus* (L.) Merr. Smooth cayenne c.v. N67-10 (pineapple), *Crassula argentea*, *Dendrobium ekapol* cv. Panda No. 1, *Hoya carnosa* R. Br., *Kalanchoe blossfeldiana* Poelln., *K. daigremontiana* R. Hamet & Perrier, *K. fedtschenkoi* Hamet & Perr, *K. gastonis-bonniere* Hamet et Perr., *K. pinnata* Pers., *Peperomia incana* A. Dietr., *Sedum praealtum*, *Vanilla fragrans* Ames. These plants were propagated by adventitious plantlets obtained from a local botanical garden in Okinawa, Japan. Plants were grown in 1/5 000 a pots containing vermiculite. Pineapple plants were propagated by crowns obtained from Okinawa Experiment Station, Nago City, Okinawa, Japan. They were grown in 1/5 000 a pots containing red soil (Kunigami Maji, pH 5.8). Five hundred mL of modified Hoagland's solution was supplied twice a week. Composition of the solution was 6 mM $Ca(NO_3)_2 \cdot 4H_2O$, 6 mM KNO_3, 2 mM KH_2PO_4, 2 mM $MgSO_4 \cdot 7H_2O$, 25 μM H_3BO_3, 10 μM $MnSO_4 \cdot 4H_2O$, 2 μM $ZnSO_4 \cdot 7H_2O$, 0.5 μM $CuSO_4 \cdot 5H_2O$, 0.5 μM H_2MoO_4, and 0.1 mM $FeC_6H_5O_7$. Plants were grown in a glasshouse under natural light

regimes. During the experiments, all plant materials were transferred into a growth chamber in photosynthesis measurement room, setting at 500 μmol m^{-2} s^{-1} PPFD and 30/25°C day/night temperature.

Measurements of CO_2 dependent O_2 evolution. The CO_2 dependent O_2 evolution was measured by a leaf-disc oxygen electrode system (Delieu and Walker, 1981, 1983 ; Buah et al., 1999 ; Kawamitsu and Boyer, 1999 ; Kawamitsu et al., 2000). According to preliminary experiments, the maximum photosynthetic rates were obtained at the uppermost, fully expanded leaves. Approximately 8 cm^2 leaf discs from leaves were cut and their fresh weights were determined. Then the leaf disc was placed in the electrode chamber and the volume of the chamber was calibrated after Delieu and Walker (1981, 1983). The CO_2 in the chamber is rapidly exhausted by photosynthesizing disc unless it is replenished (Delieu and Walker, 1981, 1983). Generally, a carbonate/bicarbonate buffer was recommended as a source of CO_2 in the system. However, we used gas mixture because CO_2 level could be altered rapidly without opening the chamber (Kawamitsu and Boyer, 1999 ; Kawamitsu et al., 2000). The procedure employed was as follows ; the tissue was illuminated in the presence of gas mixture for at least 5 min prior to sealing the chamber and measuring O_2 exchange. After sealing the chamber, to get the trace of O_2 exchange on a chart of the recorder it ran for approximately 50 s. Subsequently, the chamber was flushed with the new gas mixture for 5 min and then the O_2 evolution was re-measured in the same manner. If the O_2 evolution at 10 min was the same as that at 5 min, the rate was considered stable and the conditions could be changed for the next set of measurement. The air humidity of chamber was maintained at near saturation to avoid desiccation of the leaf disc. Photosynthetic O_2 evolution as a function of CO_2 concentration and irradiance was determined at 21% O_2. The Björkman lamp was used as a light source. The irradiance levels were altered by the use of neutral density glass filters, which were exchanged in the light source housing (LS-2, Hansatech). Irradiance was reduced stepwise from high PPFD (1 500 μmol m^{-2} s^{-1}) to darkness in nine steps. Total darkness was obtained by wrapping the electrode chamber in two layers of aluminum foil. Equilibration time to establish a steady rate of O_2 evolution under a new PPFD in the presence of CO_2 required approximately 5 min for the CAM plants used. Because disc temperature rose by illumination, the water temperature circulating the electrode was maintained at approximately 23.5°C, so that the tissue temperature in the chamber was at 25°C after sealing. This temperature was employed for all the O_2 electrode experiments reported here. In the case of the dark respiration, the temperature of water bath was adjusted at 25°C since the temperature of the water bath and the electrode chamber was the same. For the measurement of CO_2 dependent O_2 evolution, CO_2 concentration of the incoming air was controlled by mixing CO_2-free air (containing 21% oxygen and balanced nitrogen) with 10% CO_2 (containing 21% oxygen and balanced nitrogen). The CO_2 concentration was increased stepwise from 0 to 5% at 25°C disc temperature and 1 500 μmol m^{-2} s^{-1} PPFD. Each measurement was repeated 3 times and the higher value was plotted.

