Measurement of Photosynthetic Activity Using Single Cells and Discs of Tea Leaves

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Protoplasts or single cells of plants are useful material for physiological, biochemical or genetic researches. For example, photosynthetic activity of tea plants has so far been measured with whole plants or cut shoots[1]. In this case, much efforts are needed to control various environmental factors such as temperature, humidity, CO_2 and O_2 concentration, etc. In fact, it is difficult to control these factors during the measurements in most cases. Moreover, each factor exerts some effect on CO_2-diffusion resistance like stomatal aperture, so that it is difficult to know direct effects of environmental factors on photosynthetic activity.

On the contrary, the use of single cells suspended in a solution makes it easy to control environmental conditions during the measurements and enables to study more direct effects of environmental factors on photosynthetic activity. However, isolation of single cells with physiological activities from arboreus plants is known only with *Morus alba*[2], *Paulownia Forturei*, and *Populus euramericana*[3], etc., although many examples are already known with herbaceous plants. Therefore, the author has studied the method of isolating cells from tea leaves and photosynthetic characteristics of the isolated cells. In addition, photosynthetic characteristics of tea leaf discs which can be prepared easily and in plenty for the measurement was also studied.

Method of cell isolation[4,5]

Single cells were isolated enzymatically by the use of macerozyme, and they showed the maximum photosynthetic activity at the composition of enzyme solution and reaction medium shown in Table 1. Unlike tobacco and others, epidermis can not be removed from tea leaves[6]. Therefore, sample leaves were cut into small pieces of 2–3 mm width, and the enzyme solution was forced to infiltrate into their tissue by the use of aspirator. By oscillating the leaf pieces with enzyme solution at a constant temperature (25°C) cells were released from the leaf pieces at a rate linear against time up to 2 hrs (Fig. 1), giving the yields of 10–15% (from young leaves) after 2 hrs of oscillation. Yield from matured leaves was lower than those from young leaves, but it was increased to some extent by adding cellulase in addition to macerozyme.

Addition of potassium dextransulfate to the enzyme solution at 0.3–0.5% increased the photosynthetic activity. Takebe et al.[6] obtained the similar result with tobacco, but protoplast isolation with *Morus alba*[2], *Paulownia Forturei* and *populus euramericana*[3]

Table 1. Enzyme solution used for isolation of single cells and reaction medium for photosynthesis. Inmature leaves were used for isolation

Enzyme solution		Reaction medium	
0.5%	Macerozyme	5 mM	$MgCl_2$
0.4%	potassium dextransulfate	1 mM	$MnCl_2$
1.0M	sorbitol	1.0M	sorbitol
20 mM	sodium isoascorbate	1 mM	KH_2PO_4
0.5%	BSA	5 mM	DTT
8.0%	PVP-40	33 mM	Tris-HCl
2.0%	Dowex-1		
pH	5.6	pH	7.8

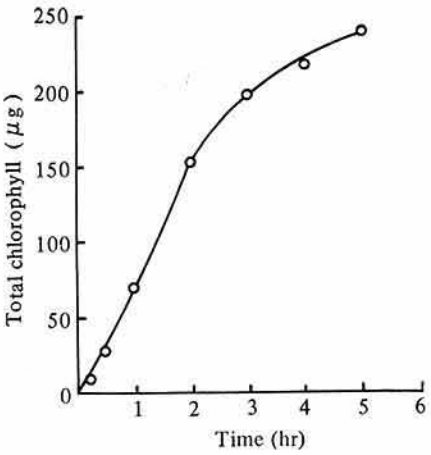

Fig. 1. Time course of the release of single cells from tea leaves

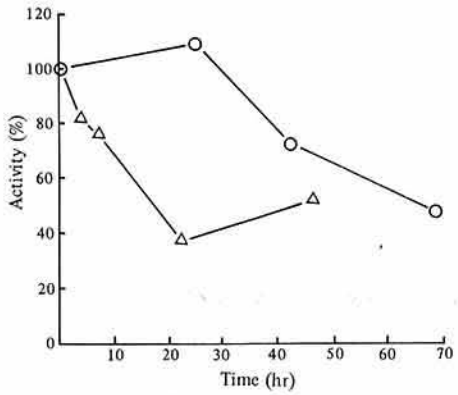

Fig. 2. Stability of single cells. Cells were kept in the dark at −4°C (○) or at room temperature (△)

was made using the concentration of 1.0% of potassium dextransulfate. However, for tea leaves the concentration of 1.0% inhibited photosynthetic activity. On the other hand, Yazawa made cell isolation with *Morus alba* without using potassium dextransulfate. Further examination will be needed on the role of this substance in cell isolation.

