EVALUATION OF PHOTOSYNTHETIC CAPACITY IN MICROPROPAGATED PLANTS BY IMAGE ANALYSIS

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1. Introduction

In micropropagation, *in vitro* environmental conditions (i.e., environmental conditions surrounding plantlets within culture vessels such as light conditions, temperature, and gaseous composition), have an important role in plantlet growth. Normally, *in vitro* environmental conditions cannot be controlled directly; instead, they are largely determined by regulated culture conditions outside the vessel. Therefore, culture conditions should be optimized for plantlet growth. It is necessary for optimization of culture conditions to understand relationships between culture conditions and *in vitro* plant growth, physiological state, or both. *In vitro* environmental conditions may change with plantlet growth during culture because the plantlet itself affects them. Therefore, non-destructive evaluation of the growth of micropropagated plantlets and their physiological state without disturbing the *in vitro* environmental conditions is desirable for investigating these relationships and considering their dynamics.

Recent studies revealed that *in vitro* cultured chlorophyllous plantlets had photosynthetic ability but their net photosynthetic rates were restricted by environmental conditions [1]. The photosynthetic properties of plantlets *in vitro* depend on culture conditions, including light intensity [2], the degree of air exchange between a vessel and the surrounding air [3], and the sugar content in the medium [4]. Photoautotrophic micropropagation which is micropropagation with no sugar added to the medium has many advantages, especially in plantlet quality [1]. For successful photoautotrophic micropropagation, *in vitro* environmental conditions should be properly controlled to enhance photosynthesis of the plantlets by manipulation of culture conditions. Successful photoautotrophic micropropagation also requires knowledge of when cultures should transit from photomixotrophic into photoautotrophic [1]. An understanding of changes in photosynthetic properties of cultured plantlets during the culture period is essential to optimize culture conditions for photoautotrophic culture to obtain high-quality plantlets.

It is difficult to evaluate photosynthetic properties of plantlets non-destructively. Carbon dioxide gas exchange rates of plantlets *in vitro* can be estimated *in situ* by measurements of the concentration of CO_2 inside and outside the culture vessel, the degree of air

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exchange between the vessel and the surrounding air, and the head space volume in the vessel [5]. However, the estimated gas exchange rates are the rates per all plantlets within the vessel, and they should be converted to the rates per unit leaf area or unit dry weight for analysis of the photosynthetic properties. This requires estimation of leaf area or dry weight of plantlets in the vessel. In addition, it should be noted that the environmental conditions could be non-uniform in a culture vessel even under controlled culture conditions. In culture vessels, air movement is limited, and as a result, there may be gradients in humidity and/or CO_2 concentration within the vessels. In addition, vertical light intensity distribution exists in slender vessels like test tubes [6]. This might cause variations in the *in vitro* microenvironment around the cultured plants and consequently cause variations in photosynthetic capacity. This variation may affect uniformity in plantlet quality, especially when propagating by cuttings, such as for potato nodal cutting cultures. An understanding of variations in photosynthetic properties within cultured plantlets may be helpful for obtaining uniform-quality plantlets.

Chlorophyll fluorescence has been a useful tool for photosynthetic research. In recent years, the value of this tool in plant physiology has been greatly increased by the availability of suitable instrumentation and an increased understanding of the processes that regulate fluorescence yield [7]. It has enabled analysis of the photosynthetic properties of plant leaves, especially characteristics related to the photochemical efficiency of photosystem II. As chlorophyll fluorescence analysis is based on photometry, i.e., measurement of light intensity, it is a promising means of non-destructive estimation of photosynthetic capacity.

In this chapter, the methods for non-destructive evaluation of photosynthetic capacity are introduced, focusing on imaging of chlorophyll fluorescence. First, the principle of photosynthetic analysis based on chlorophyll fluorescence will be outlined, and the feasibility of imaging the chlorophyll fluorescence parameters for micropropagated plants from outside the culture vessels will be discussed. Other promising indices based on spectral reflectance for imaging the photosynthetic capacity of micropropagated plants will be also discussed. In addition, estimation methods for light intensity distribution inside culture vessels will be introduced in consideration of its influence on the photosynthetic properties of cultured plants.