Measurements of CO_2 exchange. Diurnal changes in CO_2 exchange rates at normal air were determined with an infrared gas analyzer (Model Li-6251, Li-Cor) in an open system (Du et al., 1996 ; Kawamitsu et al., 1999a, b). After the plant was set in the assimilation chamber, gas exchange rate was monitored for about 1 week. Thereafter, the 24 h continued data sets were taken in the stage in which gas exchange rate was stabilized. The CO_2 exchange rates at very high CO_2 were measured at University of Delaware, College of Marine Studies, Lewes, Delaware 19958, U.S.A. with a semi-closed compensating system described in elsewhere (Graan and Boyer, 1990 ; Kawamitsu and Boyer, 1999). This system enables to increase the ambient CO_2 concentration up to 7% (70 000 ppm). The leaf area used for the experiments

was 120 cm^2. Gas exchange over 24 h was repeated twice at each CO_2 level for different plant with comparable results.

Organic acid and chlorophyll content. Organic acids were determined on the same leaf used in the measurement of CO_2 exchange under ambient CO_2 conditions. Leaf discs (2 cm^2) were weighed and then boiled for 10 min, thereafter ground in 10 mL glass-distilled water with a Potter-type tissue homogenizer. The mixture was centrifuged at 12 000$\times g$ for 10 min, and then filtered with the membrane filter (0.4 μm in diameter). Organic acid contents were determined with the liquid chromatography method (Model LC-6A, CDD-6A ; Shimadzu). Other leaf discs used for CO_2 dependent O_2 evolution were weighed and then ground with sea sand in a chilled mortar and pestle. Chlorophyll contents were determined with a spectrophotometer after extracting with 80% acetone (Arnon, 1949).

RESULTS AND DISCUSSION

Light response curves of CO_2 dependent O_2 evolution in pineapple and other CAM plants are shown in Fig. 1. All measurements were conducted during Phase III of CAM cycle (Osmond, 1978). Intercellular CO_2 concentration in CAM plants was often observed at 1-2% during Phase III (Cockburn, et al., 1979; Spalding et al., 1979). Thus we measured photosynthetic O_2 evolution at 5% CO_2, which was higher than intercellular CO_2 concentra-

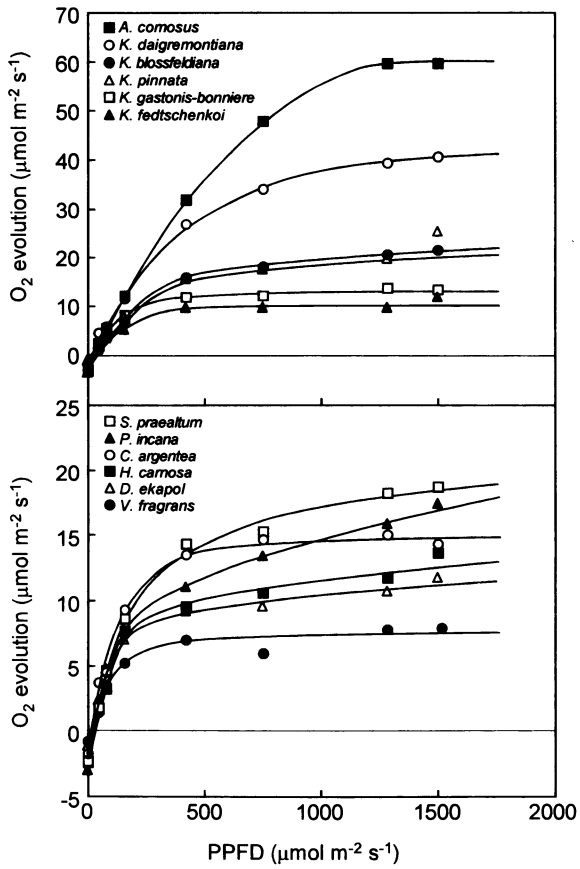

Fig. 1 Light response curves of O_2 evolution in CAM plants. Measurements were made at 5% CO_2.