The optimum concentration of sorbitol in the enzyme solution and in the reaction medium was 1.0 M, which is considerably higher than other cases. This is probably due to the fact that the leaves used were immature with fragile cells. As a matter of fact, the optimum concentration of sorbitol for mature leaves was as low as 0.4 M in the enzyme solution and 0.3 M in the reaction medium.

Stability of single cells

As tea leaves contain a large amount of polyphenol compounds which are adverse to the cell isolation procedure, removal of the compounds by adsorption with Polyclar-AT of low concentration (2%) was not sufficient, so that photosynthetic activity of cells continued to decline with the advance of the enzymatic treatment period. Concentrations of adsorbents (Polyclar-AT, PVP-40, Dowex-1, etc.) higher than 10% were required to increase and maintain activities of single cells. Especially, the mixed use of adsorbents gave a better result. BSA and sodium isoascorbate were also effective.

By these treatments, isolated cells were stabilized to a considerable extent. Namely, the cells maintained 75% and 50% of their activities after 42 and 68 hrs, respectively, at low temperature (4°C). Even at room temperature (25°C), 50% of their activity was maintained after 6 hrs.

Measuring photosynthetic activity using single cells

Rate of CO_2 fixation of single cells was measured as follows: 100 μl (about 50 μg chlorophyll/ml) of single cells was added to 180 μl of the reaction medium (Table 1), and after 5 min of pre-illumination 20 μl of ^{14}C-$NaHCO_3$ was added to make photosynthetic reaction started, at 30°C under 40 klux of white light from an incandescent lamp. At a certain time interval, the reaction was stopped by adding 100 μl of 40% acetic acid. The weighed cell samples were placed on planchets and their radioactivity of ^{14}C was measured by gas-flow counter to determine the CO_2-fixation rate.

The photosynthetic rate showed a saturation at the concentration of about 5 mM of

$NAHCO_3$, with $Km(CO_2)$ of about 40 μM (at pH 7.8). The rate was increased with an increased light intensity, reaching a saturation at about 40 klux, as was shown by the measurement with young tea plants. The rate was increased linearly with the increase of temperature from 20 to 30°C, reaching a saturation at 30–35°C. This result supports Hadfield's result[8] that the optimum temperature for photosynthesis of single leaves was 35°C.

Single cells taken from immature leaves showed the photosynthetic rate of 20–30 μmoles/mg chlorophyll·hr, whereas single cells of mature leaves gave about 1/3 of that rate. This result corresponds to the known fact that the photosynthesis measured with mature leaves was considerably lower than that of new leaves in rooted cuttings. These results indicate that the photosynthetic activity of single leaf of rooted cuttings can be estimated by knowing the photosynthesis of single cells of each leaf, and that information difficult to be obtained by using single leaf might possibly be obtained by the use of single cells, due to the easy control of environmental condition.

Measuring photosynthetic activity using leaf discs

Although environmental factors related to photosynthesis can easily be controlled by using single cells, this method has a disadvantage that it takes time to isolate single cells, so that it is difficult to handle many samples at one time. To overcome this disadvantage, the use of leaf discs was attempted. The result showed that by this method stomatal effect was minimized, and measurement of many samples (more than 10 samples for one measurement) can be made at one time, using small discs (0.69 cm^2) under relatively easy control of environmental condition, although the method does not directly reflects natural condition as the chamber-airflow method does.

Discs taken from sample leaves using cork borer were floated on distilled water, and after 1 hr of pre-illumination (3 klux) their photosynthesis was measured by Warburg apparatus. Leaf discs were floated on phosphate buffer solution (0.5 ml, pH 7.0) in a Warburg vessel with their lower surface contacting the solution. ^{14}C-$NaHCO_3$ of 0.5 ml (24–50

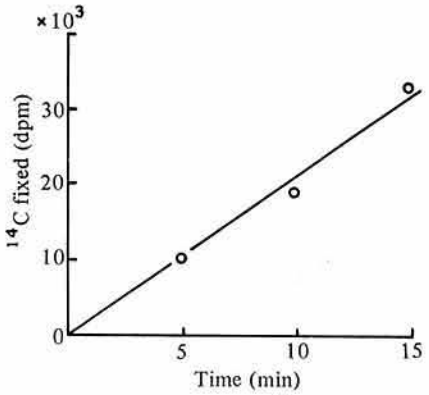

Fig. 3. Time course of ^{14}C-bicarbonate uptake
Leaf discs were adapted for 3 min under light (110 klux). ^{14}C-Bicarbonate concentration was 5 mM and temperature was 30°C