2. Basics of chlorophyll fluorescence

Chlorophyll absorbs photons for use in the photochemical reaction of photosynthesis. Excited chlorophyll can re-emit a photon and return to its ground state, and this fluorescence is called chlorophyll fluorescence. Occasionally, it is also referred to as chlorophyll *a* fluorescence, since it is due to chlorophyll *a*. The analysis of chlorophyll fluorescence provides a powerful probe of the functioning of the intact photosynthetic system [8]. It especially enables us to obtain information on the functioning of photosystem II (PSII), since at room temperature chlorophyll fluorescence is predominantly derived from PSII [9]. Methods to analyze photosynthetic properties of leaves using chlorophyll fluorescence include a method using a saturating light pulse and another method based on induction kinetics (the Kautsky curve [10]). Here, the

former method, in which fluorescence is measured while varying PSII photochemical efficiency using a saturating light pulse, is more fully explained.

After dark adaptation treatment, the yield, Φ_F of fluorescence excited by very weak irradiance is expressed by the following equation:

$$\Phi_F = \frac{k_F}{k_F + k_D + k_T + k_P} \tag{1}$$

Where k_F, k_D, k_T, and k_P are rate constants for fluorescence, thermal dissipation, energy transfer to PSI and PSII photochemistry (electron transport), respectively.

As the portion of energy transfer is very small, k_T can be neglected in the above equation [7]. This fluorescence, which occurs when the primary electron acceptor, Q_A, is fully oxidized due to excitation by weak light just after dark adaptation, is referred to as *Fo*. Then, irradiation by a saturating light pulse (of very high intensity) leads to full reduction of Q_A (sometimes the condition is referred to as "closed"). The fluorescent yield, Φ_{Fm}, of maximum fluorescence *Fm*, determined under the saturating light pulse, is expressed by the following equation:

$$\Phi_{Fm} = \frac{k_F}{k_F + k_D + k_T} \tag{2}$$

From *Fo* and *Fm*, the maximum quantum yield of PSII, *Fv/Fm*, is estimated using the following equation:

$$\begin{aligned} Fv/Fm &= \frac{Fm - Fo}{Fm} \\ &= \left\{ \frac{k_F}{k_F + k_D + k_T} - \frac{k_F}{k_F + k_D + k_T + k_P} \right\} / \left\{ \frac{k_F}{k_F + k_D + k_T} \right\} \\ &= \left\{ 1 - \frac{k_F + k_D + k_T}{k_F + k_D + k_T + k_P} \right\} \\ &= \frac{k_P}{k_F + k_D + k_T + k_P} \end{aligned} \tag{3}$$

Fv/Fm is a measure of photoinhibition and has been used for photosynthetic capacity evaluation in photosynthetic research (e.g., [11]) and cultivar screening (e.g., [12]). Under light conditions without dark adaptation (hereafter, the light is referred to as actinic light to distinguish from the light for fluorescent measurements), the actual quantum yield of PSII, Φ_{PSII}, can be also estimated using the following equation:

$$\Phi_{\mathrm{PSII}} = \Delta F/Fm' = \frac{Fm' - F}{Fm'} \tag{4}$$

Where F is the fluorescence excited by the measuring light under the actinic light, and *Fm'* is the fluorescence excited by the measuring light while irradiating with the saturating light pulse (that is, when Q_A is fully closed) under the actinic light. As for the other parameters, photochemical quenching, *qp*, which shows the extent to which Φ_{PSII} is restricted by photochemical capacity at PSII, and indices of non-photochemical quenching, *qN* and *NPQ*, which are related to heat dissipation, can be derived by fluorescence measurement using a saturating light pulse. Also, the linear electron transport rate, *ETR*, can be estimated if the number of photons absorbed is known [13]. These parameters were reviewed by Maxwell and Johnson in detail [14]. The chlorophyll fluorescence parameters can be measured by a pulse amplitude modulation (PAM) fluorometer. In this fluorometer, the excitation light (pulsed light of low intensity; hereafter, measuring pulse) used to measure chlorophyll fluorescence is separately applied to the actinic light, which drives the photosynthetic light reaction [15]. Due to the selective pulse-amplification system, only fluorescence excited by the measuring pulse is recorded in the presence of the actinic light [15]. Although in some cases the parameters can be obtained non-destructively with PAM fluorometer, there are some limitations in the measurements, for example due to the short distance (10-15 mm) between the sensor probe of the fluorometer and the leaf surface.