tions reported. The rates of photosynthetic O_2 evolution in other CAM plants were also measured under the same conditions. Pineapple showed the highest O_2 evolution rate at higher PPFD (Fig. 1). The light saturation point of the O_2 evolution was approximately 1 500 μmol m^{-2} s^{-1} PPFD. *K. daigremontiana* showed an intermediate rate of O_2 evolution between pineapple and the other CAM plants (below 20 μmol m^{-2} s^{-1}). In addition, the light saturation points were lower than 500 μmol m^{-2} s^{-1} PPFD in those CAM plants. Sixty μmol m^{-2} s^{-1} of O_2 evolution in pineapple was 7.5-12 times of the value of 5-8 μmol m^{-2} s^{-1} obtained at ambient CO_2 conditions with the conventional CO_2 exchange system (Fig. 6).

The photosynthetic O_2 evolutions were also expressed on a chlorophyll content basis (Fig. 2). Pineapple still showed higher photosynthetic rate than the other CAM plants. However, *K. gastonisbonniere*, which had a low value on a leaf area base in Fig. 1, showed the highest rate on a unit of chlorophyll base (Fig. 2). Growth rate of this species was higher than those of the other CAM plants, except pineapple (data not shown).

During the decarboxylation phase of CAM mode (Phase III), the release of CO_2 from stored malic acid generates high intercellular CO_2 concentration in the stomata-closed leaf, so that photosynthetic O_2 evolution occurs even when measured at CO_2 free, ambient air (Fig. 3). Although some CO_2 had escaped when the disc sample was punched from the intact leaf, O_2 evolution at 1 500 μmol m^{-2} s^{-1} PPFD was approximately 10 μmol m^{-2} s^{-1}, which was one-sixth of the maximum O_2 evolution at 5% CO_2 (Fig. 1). When CO_2 concentration around the leaf disc was increased to the normal ambient air (0.03%), the rate of photosynthetic O_2 evolution remained unchanged (Fig. 3). It meant that the stomatal conductance was unusually low during Phase III, so that 0.03% CO_2 could not penetrate through epidermis or alternatively, disc intercellular CO_2 concentration was higher than the CO_2 concentration in the electrode chamber. Usually, in C_3 and C_4 plants, photosynthetic rate increases with increasing ambient CO_2 concentration. But the saturation point in C_4 species is lower than that in C_3 species because of CO_2 concentrating mechanism based on PEPCase and the lack of photorespiration (Long, 1999). In CAM plants, if the intercellular CO_2 concentration were maintained at 1-2% during Phase III, photosynthetic rate may not be influenced with altering CO_2 concentration up to 2% around the leaf disc. In pineapple, however, photosynthetic O_2 evolution was increased with increasing CO_2 up to 3% and subsequently decreased above 4% CO_2 (Fig. 4). Cockburn et al. (1979) showed 0.5% of intercellular CO_2 concentration in pineapple when leaves were illuminated. As shown in the present study (Fig. 4) and

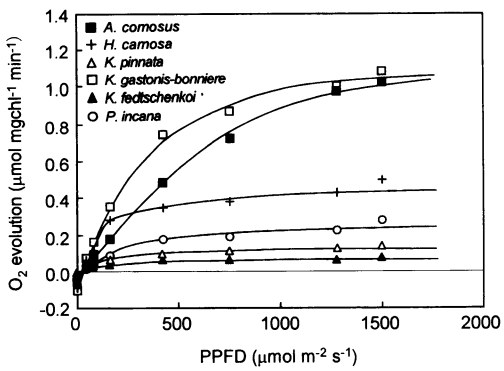

Fig. 2 Light response curves of O_2 evolution in CAM plants.
Measurements were made at 5% CO_2 and expressed on a chlorophyll base.

