Response of Liverwort Cells to Peroxidizing Herbicides

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Cell suspension cultures of the liverwort, *Marchantia polymorpha* L. were found useful to study the influence of peroxidizing herbicides either on the greening process or on the fully green cells. The cells of both physiological stages exhibit a characteristic sensitivity to the herbicides. The sensitivity increased rapidly during the exponential phase of growth, reached a maximum during the late exponential phase, and then decreased in the stationary phase.

We investigated the kinetics of accumulation of protoporphyrin IX (PPIX) in *Marchantia* cells treated with several peroxidizing herbicides at various stages of cell growth, and observed a correlation between accumulation of PPIX and herbicidal damage.

The glutathione (GSH) content in the cell was also investigated to examine the role of GSH against herbicide treatment. In the light, GSH levels in the cells treated with AFM rose rapidly reaching a peak after 8 h, and rapidly decreased subsequently. The beginning of PPIX accumulation coincided with the decline of GSH after 8 h of treatment. Obviously, GSH plays a key role in protection against oxidative damage caused by AFM in the early treatment period. In the dark, AFM also induced an accumulation of GSH and PPIX, followed by a decline in GSH and PPIX contents during a 20 h incubation. The decline of PPIX was observed several hours after GSH starts to decrease, remaining at a constant level for the following 40 h, leading to accumulation of an other fluorescent still-unknown pigment.

Introduction

Tissue cultures of higher plants are often used to study the influence of photobleaching herbicides, because they exhibit several advantages as compared to intact plants [1–3]. However, the chlorophyll content of most of these green cultures of vascular-plant cells is considerably lower than that of intact tissues [4, 5]. Moreover, the growth of the cultured cells is not always accompanied by chloroplast development, indicating an incompatibility of these cellular activities [6]. Callus tissues obtained from liverworts are rich in chlorophyll and grow actively under both mixotrophic and autotrophic conditions [7–9]. Ohta *et al.* have reported the establishment of a green suspension culture of the liverwort *M. polymorpha* in a chemically defined medium [8]. This liverwort cell suspension culture having a high chlorophyll content apparently is an appropriate tool for the study of peroxidizing herbicides affecting the photosynthetic apparatus. Although not yet fully recognized, these cultures exhibit several advantages: (a) *M. polymorpha* cells can easily be cultured in well defined simple liquid media with high growth rates under continuous illumination. (b) Controlled growth conditions can be maintained. (c) Herbicides can be exactly applied *via* the culture medium. (d) Inhibitory effects develop soon after application of herbicides. (e) Determination of uptake of compounds, accumulation and excretion of metabolites can be followed.

This paper reports on this biological system in which the effects caused by the so-called peroxidizing (photobleaching) herbicides can be reliably determined. We demonstrate that accumulation of

Abbreviations: Acifluorfen-methyl (AFM), methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; chlorophthalim, N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide; oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene; GSH, glutathione; PPIX, protoporphyrin IX.

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PPIX caused by the herbicides in the light during the exponential phase of cell growth is a rapid effect, but is not observed during the stationary phase of growth.

Furthermore, PPIX, although accumulating in the cells in the light when peroxidizing compounds are present cannot induce peroxidative degradations of cellular membrane as long as a certain concentration of reduced glutathione is present. The major role proposed for glutathione in higher plants is to protect the cellular function against oxidative damage in the chloroplasts [10–12].

Moreover, this paper reports that some PPIX converts into another fluorescent pigment. These two fluorescent pigments accumulated in the cells, and apparently induced a light-dependent peroxidative degradation of cell membranes on exposure to light.

Materials and Methods

Chemicals

Acifluorfen-methyl (AFM), oxyfluorfen and chlorophthalim were produced in our laboratory. AFM was synthesized according to the method of Johnson [13]. Oxyfluorfen and chlorophthalim were prepared by the method of Wakabayashi *et al.* [14]. PPIX was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Other chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Cell culture and herbicide treatments

A chlorophyllous cell suspension culture of *M. polymorpha* L., has been routinely subcultured every two weeks using a modified medium of Murashige and Skoog [15] (Table I) for more than ten years. The suspension cultures were grown in 100 ml flasks containing 40 ml of medium on a rotary shaker (Model LR-3: Iwashiya K. Sawada Co., Tokyo) at 110 rpm/min at 25 °C. The cultures were continuously illuminated from the bottom with fluorescent lamps (Matsushita FL40W. "natural white", Matsushita Electric Works, Osaka) with an average light intensity of about 8.7 W/m^2 at the bottom of the flasks. Suspension cultures in the dark were grown by placing the flasks in black bags.

For experimental purposes, 6 day old cells (cells at exponential growth phase) or 20 day old cells (cells at stationary growth phase) were adjusted to a cell density of 1 mg dry weight/ml culture medium. Aliquots were treated with herbicide or GSH at the desired concentrations and incubated either in the dark or in the light for 72 h at 25 °C.

Table I. Composition of culture medium for *Marchantia polymorpha*.

Composition	Components
Mineral salts	according to Murashige and Skoog [15]
Carbon source	Glucose, 111 mM
pH (before autoclaving)	5.6
Fumaric acid	10 mM
Rhamnose	13.7 μM
Vitamins:	
Inositol	5.5 mM
Nicotinamide	8.2 μM
Pyridoxine·HCl	2.66 μM
Thiamine·HCl	2.96 μM
Cyanocobalamine	1.48×10^{-2} μM
Biotin	4.1×10^{-2} μM

Measurement of growth

Cell growth was followed by estimation of dry weight increase at appropriate intervals. Cells were collected by vacuum filtration on a polytetrafluoroethylene filter (10 μm pore size, 25 mm diameter; Floropore 1000, Sumitomo Electric Industries, Osaka) and dried in an oven at 105 °C for 3 h prior to dry weight estimation.

