Photosynthesis Measurement by Oxygen Electrode as a Simple Bioassay Method

By HITOSHI SAKA* and HIDEO CHISAKA**

* Department of Environmental Biology, National Institute of Agro-Environmental Sciences
(Yatabe, Ibaraki, 305 Japan)
** Agriculture, Forestry and Fisheries Research Council Secretariat, Ministry of Agriculture, Forestry and Fisheries
(Kasumigaseki, Tokyo, 100 Japan)

Photosynthetic reaction performed in chloroplasts of green plants is composed of a photochemical reaction (light reaction) occurring in the thylakoid membrane system and an enzymatic CO_2 fixation pathway (dark reaction) in soluble stroma (Fig. 1). The light reaction is the processes of light-dependent productions of ATP and $NADPH_2$ and of oxidation of water to evolve oxygen. The dark reaction reduces CO_2 to produce carbohydrate by utilizing ATP and $NADPH_2$. The both reactions are closely linked with each other.

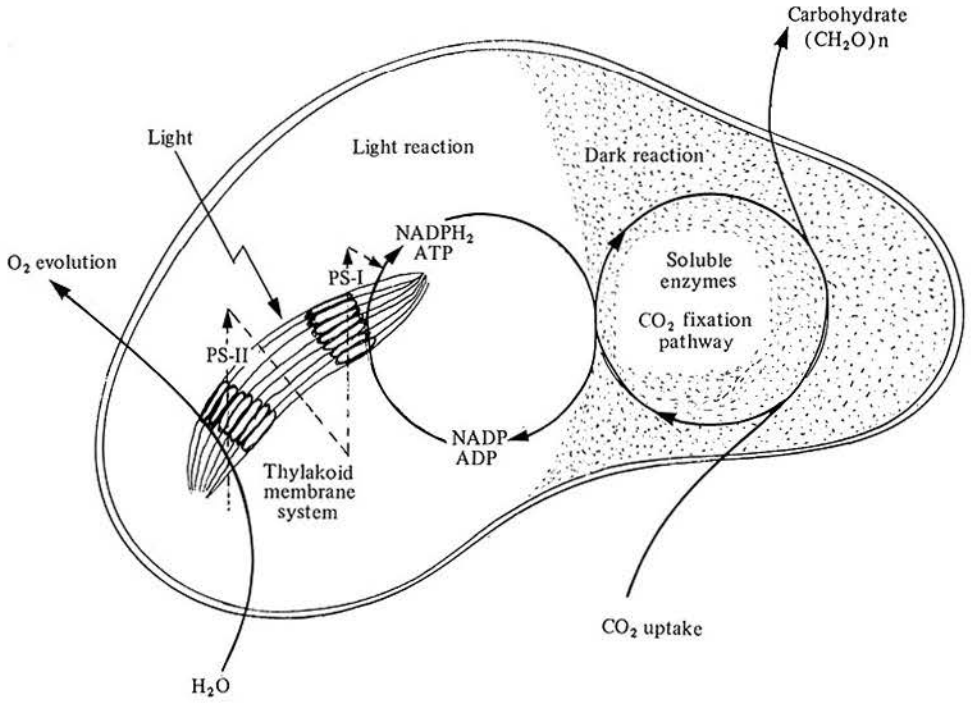

Fig. 1. Photosynthetic function in chloroplast
Light reaction which is located in the thylakoid membranes converts light energy into chemical energy (ATP, $NADPH_2$) via two photochemical systems (PSI and PSII). Dark reaction in the soluble stroma utilizes these ATP and $NADPH_2$ to form the metabolic intermediates of CO_2 fixation pathway and finally carbohydrate.

Accordingly, the photosynthetic rate of plants can be determined basically by measuring the rate of CO_2 uptake by leaves, or the rate of O_2 evolution from leaves. Measurement of CO_2 uptake by using the infrared gas analyzer has usually been employed so far to express photosynthetic rates. As to the measurement of O_2 evolved, it has been re-