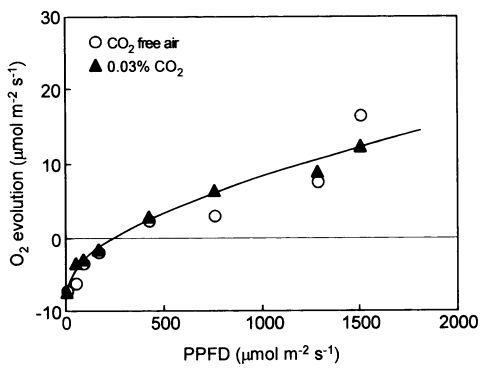

Fig. 3 Light response curves of O_2 evolution in pineapple at CO_2 free air and 0.03% CO_2.

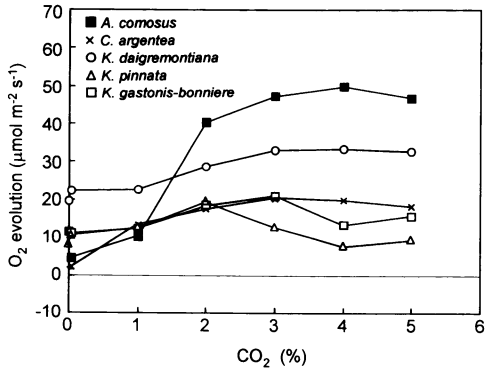

Fig. 4 Effects of CO_2 concentration on photosynthetic O_2 evolution in CAM plants.

Measurements were made at 1 500 μmol m^{-2} s^{-1} PPFD, 21% O_2 and 25°C disc temperature.

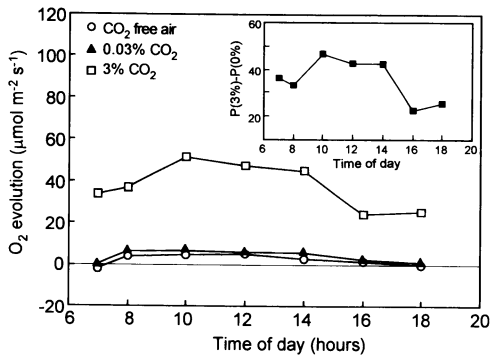

Fig. 5 Time courses of photosynthetic O_2 evolution at different CO_2 concentration in pineapple.

The inset indicates the difference in O_2 evolution between at 3% CO_2 and CO_2 free air.

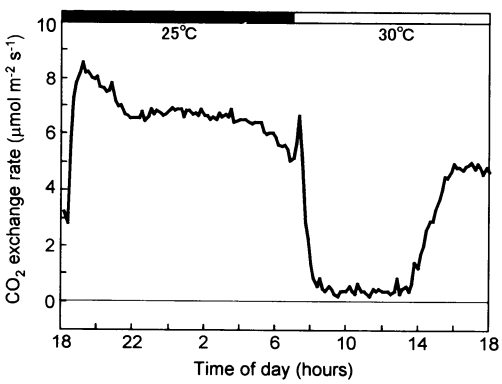

Fig. 6 Diurnal courses of CO_2 exchange rate at ambient CO_2 concentration in pineapple.

Day/night temperature was set at 30/25 °C, and PPFD was 600 μmol m^{-2} s^{-1} during the light period.

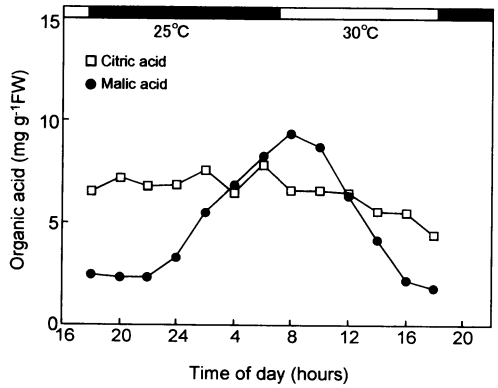

Fig. 7 Diurnal courses of organic acid contents in pineapple leaf.

Conditions were the same as in Fig. 6.

some other studies (Cockburn et al., 1979 ; Spaldings et al., 1979), we assume that the intercellular CO_2 concentration in pineapple in Phase III is at around 0.5%. Moreover, maximum O_2 evolution at high CO_2 concentration in pineapple is higher than those of other CAM plants. Due to insufficient CO_2 diffusion at the most inner side of thick leaf in pineapple, only the mesophyll cells near the stomatal cavities may be actively fix CO_2 from the ambient air. In the other CAM plants, the degrees of increment in photosynthetic O_2 evolution with increasing CO_2 concentration were small, indicating that photosynthesis are already saturated at the intercellular CO_2 concentration. If so, the intercellular CO_2 concentration in those CAM plants was probably lower than that of pineapple.