3. Imaging of chlorophyll fluorescence for micropropagated plants

3.1. CHLOROPHYLL FLUORESCENCE IN *IN VITRO* CULTURED PLANTS

In research on micropropagation, the chlorophyll fluorescence parameter *Fv/Fm* has been used to evaluate photosynthetic capacity, though applications are limited to a few studies. The nutrient composition of the medium affects *Fv/Fm* of *in vitro* cultured *Pinus radiata* [16]. *Ex vitro* transfer for acclimatization causes a decrease in *Fv/Fm* of plantlets and the degree of the reduction in *Fv/Fm* depended on culture conditions [17,18]. In general, plants grown under low light intensity are more sensitive to photoinhibition caused by high light intensity [19]. Therefore, *Fv/Fm* of micropropagated plantlets may be subject to change according to culture conditions.

Table 1. Fv/Fm of potato plantlets of different sucrose content treatments (Exp.1). Reproduced from Ibaraki, Y. and Matsumura, K. (2004) [20].

	Fv/Fm	
	Average	CV*
30 g/L	0.795 b**	0.032 ab**
10 g/L	0.750 c	0.055 a
0 g/L	0.818 a	0.020 b

* Coefficient of variation in a single plantlet, ** Different letters within row show significant differences by Tukey multiple range test at 1% level

Table 2. Fv/Fm of potato plantlets of different sucrose content treatments (Exp.2).

	Fv/Fm	
	Average	CV*
30 g/L	0.77 a**	0.032 b**
0 g/L	0.72 b	0.115 a

* Coefficient of variation in a single plantlet, ** Different letters within row show significant differences by Tukey multiple range test at 1% level.

To investigate sensitivity of *Fv/Fm* to culture conditions, two experiments were conducted to determine *Fv/Fm* for potato plantlets cultured under various environmental conditions [20]. In one experiment, potato nodal cuttings were transplanted into glass tubes containing MS medium [21] with different contents of sucrose (30 g/L, 10 g/L, and 0 g/L). In the case of the sugar-free treatment, a hydrophobic Fluoropore® membrane filter (Milliseal®, Millipore®) was attached to the plastic cap of the glass tube to enhance gas exchange for photoautotrophic growth. In another experiment, *Fv/Fm* values of plantlets cultured in medium with 30 g/L sucrose or in sugar-free medium were compared under conditions where gas exchange was suppressed using normal plastic caps for both treatments. At the end of culturing (35d and 40d after transplanting for experiment 1 and experiment 2, respectively), plantlets were transferred *ex vitro*, and *Fv/Fm* was measured randomly for all measurable leaves of the plantlets using a PAM fluorometer (MINI-PAM, Walz, Germany) after a 60 min dark adaptation treatment. For each treatment, 8 plantlets were tested. Average *Fv/Fm* values were affected by culture conditions (Tables 1 and 2). Without promoting gas exchange of culture vessels, *Fv/Fm* values of plantlets cultured in sugar-free medium were lower than for plantlets in 30 g/L sucrose treatment, which is a conventional medium formulation. In contrast, plantlets cultured with sugar-free medium in culture vessels promoting gas exchange showed

higher *Fv/Fm* than plantlets cultured in medium containing 30 g/L sucrose, indicating a higher photochemical efficiency. Combined effects of enhanced gas exchange and omission of sucrose from the medium might improve photosynthetic capacity. In comparisons between sucrose-containing treatments (experiment 1), plantlets of the 10 g/L treatment showed a lower *Fv/Fm* than plantlets of the 30 g/L treatment, and also suppressed growth. Variations in *Fv/Fm* values were observed among the plantlets and the distribution patterns in a plantlet changed slightly with sucrose content (Figures 1 and 2).