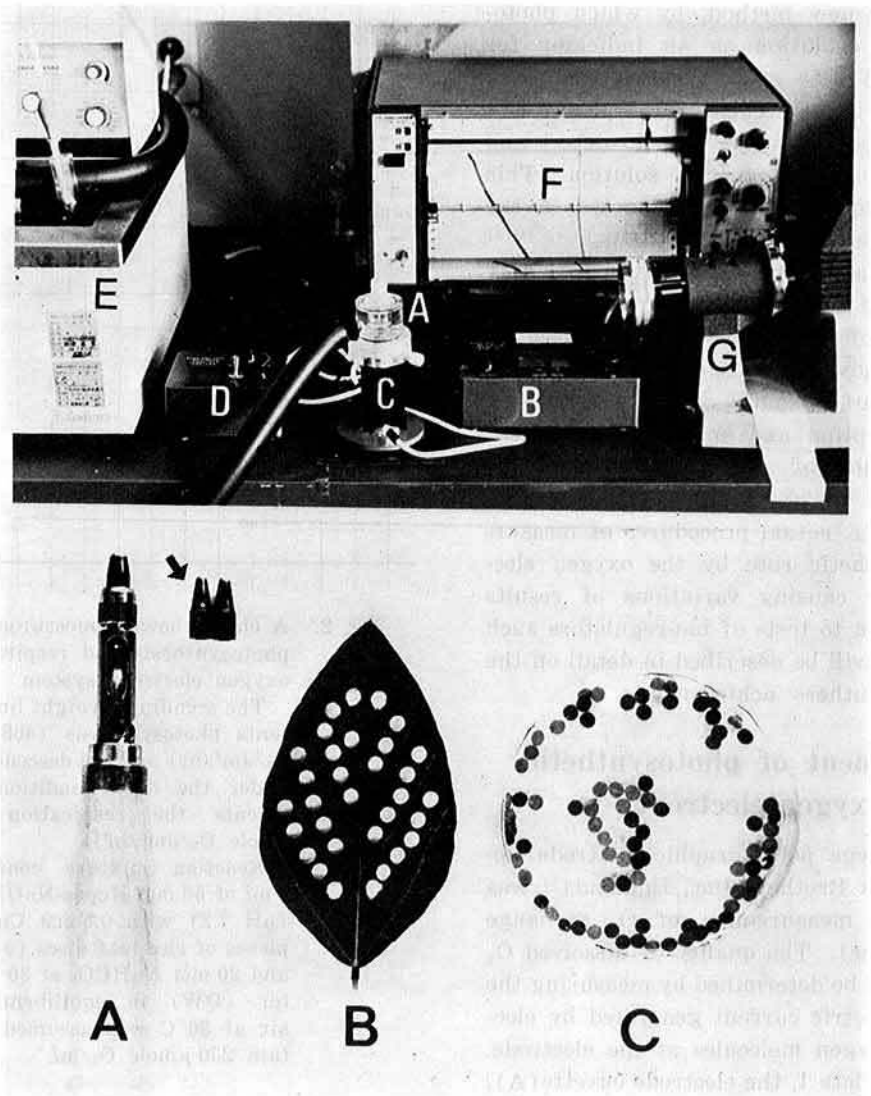

Plate 1. Oxygen electrode system (upper) and leaf disc preparation by screw punch (lower)
Upper: A: oxygen electrode, B: polarizing circuit, C: magnetic stirrer, D: power stabilizer of magnetic stirrer, E: water bath connected with oxygen electrode jacket, F: recorder, G: light sourse (slide projector, 1 kW).
Lower: A: screw punch with substitutive tips (arrow), B: soybean leaf after leaf discs (4.0 mm) were taken by screw punch, C: vacuum infiltrated soybean leaf discs in the Hepes-NaOH buffer.

garded extremely difficult to analyze small changes of O_2 concentration caused by photosynthesis in the gas phase, because O_2 presents at a high concentration (ca. 20%) in the atmosphere.

Recently, a new method, by which photosynthetic O_2 evolution as an indicator for photosynthetic rate can be determined in a water phase, was developed by utilizing the oxygen electrode which is able to detect and measure dissolved O_2 in the solution. This method has now come to be adopted in the research fields on photosynthesis,[1,3,4,5,11,13] This method has many advantages such as easy procedure and operation, relatively high reproducibility of measurement and low cost.[4,8] This is also advantageous on the elimination of the effect of stomatal diffusion resistance on CO_2 absorption and on the measurement of O_2 exchanges of small part of plant organs.[4,5]

In this paper, actual procedures of measuring photosynthetic rate by the oxygen electrode, factors causing variations of results and application to tests of bio-regulators such as herbicides will be described in detail on the basis of the authers' achievements.