Diurnal changes in photosynthetic O_2 evolution at very high CO_2 concentration were measured during the light period (Phases II, III and IV) (Fig. 5). As a comparison, the diurnal course in CO_2 exchange of the different leaf of the same plant at ambient CO_2

conditions was also measured (Fig. 6). In pineapple, CO_2 uptake occurred during Phases I, II and IV, and its mode was classified as full CAM type (Kluge and Ting, 1978). Maximum CO_2 exchange rate in Phase I was approximately 7 μmol $m^{-2} s^{-1}$ (Fig. 6) and a little CO_2 uptake was detected during Phase III. Diurnal changes in malic and citric acids of the same leaf exhibited a typical CAM pattern (Fig. 7). At the end of Phase I, malate content reached the peak value, and thereafter decreased to the minimum value at Phase IV. However, Fig. 5 showed that O_2 evolution occurred throughout the light period (Phases II, III and IV) and furthermore, photosynthetic O_2 evolution at 3% CO_2 was 8–10 times higher as compared with those at 0.03% CO_2. There were no differences in photosynthetic O_2 evolution between at 0.03% CO_2 and CO_2 free air because of the difficulties of CO_2 penetration through the epidermis of pineapple leaf. At 3% CO_2, the photosynthetic O_2 evolution had a peak at around 10 : 00 (Phase III) and decreased at Phases II and IV. Under CO_2 free, ambient air, there are no carbon sources around the leaf disc, but O_2 evolution occurred during the light period, this indicated that malate transported from the vacuole was decarboxylated to supply CO_2 to Calvin cycle with high concentration levels. Under the identical conditions, terrestrial C_3 and C_4 species showed no evidence of this type of O_2 evolution (Kawamitsu and Boyer, 1999). The O_2 evolution was decreased with time probably due to depletion of the stored malate. The inset in Fig. 5 indicates the difference in photosynthetic O_2 evolutions between at 3% CO_2 and CO_2 free air. Interestingly, the difference was high at mid-day and low at the beginning and end of light period, suggesting that the activities of Rubisco, a predominant CO_2 fixing enzyme, were not only dependent on carbon dioxide concentration inside the leaf but also regulative by a diurnal rhythm of CAM.

To ensure the effects of very high CO_2 concentration on CO_2 exchange rate in a whole day, we measured diurnal changes in CO_2 exchange rate at 0.0392, 0.1078 and 1.017% CO_2 with a novel gas exchange system (Graan and Boyer, 1990; Kawamitsu and Boyer, 1999) (Fig. 8). Diurnal patterns of CO_2 exchange at 0.0392% CO_2 was a typical CAM mode and Phase IV was clearly identified as in Fig. 5. When CO_2 concentration increased to 0.1078 or 1.017%, CO_2 uptake was increased not only during the light period but also during the dark period (Table 1). Especially at 1.017% CO_2, the CO_2 uptake was significantly enhanced during Phases II

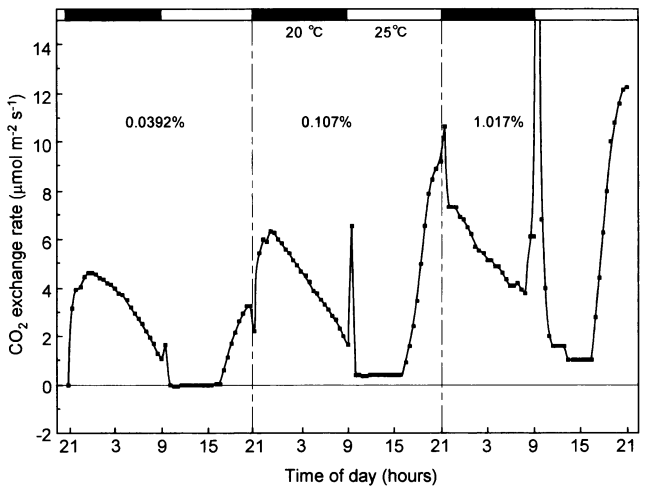

Fig. 8 Effects of CO_2 concentration on the time courses of CO_2 exchange in pineapple.
Day/night temperature was set at 25/20°C, and PPFD was 650 μmol $m^{-2} s^{-1}$ during the light period.