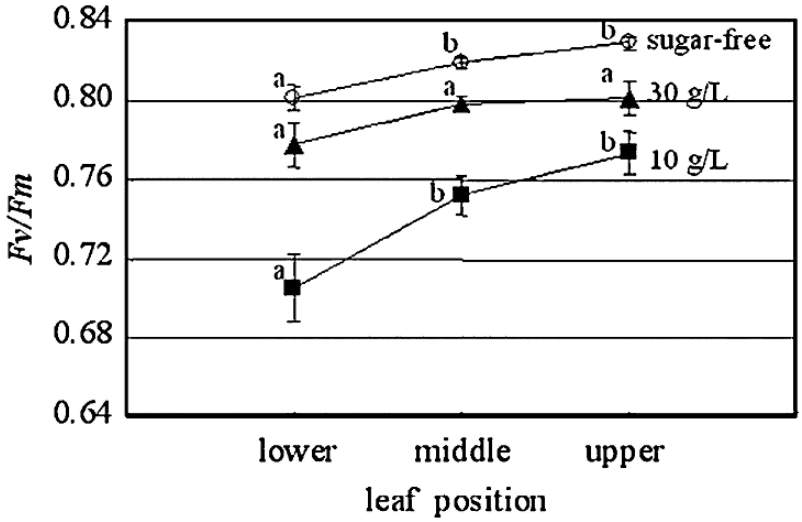

Figure 1. Fv/Fm distribution in potato plantlets cultured in MS medium contained 30 g/L, 10 g/L, or 0 g/L sucrose for 35 d (Exp. 1). Reproduced from Ibaraki, Y. and Matsumura, K. (2004) [20]. In sugar-free treatment, gas exchange was promoted by using the cap attached a hydrophobic Fluoropore (R) membrane filter. Lower 3 leaves, upper 3 leaves, and other leaves were classified into lower, upper, and middle in leaf position, respectively. Bar, SE. Different letters on graph lines show significant differences among leaf positions by Tukey multiple range test at 1% level.

These results suggest that *Fv/Fm* may change according to culture conditions, and that analysis of *Fv/Fm* for evaluation of photosynthetic capacity of cultured plantlets is effective for optimization of culture conditions.

Although *Fv/Fm* measurement is simple with the PAM fluorometer, there are some difficulties in measurements of plantlets within the culture vessel through the culture vessel wall. The measurement requires fixing the short distance between the sensor probe of the fluorometer and the leaf surface. This is a difficult requirement for plantlet leaves in a culture vessel. In addition, measurements for small leaves of plantlets with the fluorometer were subject to errors [20]. Non-destructive methods suited for micropropagated plants are desirable.

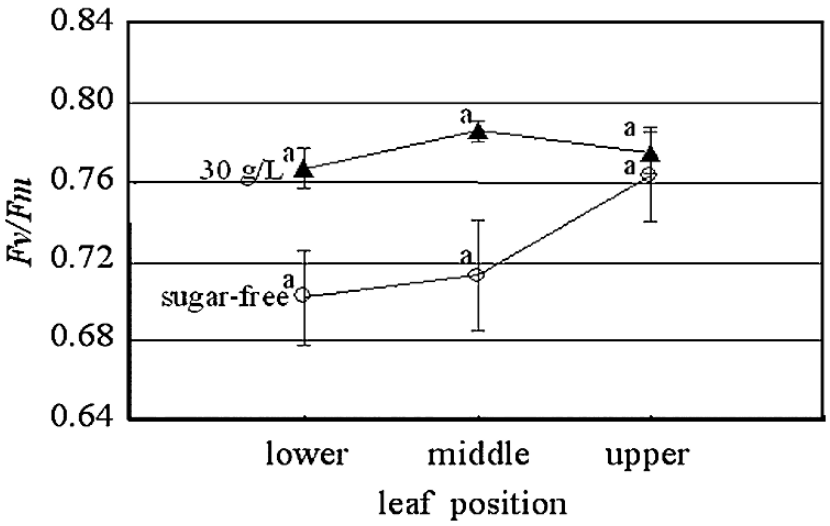

Figure 2. Fv/Fm distribution in potato plantlets cultured in MS medium contained 30 g/L or 0 g/L sucrose for 40 d (Exp. 2). Lower 3 leaves, upper 3 leaves, and other leaves were classified into lower, upper, and middle in leaf position, respectively. Bar, SE. Different letters on graph lines show significant differences among leaf positions by Tukey multiple range test at 1% level.

In a few studies, the chlorophyll fluorescence parameter *ΔF/Fm'*, determined under actinic light by PAM fluorometer, has been used in micropropagation research. Since *ΔF/Fm'* depends on the level of light irradiating a leaf, and it is difficult to know the exact irradiation level, careful consideration is required to determine photosynthetic properties from values of *ΔF/Fm'*. If the same light intensity were set for all plantlets tested, or if the light intensity distribution could be determined in culture vessels, *ΔF/Fm'* would offer information on plantlet photosynthetic capacity.