Measurement of photosynthetic rate by oxygen electrode

The clark-type polarographic electrode apparatus (Rank Brothers Inc., England)[1] was used for the measurement of O_2 exchange (Plate 1, upper). The quality of dissolved O_2 in solution can be determined by measuring the changes of electric current generated by electrolysis of oxygen molecules at the electrode. As shown in Plate 1, the electrode cuvette(A), a cylinder with the diameter of 1.5 cm (maximum cuvette volume of 8 m*l*), is covered by a water jacket to be connected with water bath (E) circulating water in a constant temperature and is separated by a thin Tefron membrane (25 μm) from the electrode well filled with saturated KCl at the bottom. This electrode cuvette is placed on a magnetic stirrer(C) equipped with a power stabilizer (D). Oxygen diffusing from the cuvette solution through the membrane is reduced at the platinum surface (cathode) and a current flows through the polarizing circuit(B). The current is recorded on a recorder(F).

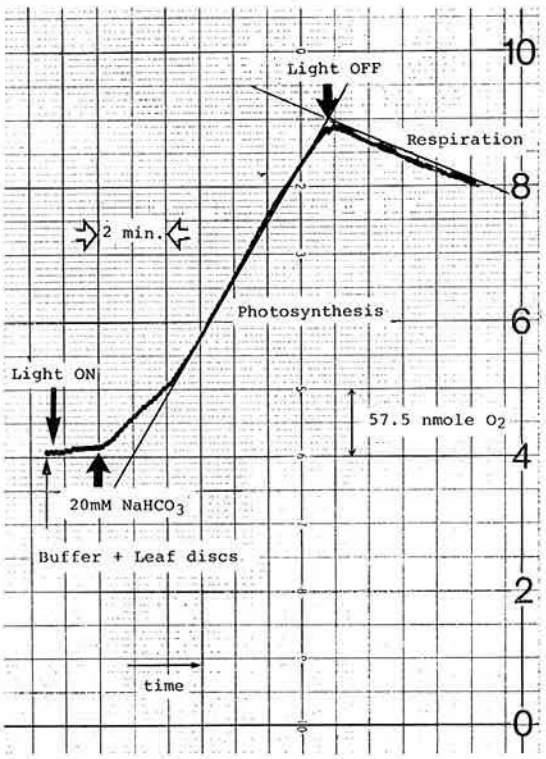

Fig. 2. A chart showing measurements of photosynthesis and respiration by oxygen electrode system
The scending straight line represents photosynthesis (498.1 μmole $O_2/dm^2/hr$) and the descending one under the dark condition represents the respiration (117.2 μmole $O_2/dm^2/hr$).
Reaction mixture consists of 3 m*l* of 50 mM Hepes-NaOH buffer (pH 7.2) with 0.5 mM $CaSO_4$, 25 pieces of rice leaf discs (ϕ3.0 mm) and 20 mM $NaHCO_3$ at 30°C. Water (DW) in equilibrium with air at 30°C was assumed to contain 230 μmole O_2/m*l*.

Leaf discs of a definite size were cut from fully expanded upper leaves, omitting the major vein, by using a screw punch (available for leathercraft use) (Plate 1, lower). The discs were immediately placed into the 50 mM

Hepes-NaOH buffer solution (pH 7.2) containing 0.5 mM $CaSO_4$, and vacuum-infiltrated, so that they were completely submerged in the solution within a few minutes. The disc sample thus prepared was placed under fluorescent light of ca. 6 klux until use.

The measurement of the rate of photosynthesis and respiration was carried out as follows. A necessary number of the discs of a definite diameter with 3 m*l* of the buffer solution mentioned above were put into the electrode cuvette (A in Plate 1, upper) at 30°C, and then the cuvette was capped and illuminated (ca. 70 klux, G in Plate 1, upper) from one side. After the pre-illumination for a few minutes, the $NaHCO_3$ (to make 20 mM final concentration) was added through a pinhole of the cap to get photosynthetic reaction started. After given hours (6 to 8 min), the light was turned off and respiratory O_2 uptake was measured (Fig. 2).