Table 1 Effect of CO_2 concentration on CO_2 balance (relative to 0.0392% CO_2) in pineapple.

Phase	CO_2 concentration (%)		
	0.0392	0.1078	1.017
Total	100	178	272
I	100	133	147
II and III	100	400	2 400
IV	100	400	560

and III because incoming CO_2 from the atmosphere was fixed by Rubisco. Interestingly, CO_2 fixation during the dark period was also increased even though during which PEPCase with higher affinity to CO_2 mainly regulated CO_2 fixation (Kluge and Ting, 1978; Ting, 1994; Winter and Smith, 1996). As in C_4 plants, PEPCase in CAM plants during Phase I was thought to be saturated at near-ambient CO_2 levels (Ting, 1994; Winter and Smith, 1996). However, Zhu et al. (1997) showed that Phase I CO_2 uptake by PEPCase in pineapple was not CO_2 saturated at ambient CO_2 levels, and that the enhancement of CO_2 dark fixation by elevated CO_2 was probably due to the CO_2 un-saturation of PEPCase, the reduced dark respiration and the improvement of the low mesophyll conductance.

In addition to these factors, since the capacity of vacuoles as reservoir in mesophyll cells was substantially restricted, then increments in CO_2 fixation by means of CO_2 enrichment have not been expected to enhance the CO_2 uptake during Phase I (Black, 1986; Ting, 1994). However, as shown in Fig. 8 in our study and some other studies (Zhu et al., 1997, 1999), high CO_2 concentration significantly increased the dark CO_2 fixation. For this, we emphasize again that the CO_2 diffusion will be insufficient at the most inner side of the succulent leaf of pineapple, and only the mesophyll cells near the stomatal cavities actively fix CO_2 from the ambient air. Thus the high CO_2 concentration overcomes the slower CO_2 diffusion in inner mesophyll cells in pineapple.

In CAM plants stomata close with increasing CO_2 (Kawamitsu et al., 1999b), so that the effect of CO_2 enrichment on CO_2 uptake seems to be simultaneously restricted with the stomatal closure. However, even though the stomata are closed at high CO_2, the very high ambient CO_2 enhances CO_2 uptake not only during the light period, but also during the dark period. The increased CO_2 uptake and reduced stomatal conductance under CO_2 enrichment increased the water-use efficiency in pineapple and also other CAM plants (Zhu et al., 1999). Moreover, as mentioned above, the very high CO_2 significantly improves the CO_2 supply to the thicker mesophyll cell, which provides higher capability to fix CO_2.

Based on the photosynthetic O_2 evolution or CO_2 exchange rates presented here, the more increased CO_2 uptake in pineapple at very high CO_2 concentration will be attained both in the light and dark periods. It is necessary, therefore, to carry out the nighttime CO_2 enrichment in pineapple cultivation under greenhouse conditions when windows are closed at night in winter season.

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〈和文抄録〉

CAM植物における明期の光合成の制御
——気相型酸素電極および補償型赤外線 CO_2 分析装置による評価——

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通常の CO_2 交換測定装置では評価が不可能なCAM植物（パインアップル（*Ananas comosus*）を含む12種）の明期における葉内部の光合成活性を，気相型酸素電極および補償型赤外線 CO_2 交換測定装置を用いて検討した．5%の CO_2 濃度下で測定した場合，パインアップルの酸素放出速度は1 500 μmol m^{-2} s^{-1} PPFDで光飽和し，その最大値は通常のガス交換速度で得られる値の約10倍の60 μmol m^{-2} s^{-1} であった．また，飽和光下の酸素放出速度は CO_2 濃度が3%までは上昇を続け，4%以上では低下した．しかしながら，他のCAM植物ではそのような CO_2 濃度上昇に伴う酸素放出速度の増加は小さかった．次に，補償型 CO_2 交換測定装置を用いて，高 CO_2 濃度条件下でパインアップルの CO_2 交換速度を測定したところ，CO_2 の取り込みは明期のPhase IIIだけではなく暗期のPhase Iにおいても増大することがわかった．以上の結果に基づき，CAM植物，特にパインアップルにおける CO_2 取り込み速度の更なる上昇の可能性を，気孔の機能やリンゴ酸の貯蔵容量の観点から考察した．