3.2. IMAGING OF CHLOROPHYLL FLUORESCENCE

Imaging of chlorophyll fluorescence was first reported by Omasa *et al*. [22]. In this study, the kinetics of chlorophyll fluorescence was analyzed using fluorescent images. For cultured callus and plantlets of *Daucus carota,* images of chlorophyll fluorescence induction were also used to analyze the development of photosynthetic apparatus [23]. Although several studies on chlorophyll fluorescence imaging had been reported, these primary studies required empirical calibration of the fluorescent signal using other methods, such as gas exchange, when the fluorescence images were converted to images of photosynthesis [24]. Recently, several reports showed the possibility of imaging chlorophyll fluorescence parameters based on a saturating light pulse method in order to obtain an image of photochemical efficiency over a leaf. Genty and Meyer [24] developed a method to construct the topography of the photochemical quantum yield of PSII and showed the effectiveness of the method by mapping the heterogeneous

distribution of photosynthetic activity after treatment with an herbicide, with abscisic acid, or during the course of induction of photosynthesis. Oscillations in photosynthesis initiated by a transient decrease in light intensity could be imaged over the leaf [25]. The sink-source transition of developing tobacco leaves was analyzed using images to evaluate electron transport rates [26]. Oxborough and Baker [7] proposed a method to image not only photochemical quantum yield but also non-photochemical quenching, assumed to correspond mainly to heat dissipation. In addition, Oxborough and Baker [27] developed a system to image *Fo* and consequently obtain an *Fv/Fm* image using a fluorescence microscope and a cooled charge coupled device (CCD) camera.

Chlorophyll fluorescence parameters can be imaged by considering the following points: 1) to distinguish between fluorescence and reflection by use of optical filters, and 2) to measure fluorescent quantum yield. Basic device arrangements for imaging of chlorophyll fluorescence include a light source for excitation of fluorescence, a camera, and optical filters for controlling excitation light intensity and separating reflected light and fluorescence. Normally, fluorescent intensity can be imaged as the grey level in each pixel by the camera. Therefore, it is necessary to convert fluorescent intensity into fluorescent yield to construct images mapping chlorophyll fluorescence parameters. If the irradiance distribution on a leaf were determined exactly, it would be possible to convert the fluorescent intensity to fluorescent yield. Actually, the conversion is done by controlling exposure time according to excitation light intensity [24], by imaging a fluorescent standard at the same time [25], or by imaging a reference leaf at the same time [20]. Recently, a PAM-based fluorescence imaging system (IMAGING-PAM, Walz, Germany) has been developed, which is now available. Although there have been few studies using the system to date, it is promising for non-destructive evaluation of plant photosynthetic properties.

For selection of cameras to image fluorescence, some considerations are required. In *F*v/*F*m measurements, *F*o is not intense because it is excited by very low irradiance, so highly sensitive cameras such as expensive cooled CCD cameras are needed. Although low-cost CCD cameras with high sensitivity have become available recently, the images acquired by most have reduced numbers of distinct grey levels. It is necessary to discuss whether the number of distinct grey levels in an image is sufficient for calculations used to derive chlorophyll parameters. In addition, gamma and auto-gain features of cameras should be carefully treated because they affect the relationship between light intensity and the pixel grey level value. The relationship between light intensity and the pixel grey level value in the image should be calibrated using a fluorescent or grey standard.

3.3. IMAGING OF CHLOROPHYLL FLUORESCENCE IN MICROPROPAGATED PLANTS

A system for imaging chlorophyll fluorescence of leaves of *Solanum tuberosum* plantlet from the outside of culture vessels and for estimating the fluorescence parameter *Fv/Fm* was developed [20].

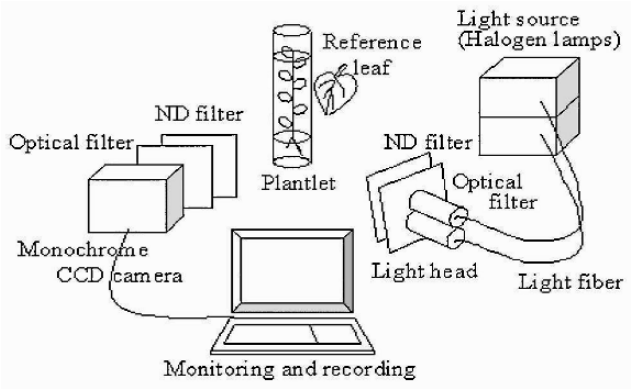

Figure 3. Schematic layout of a chlorophyll fluorescence imaging system. Reproduced from Ibaraki, Y. and Matsumura, K. (2004) [20].