Assay of the metabolic inhibitors like herbicide etc. was made as follows. After the measurement of photosynthetic rate without inhibitors (the control), the discs and buffer solution were removed from the electrode cuvette. Then, the cuvette was washed with distilled water (DW) several times. After that, using new discs taken from the same disc sample and the buffer solution containing an inhibitor, the similar measurement as above was made. Comparison of the values obtained with and without the inhibitors shows the degree of the inhibiting activity as a percent of the control.[8,9] That is, the effect of the inhibitors on photosynthesis in this experiment was judged from the inhibition expressed within a very short period after the addition of the chemicals.[11]

Chlorophyll content was determined as follows. Leaf discs (for example, 10 discs with 3.0 mm diameter) after being used for measuring photosynthetic rate were placed into a test tube together with 5 m*l* of 80% acetone. The test tube was tightly sealed by silicone cap and allowed to stand in darkness at room temperature until disc-bleaching for about two days. The extract was submitted to double beam spectrophotometer and chlorophyll (a + b) content was calculated by Mackinney method.[6]

Establishment of photosynthesis measurement system

When leaf discs of 3.0 mm in diameter were used, their photosynthetic and respiratory rates increased in parallel with the increase of the number of discs used up to 25 pieces.[9] Then, different groups of discs with different diameters (1.5–6.0 mm) were prepared and the number of discs to be used for each group was adjusted so as to reach the almost same total leaf area 141.3 mm^2, which corresponds to the area of 20 discs of 3.0 mm in diameter. Photosynthetic rate and effect of a photosynthesis-inhibitory herbicide, atrazine, were examined with each group.[9]

The result given in Table 1 shows clearly that photosynthetic rate and its inhibition

Table 1. Effect of leaf disc size on photosynthesis and its inhibition by atrazine (3.3×10^{-5} M)

Diameter of leaf discs (mm)	Number of discs	Total leaf area (mm^2)	Total circumference length (mm)	Photosynthetic O_2 evolution (μmole $O_2/dm^2/hr$)		
				Control	+ Atrazine	% of control
1.5	80	141.3	376.8	303.4	164.8	54.3
2.0	45	141.3	282.6	350.6	177.8	50.6
3.0	20	141.3	188.4	350.0	178.5	51.6
4.0	11	138.2	138.2	310.0	160.8	51.9
5.0	7	137.4	109.9	366.2	193.0	52.7
6.0	5	141.3	94.0	300.4	164.8	54.8

Leaf discs were prepared from the fully expanded top leaf blade at 7.8th leaf stage of rice plant (cv: Koshihikari) grown in the field in summer.

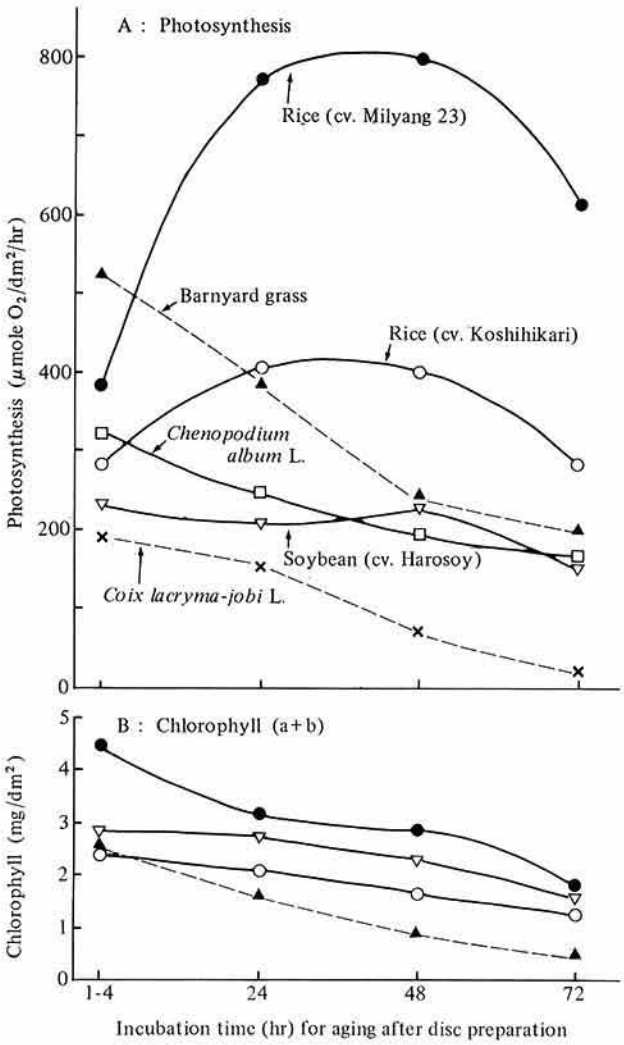

Fig. 3. Time-dependent changes of photosynthesis (A) and chlorophyll (a+b) content (B) during the aging of leaf discs in various plants