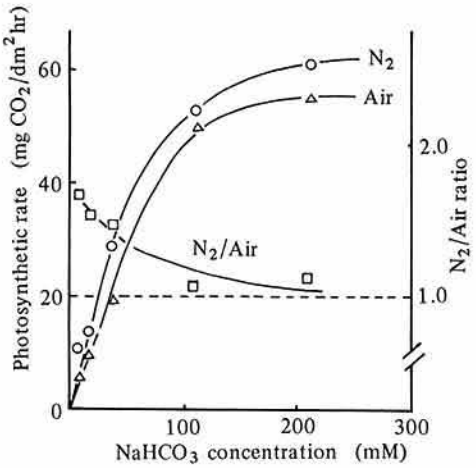

Fig. 4. Effect of sodium bicarbonate concentration on the magnitude of photorespiration (N_2/Air ratio, □) by leaf discs. ^{14}C-Bicarbonate uptake was measured for five min following three min adaptation in light (110 klux) under N_2 (○) and air (△) atmosphere. Temperature was 30°C

Table 2. Stability of leaf disc photosynthesis

Time (hr) after punching	Photosynthetic rate (mg CO_2/dm^2 hr) in leaf disc
0	33.5 (100.0)
1	35.7 (106.5)
3	27.2 (81.2)
6	24.4 (72.9)
24	26.7 (79.6)
48	23.7 (70.6)

Note: Leaf discs were floated on the distilled water under light (3,000 lux) at room temperature. At each time indicated on the table, discs were used for the measurement of the photosynthetic rate.

μCi/mmol) was pipetted into the side room of the vessel, and the vessel was closed. After 3 min of pre-illumination, $NaHCO_3$ in the side room was poured into the main room to make photosynthesis started. After a certain period, discs were taken out and immersed into 10% acetic acid to stop the photosynthesis. ^{14}C-uptake increased linearly within 15 min. (Fig. 3).

Stability of leaf disc photosynthesis is shown in Table 2. The discs maintained 80% and 70% of their original activity after 24 and 48 hrs, respectively, even at room temperature.

Light-saturation of photosynthesis of leaf discs occurred at about 30 klux with saturated concentration of $NaHCO_3$, and at 20 klux with unsaturated $NaHCO_3$ concentrations. The photosynthesis of discs showed a saturation at the concentration of $NaHCO_3$ of 110 mM, and the optimum temperature was 30–35°C.

Inhibition of photosynthesis by oxygen[10]

Photosynthesis is reversibly inhibited by oxygen. Effects of some environmental factors on that reaction were studied. Inhibition of disc photosynthesis by oxygen was small at saturated $NaHCO_3$ concentration, showing the N_2/Air ratio of about 1.1, but the inhibition

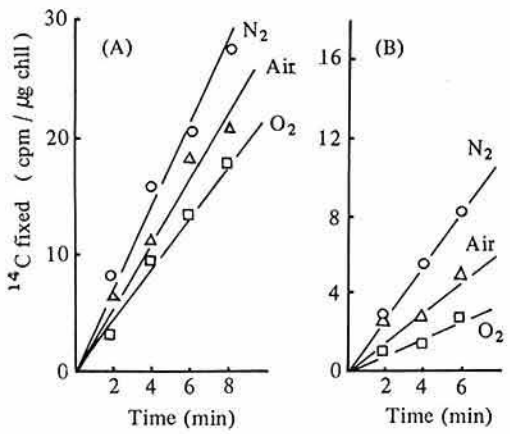

Fig. 5. Effect of oxygen on the photosynthetic rate by single cells isolated from tea leaves.
$NaH^{14}CO_3$ (A : 6.67 mM, B : 1.33 mM) was added following five min adaptation in light (40 klux) under various atmospheres (N_2 : ○, air : △, O_2 : □). Temperature was 31°C

increased with the decrease in $NaHCO_3$ concentration, giving the N_2/Air ratio higher than 1.5 i.e., about 40% inhibition of photosynthetic rate.

The remarkable inhibition of photosynthesis at low concentration of $NaHCO_3$ was also observed with single cells (Fig. 5). This inhibition occurred more remarkably in oxygen gas than in air, indicating that the inhibition depends on oxygen concentration.

Furthermore, it was shown that under the conditions which promote photosynthetic rate, such as strong light intensity or high temperature, the oxygen inhibition took place more remarkably.

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