Figure 3 shows the schematic layout of the system. The plantlets in glass test tubes were illuminated by a halogen lamp with a light fiber (HL-150, Hoya-Schott, Japan), and the light intensity for fluorescence excitation was controlled by neutral density filters (S-73-50-3,-13, Suruga, Japan). Fluorescence was imaged by a highly sensitive monochromatic CCD camera (WAT-120N, Watec, Japan) with long path filters. *Fv/Fm* was estimated from the *Fo* image, which was a fluorescent image acquired under low intensity illumination (0.15 μmol m^{-2} s^{-1}) after a 60 min dark adaptation treatment, and the *Fm* image, which was then acquired under high intensity illumination (2500 μmol m^{-2} s^{-1}). A detached *Epipremnum aureum* leaf, with a predetermined *Fv/Fm*, was imaged together as a reference leaf, and used to calibrate the fluorescence image. The *Fv/Fm* image (I_{FvFm}) was constructed as a pixel-by-pixel calculation of the *Fo* image (I_{Fo}) and the *Fm* image (I_{Fm}) by the following equation:

$$I_{FvFm} = \frac{\left(I_{Fm} - kI_{Fo}\right)}{I_{Fm}} \tag{5}$$

Where, *k* is a coefficient that is used to convert fluorescent intensity into fluorescent yield and was determined so as to fit the estimated *Fv/Fm* of the reference leaf by equation 1 to the *Fv/Fm* measured before imaging by the fluorometer (MINI-PAM, Walz, Germany).

Figure 4 shows examples of chlorophyll fluorescence images, and *Fv/Fm* images derived from them, of potato plantlets using the system. For a few leaves of the plantlets, *Fv/Fm* could be imaged at the same time. Therefore, using images acquired repeatedly after dark-adaptation treatment, the *Fv/Fm* distribution in an individual plantlet could be determined. Changes in *Fv/Fm* of an individual leaf over a culture period could also be detected using the system. Figure 5 shows the changes in *Fv/Fm* of the 5th leaf determined by the fluorescence imaging system developed. The leaf just expanded (14 d after transplanting) showed a lower *Fv/Fm* (<0.8). Then, *Fv/Fm* increased and decreased

again after a peak at 14 d after leaf expansion. This was a reasonable pattern in *Fv/Fm* changes, since a decline of *Fv/Fm* was reported in young leaves and older leaves [28]. The system enabled gathering of information on photosynthetic capacity of cultured plantlets from the outside of culture vessels non-destructively. The system should be useful for optimizing culture conditions.

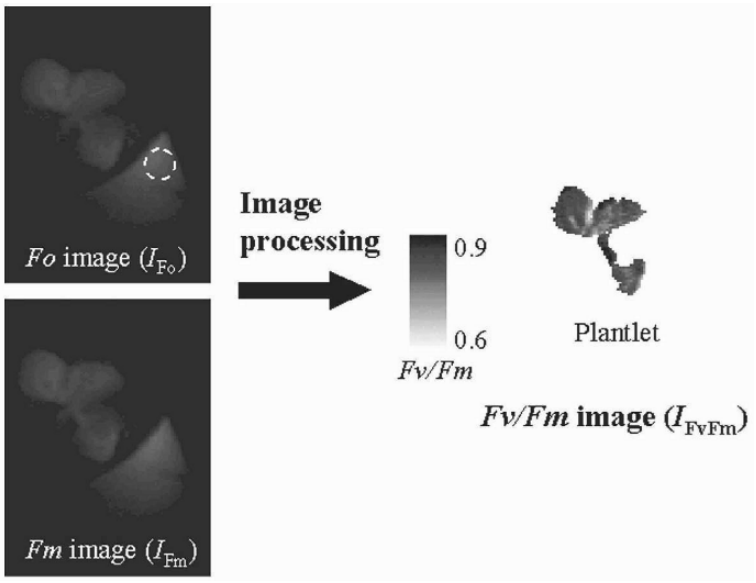

Figure 4. An example of Fv/Fm images constructed from Fo image and Fm image acquired by the chlorophyll fluorescence imaging system. Reproduced from Ibaraki, Y. and Matsumura, K. (2004)[20]. A circle in Fo image is an area to be used as the reference in the potato leaf.

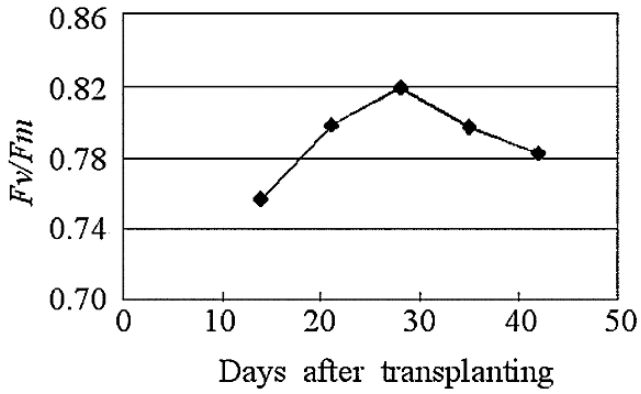

Figure 5. Changes in Fv/Fm of the 5th leaf of a potato plantlet at intervals of 7d. Reproduced from Ibaraki, Y. and Matsumura, K. (2004) [20].