The discs were prepared from the fully expanded top leaf blade of each plant grown in the field in summer.

were not influenced by the size of discs. It implies that penetration (absorption) of CO_2 and atrazine into leaf tissues is not dependent on the extent of cut edge area of discs, but on the total area of both surfaces of the discs. The similar result was obtained with leaf discs taken from soybean plants.[9]

Photosynthetic rate varies markedly with leaf position and age of individual leaves in a plant. It was found out that the rate is also strongly influenced by the environmental conditions such as light and temperature during the plant growth.[10,12] In addition, the photosynthesis in the prepared leaf discs showed a pronounced change with the elapse of time after submerged in the buffer solution, i.e. with the aging of the discs (Fig. 3).[12] When the submerged leaf discs taken from rice

Table 2. Photosynthetic rates of some selected plant species and their *inhibition* inhibition by atrazine (3.3×10^{-5} M)

Plants	Photosynthesis (μmole O_2/dm^2/hr)			
	Control	+Atrazine	%	Chlorophyll*
C_3 plant				
Rice (cv. Nihonbare)	266.0	130.9	49.2	3.44
Soybean (cv. Enrei)	228.1	102.6	45.0	2.71
Chenopodium album L.	294.9	84.0	28.5	3.29
Solidago altissima L.	173.9	106.5	61.3	2.37
Phytolacca americana L.	213.1	87.9	41.2	3.30
Artemisia princeps PAMP.	183.1	56.3	30.7	3.14
C_4 plant				
Echinochloa oryzicola Vasing	433.3	256.5	59.2	2.05
Grain sorghum	107.1	—	—	4.99
Corn (cv. Golden cross bantum)	128.2	50.5	39.5	2.41
Digitaria ciliaris Koel.	280.8	96.3	34.3	3.01
Coix lacryma-jobi L.	166.0	55.8	33.6	2.56

* Chlorophyll content in mg/dm^2
Experiments were performed by using leaf discs taken from plants grown in the field in mid-summer.

plants grown in the field in summer were kept under 6–8 klux of illumination, for example, their photosynthetic rate showed a marked increase within 4–24 hr and then gradually decreased. In the discs under dark condition, or in the discs from the plant grown in winter season, however, such an increase of photosynthetic rate never occurred.[12] The increase under the light was hardly influenced by temperature (4–27°C).[12] In other crops, photosynthetic rate of discs decreased with the aging of discs[8,10] (Fig. 3). The transition of chlorophyll content was independent of such a dramatic change in the photosynthetic rate (Fig. 3).

These results indicate that photosynthetic O_2 evolution rate by oxygen electrode method shows a wide variation, depending on conditions of plant growth and of leaf discs prepared. Therefore, careful attention must be paid to this fact when absolute values of photosynthetic rate are compared among species or varieties.

Table 2 shows photosynthetic rate and effect of atrazine on different plant species.[9] Although further examinations on the above mentioned fact will be needed, it can be said that there exist conspicuous difference among species. The photosynthetic rates of C_4 plants were generally similar to or rather lower than the rates of C_3 plants, except *Echinochloa oryzicola* Vasing (barnyardgrass). This result is quite different from the results[7] so far obtained in which the photosynthetic rates were determined by measuring CO_2 uptake by leaves among plant species. These contradiction between the results of photosynthetic rate measured by O_2 evolution and by CO_2 uptake, remains to be clarified by further studies. The treatment of atrazine reduced the photosynthetic rate to 30–60% of the control in each species, irrespective of C_3 and C_4 plants classification. Table 2 also shows a very low correlation between the photosynthetic rate and chlorophyll content.

Assay of bio-regulators by oxygen electrode

As described above, the activity of bio-regulators to inhibit photosynthesis is expressed by relative values of photosynthetic rate (percent of the control). Therefore, in the assay of bio-regulators by this method, absolute values of photosynthetic rate offer no problem. Variations of photosynthetic rate,

depending on the growth environment of the plants and aging of leaf discs etc, are not involved as a fatal factor in the assay. Thus, this method can be regarded as a simple and handy assay method for chemicals.

Furthermore, it is noteworthy that the selective action of atrazine did not appear in the short time assay as shown in Table 2, inspite of the fact that in practice, atrazine shows a remarkable selectivity in herbicidal action between maize or sorghum and other plant species including soybean. The similar result was shown with propanil applied to rice (Table 3). It implies that any plant species can be used for the assay, as far as a given species is used for a given series of experiments.

Table 3. Photosynthesis inhibition by herbicides measured by the oxygen electrode method using leaf discs of rice plants (CV.: Milyang 23)

Herbicides	PI_{50} (M)	HI_{50} (M) (cited from literatures[11])
Hill reaction inhibitor (A)		
DCMU (PC)	4.5×10^{-6}	3.3×10^{-7}
prometryn (Tech)	1.5×10^{-5}	3.6×10^{-7}
simetryn (PC)	2.0×10^{-5}	4.9×10^{-7}
metribuzin (Tech)	2.5×10^{-5}	7.0×10^{-7}
linuron (50% WP)	3.8×10^{-5}	2.0×10^{-7}
atrazine (47.5% WP)	5.0×10^{-5}	4.6×10^{-7}
propanil (PC)	7.2×10^{-5}	2.2×10^{-6}
simazine (50% WP)	1.3×10^{-4}	7.0×10^{-6}
pyrazon (65% WP)	1.3×10^{-4}	7.0×10^{-6}
S-52* (Tech)	1.5×10^{-4}	2.4×10^{-7}
swep (40% WP)	1.7×10^{-4}	3.3×10^{-6}
pentanochlor (PC)	1.8×10^{-4}	1.1×10^{-6}
bentazon (PC)	4.3×10^{-3}	4.0×10^{-5}
Respiration inhibitors (B)		
dinoseb (Tech)	6.6×10^{-4}	1.7×10^{-5}
antimycin A	7.0×10^{-4}	3.0×10^{-5}
KCN	8.5×10^{-4}	$>10^{-2}$
PCP (Tech)	1.2×10^{-3}	1.5×10^{-5}
Mixed action inhibitors**		
bromacil (Tech)	1.2×10^{-4}	1.4×10^{-6}
ioxynil (Tech)	1.8×10^{-3}	7.0×10^{-7}
CIPC (45.8% EC)	1.1×10^{-3}	2.3×10^{-4}
methazol (75% WP)	2.9×10^{-3}	- - - -
Electron acceptors		
paraquat (PC)	$10^{-2}\sim10^{-1}$	- - - -
diquat (PC)	$10^{-2}\sim10^{-1}$	- - - -

* N-4-(4-methylphenethyloxy) phenyl-N-methylurea
PC: active ingredient with purity more than 98%, Tech: active ingredient with purity less than 98%, WP: wettable powder, EC: emulsifiable concentrate, PI_{50}(M): molar conc. for 50% inhibition of photosynthesis, HI_{50} (M): molar conc. for 50% inhibition of Hill reaction activity.

** (B)+(A) action or (B)+nucleic acid synthesis inhibition

Of more than 50 kinds of herbicides and metabolic inhibitors used in this study, those which inhibited photosynthetic O_2 evolution rate are shown in Table 3 with their concentrations for inhibition (PI_{50}).[11] The concentrations for 50% inhibition of Hill reaction activity (HI_{50}) which were determined by broken chloroplast suspension are cited from literatures and also shown in Table 3.[11]

Our data shown in Table 3 were obtained by using flag leaf discs of rice at the flowering stage (CV. Milyang 23). Although the PI_{50} values were as 5–200 times as HI_{50} values, a very high positive correlation was found between PI_{50} and HI_{50}, when the results of bentazon and ioxynil were neglected. Moreover, although the PI_{50} value may possibly vary depending on plant species and their growth stages,[3] all the chemicals used in this study including those shown in Table 3 can be divided into 4 groups on the basis of the level of PI_{50}, with the exception of some chemicals (2,4-DNP, bentazon, etc.) as shown in Table 4. It is expected that this classification may serve as a guide to identify mode of action and even a part of action mechanism of unknown chemicals more simply than the Hill reaction inhibition test. Needless to mention, chemicals which show no effect on photosynthesis (Group 4 in Table 4) must be tested by other assay method.