4. Techniques for image-analysis-based evaluation of photosynthetic capacity

Spectral reflectance has been used to obtain plant growth information, especially in the research area of remote sensing. As spectral reflectance measurements are based on photometry, they have potential for non-destructive evaluation of plant growth and physiological state. The normalized difference vegetation index (NDVI), which can be calculated by reflectance at red and near infrared (NIR) wavelengths, has been widely used for monitoring, analyzing, and mapping temporal and spatial distributions of physiological and biophysical characteristics of vegetation [29]. It is applied not to an individual leaf, but to a plant canopy or wider area such as a forest, and is used mainly for quantification of vegetation, such as estimation of specific leaf area and evaluation of plant activity. The chlorophyll content of leaves can be estimated using the ratio of reflectance at 675 nm and 700 nm [30] or at 695 nm and 760 nm [31]. Although these indices are not a direct measure of photosynthetic capacity, they would be usable if empirical relationships between indices and photosynthetic capacity estimated by other methods could be determined.

Recently, the photochemical reflectance index (PRI) was proposed for estimation of photosynthetic radiation use efficiency [32]. This index is derived from reflectance at 531 nm and 570 nm, and is a measure of the degree of the photo-protective xanthophyll cycle pigment, zeaxanthin. The xanthophyll cycle, where the carotenoid pigment violaxanthin is converted to antheraxanthin and zeaxanthin via de-epoxidase reactions [33], is related to heat dissipation. The PRI is highly correlated with quantum yield of PSII determined by chlorophyll fluorescence for 20 species representing three functional types of plants [32]. Stylinski *et al*. [34] also reported a strong correlation of PRI to the chlorophyll fluorescence parameter *ΔF/Fm'* across species and seasons. As described previously, light use efficiency can vary with incident light intensity. Although several limitations still remain, the use of PRI is promising for evaluating photosynthetic capacity by a machine vision system.

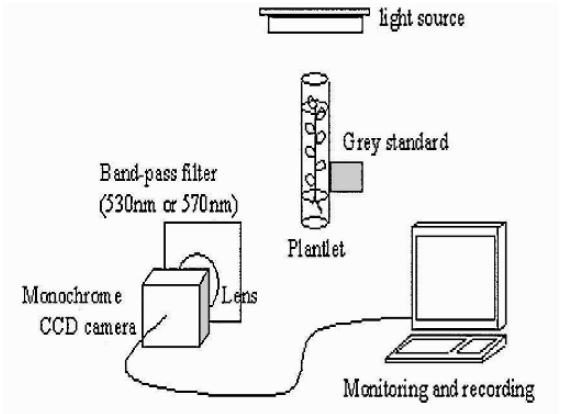

Figure 6. A concept illustration of a PRI imaging system.

Figure 6 shows a concept for a hypothetical PRI imaging system. In measurement of PRI, reflectance images should be acquired at two different wavelengths (531 and 570 nm). For this purpose, each image is taken with a grey standard by the CCD camera with a narrow-band-pass filter for the respective wavelength. The grey standard has nearly constant reflectance over the visible spectrum and is used to determine relative reflectance from light intensity. Configurations of the light source, the object (the culture vessel), and the camera should be carefully determined to collect the diffuse reflectance while reducing total internal reflection. Carter *et al*. [35] proposed a system using the same concept for reflectance imaging for early detection of plant stress.

5. Estimation of light distribution inside culture vessels

5.1. UNDERSTANDING LIGHT DISTRIBUTION IN CULTURE VESSELS

One of the most important factors for photosynthesis of cultured plantlets during micropropagation is the light environment, especially light intensity. High light intensity with sufficient CO_2 supply can enhance plantlet growth [36] and has the potential to facilitate acclimatization. From the viewpoint of photosynthesis, light intensity should be evaluated by photosynthetic photon flux density (PPFD) on the plantlet. However, since PPFD on plantlets is difficult to measure in a small culture vessel, it is usually represented by the value determined outside the vessel. PPFD on plantlets depends on the material and shape of culture vessels, the position of the vessel on the culture shelf, the position of the light sources, the optical characteristics of the shelf, etc [37]. It should be noted that PPFD in culture vessels with a closure, even with a high light transmissivity, was significantly lower than that on the empty shelf [38]. Moreover, when long culture vessels such as test tubes are used, light intensity can differ greatly between the top and bottom of the vessel. Non-uniform light distribution in a culture vessel may be responsible for differences in photosynthetic capacity and/or growth among leaves in the plantlet. As a result, this may lead to variations in plantlet quality in the case of a nodal cutting culture such as potato [6]. The estimation of light intensity distribution inside culture vessels is important for understanding the relationship between culture conditions and cultured plantlet growth properly. The use of information on light distribution in a culture vessel with information on photosynthetic capacity determined non-destructively would be helpful for optimization of culture conditions.

5.2. ESTIMATION OF LIGHT DISTRIBUTION WITHIN CULTURE VESSELS

A recently developed sensor film for measuring integrated solar radiation (Optleaf®), Taisei Chemical Co. Ltd., Japan) potentially offers a simple technique to estimate light intensity distribution. It has been used previously to estimate light intensity distribution in plant canopy (e.g., [39]). Here, the method [6] to estimate light intensity distribution inside a small culture vessel using the small piece of the sensor film is introduced.
This method enabled us to estimate light intensity distribution inside a culture vessel using a plantlet model whose leaves were constructed from sensor film. A plantlet

model simulating a potato plantlet consisted of 8 model leaves fabricated from sensor films (Optleaf R-2D, Taisei Chemical Co. Ltd., Japan) for measuring integrated solar radiation and a wire stem. A leaf-shaped piece of sensor film (dimensions 10 mm x 7 mm) was attached to an identically shaped piece of white paper and fixed to the wire stem at an angle of 30°. Each leaf was set at vertical intervals of 12 mm and at a horizontal angular interval of 120°. The total height of the plantlet model was 135 mm. A glass tube (25 mm x 150 mm) with a transparent plastic cap was used as the culture vessel. The sensor film was a cellulose acetate film coloured by azo dyes. Integrated radiation was estimated based on the degree of fading of the sensor film, which was quantified by measuring transmittance at 470 nm with a photometer (THS-470, Taisei Chemical Co. Ltd., Japan). Normally, measurements are performed while the film is set to a film mount (accessory of the photometer), but the model leaf was so small that the film mount could not be used. Therefore, the model leaf was set on 100% transmittance adjustment film (accessory of the photometer). The linear model determined previously could be used to correct the transmittance of model leaves. The sensor film absorbance was calculated from the sensor film transmittance and the ratio of the sensor film absorbance after exposure to that before exposure (film fading ratio) was determined. Integrated radiation was determined from the film fading ratio using a calibration curve provided by the film manufacturer (Taisei Chemical Co. Ltd., Japan).

Culture vessels with plantlet models were set on the shelf being surrounded with vessels containing potato plantlets in a temperature-controlled growth chamber at 24°C. Fluorescent tubes illuminated the growth chamber from the top (downward lighting) and the distance between the surface of fluorescent tubes and the top of vessels was 10 mm. In downward lighting condition, PPFD decreased toward the bottom of the vessel and was reduced to 50% and 30% of the maximum at the middle and the lower leaves, respectively. As compared with the PPFD measured with the photon sensor at the same position as each leaf position outside the vessel without the surrounding vessels, the steeper decline in PPFD inside the vessel could be observed. This might be due to interception of light by upper leaves and the surrounding vessels. PPFD distribution pattern inside the vessel can differ from that outside the vessel.

The results demonstrate that the use of sensor film plantlet models enables light intensity distribution inside a small culture vessel to be estimated, which was previously assumed to be too difficult to measure. This method could be applied to the determination of light intensity distribution patterns inside various types of culture vessels and under various lighting conditions, and thus would be of value in the optimization of culture conditions.

6. Concluding remarks

Non-destructive measurements of photosynthetic properties of plants in culture vessels are useful for understanding relationships between culture conditions and photosynthetic capacity, offering data on changes in physiological state of the plants during culturing without disturbing the *in vitro* microenvironment. Chlorophyll fluorescence has potential for non-destructive evaluation of leaf photosynthetic properties because the measurement can be conducted based on photometry. Parameters derived from chlorophyll fluorescence measurements relate to the functioning of PSII, including the

maximum quantum yield. Image analysis yielding these parameters is promising for non-destructive evaluation of photosynthetic capacity of micropropagated plants.

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