Conclusion

The technique to measure photosynthetic activity by taking advantage of the oxygen electrode which has been widely employed in fields of physiology, biochemistry and enzyme chemistry is regarded as a new simple system to assay photosynthesis inhibitory chemicals

Table 4. Classification of herbicides based on photosynthesis inhibition measured by the oxygen electrode method

Group	Range of PI_{50} (m)	Herbicides and metabolic inhibitors
1	$4.5\times10^{-6}\sim1.8\times10^{-4}$	Hill reaction inhibitors: see the list in Table 3 except bentazon
2	$6.6\times10^{-4}\sim1.2\times10^{-3}$ $10^{-3}\sim10^{-2}$	Respiration system inhibitors: see the list in Table 3 Mixed action inhibitors: see the list in Table 3
3	$10^{-2}\sim10^{-1}$	Electron acceptors: see the list in Table 3
4	No inhibition even in 10^{-3} m	Inhibitors of protein and nucleic acid synthesis, mitosis, auxin action etc.: alachlor, butachlor, thiobencarb, diphenamid, molinate, piperophos, alloxydim, sethoxydim, asulam, metolachlor, trifluralin, pendimenthalin, 2, 4-D, 2, 4, 5-T, MCPA, chloramben, dicamba, diclofop-methyl, amitrol, bifenox, nitrofen, oxadiazon, chlometxynil, pyrazolate, glyphosate, SL-501

in a non-destructive manner, unlike the measurement of Hill reaction activity by the macerated chloroplast preparation. However, for the classification of photosynthetic activity, a detailed study on the various factors causing variations of O_2 evolution from leaf discs will urgently be needed. A new apparatus, recently developed by an improvement of this oxygen electrode apparatus, which can measure simultaneously both photosynthetic rate and chlorophyll-a fluorescence[2] may throw a new light to the improvement of our proposed simple bioassay technique.

References

1) Delieu, T. J. & Walker, D. A.: An improved cathode for the measurement of photosynthetic oxygen evolution by isolated chloroplasts. *New Phytol.*, 71, 201–225 (1972).
2) Delieu, T. J. & Walker, D. A.: Simultaneous measurement of oxygen evolution and chlorophyll fluorescence from leaf pieces. *Plant Physiol.*, 73, 535–541 (1983).
3) Hyeon, S.-Be. et al.: A simple bioassay for chemicals active to the photosynthetic or respiratory systems of plants. *Agric. Biol. Chem.*, 46, 811–812 (1982).
4) Ishii, R. et al.: On a method for measuring photosynthesis and respiration of leaf slices with oxygen electrode. *Jpn. J. Crop. Sci.*, 46, 53–57 (1977).
5) Jones, H. G. & Osmond, C. B.: Photosynthesis by thin leaf slices in solution. I. Properties of leaf slices and comparison with whole leaves. *Aust. J. biol. Sci.*, 26, 15–24 (1973).
6) McKinney, G.: Absorption of light by chlorophyll solutions. *J. Biol. Chem.*, 140, 315–322 (1941).
7) Matsunaka, S. & Saka, H.: C_3 and C_4 plants-classification and weed control. *Weed Res. Jpn.*, 22, 131–139, 177–183 (1977) [In Japanese].
8) Saka, H. & Chisaka, H.: A simple method for the detection of photosynthesis inhibitors by oxygen electrode. *Weed Res. Jpn.*, 26, 79–84 (1981) [In Japanese].
9) Saka, H. & Chisaka, H.: Determination of leaf disc photosynthesis and respiration with oxygen electrode and its application for a simple assay of herbicidal activities. *Weed Res. Jpn.*, 26, 304–310 (1981) [In Japanese with English summary].
10) Saka, H. & Chisaka, H.: On the photosynthesis of higher plants by oxygen electrode. I. Changes of the photosynthesis during the growth and the aging of leaf discs in plants. *Rep. Tokai Br. Crop Sci. Soc. Jpn.*, 93, 41–44 (1982) [In Japanese].
11) Saka, H. & Chisaka, H.: Determination and comparison of photosynthesis inhibition by herbicides with oxygen electrode. *Weed Res. Jpn.*, 27, 63–70 (1983) [In Japanese with English summary].
12) Saka, H. & Chisaka, H.: Some environmental factors affecting photosynthetic rate of leaf discs. *Jpn. J. Crop Sci.*, 52 (Extra issue 1), 217–218 (1983) [In Japanese].
13) Yamashita, T. et al.: The simultaneous measurement of O_2-evolving and CO_2-fixing activities in fresh leaves. *J. Fac. Agr., Kyushu Univ.*, 22, 107–118 (1978).

(Received for publication, August 22, 1984)