Extraction and determination of chlorophyll, fluorescent pigments and glutathione

Cells of 1 ml culture aliquots were collected by centrifugation (5 min at $3000 \times g$) and the pellet was extracted 3 times with boiling 90% methanol to extract total chlorophyll. Optical densities at 653 nm (A_{653}) and 666 nm (A_{666}) were measured on a model-210A spectrophotometer (Shimadzu, Kyoto) and total chlorophyll (μg/ml) was calculated using the equation, $c = 23.6 \times A_{653} + 2.57 \times A_{666}$ [16]. Extraction and estimation of fluorescent pigments including PPIX were carried out as described [3]. Cells collected by centrifugation were extracted with 0.1 M Tris-HCl buffer (pH 7.5) containing 1% Tween 80 by sonication for 1 min (Branson sonifier, model 250). After centrifugation (2 min in a Beckman Microfuge), the clear su-

pernatant was collected and its fluorescence measured with a Shimadzu Rf-fluorimeter, using excitation and emission wavelengths of 410 and 633 nm, respectively. Amounts of PPIX were then calculated by comparison with a reference fluorescence curve using authentic PPIX dissolved in Tris-HCl-Tween buffer.

Cells to be assayed for GSH were collected by centrifugation (3000 × *g*, 5 min). Extracts were prepared by adding 3 ml of 10% (w/v) 5-sulfosalicylic acid and sonication for 1 min. The homogenate was centrifuged at 12,000 × *g* at 4 °C for 5 min, and the acid-soluble supernatant was assayed. GSH was separated by HPLC on a Merck Lichrospher 100 RP-18 (e) column using a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The gradient program was 0% acetonitrile for 2 min, 0 to 10% acetonitrile in 2 min, 10 to 20% acetonitrile in 20 min. The column eluent was derivatized with 75 μM 5,5-dithiobis(2-nitrobenzoic) acid in 50 mM potassium phosphate (pH 7.6) at a flow rate of 2 ml/min and monitored at 412 nm. Retention times and peaks areas were determined with a Shimadzu C-R3A integrator. GSH determinations were based on peak areas of GSH standards.

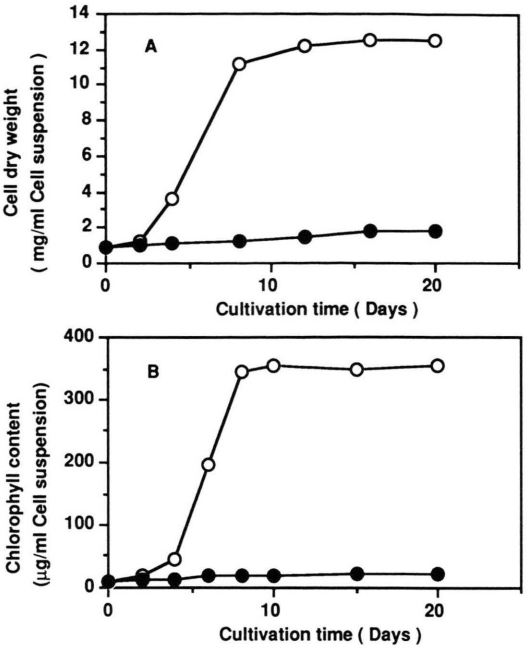

Fig. 1. Growth and chlorophyll content of *Marchantia polymorpha*. (A) dry weight and (B) chlorophyll contents in light (○–○) and darkness (●–●).

Results

Effects of herbicides on Marchantia polymorpha cells at different growth phases

Fig. 1(A) shows growth curves of the green suspension cultures grown under continuous light and in the dark. The cells started to divide after a lag of 2 days in the light, cell growth reached a stationary phase after 8 days. In the dark the cells grew quite poorly and at most the dry weight doubled that of the inoculum after a 4 weeks culture. As shown in Fig. 1(B), the amount of chlorophyll in illuminated cultures increased roughly in parallel with growth and reached a maximum value of 362 μg/ml after 20 days of culture. When chlorophyll content was plotted against cell dry weight it increased to an almost constant value of 30 μg/mg cell dry weight after one week. The effects of the peroxidizing herbicides AFM, oxyfluorfen and chlorophthalim, on growth and chlorophyll content were measured by using samples of two different physiological cell states. One sample were greening *immature* cells subcultured from 6 day old cultures at the exponential phase. The other sample represented fully green-pigmented *mature* cells of subcultured 20 day old cells of full stationary phase. In both cases, loss of chlorophyll was much more rapid and sensitive against the herbicide treatment than the change in cell dry weight. Growth of immature cells in the light was completely inhibited by AFM (15 μM), oxyfluorfen (15 μM) and chlorophthalim (20 μM) within 24 h (Fig. 2). After a 24 h exposure to the herbicide in the dark, chlorophyll reduction of approximately 10% was caused by 15 μM AFM (Fig. 2). By a 15 μM AFM treatment for 24 h in the light, cellular disruption was complete in many cells as seen by plasmalemma and chloroplast membrane disruption, and visualized by light microscopy. The membrane components of most cells of incubated with the herbicide in the dark appeared normal as compared to control cells. In contrast, AFM was less phytotoxic to mature cells and did not caused the cellular disruption until 5 days after light incubation, even at 15 μM concentration (Fig. 2).

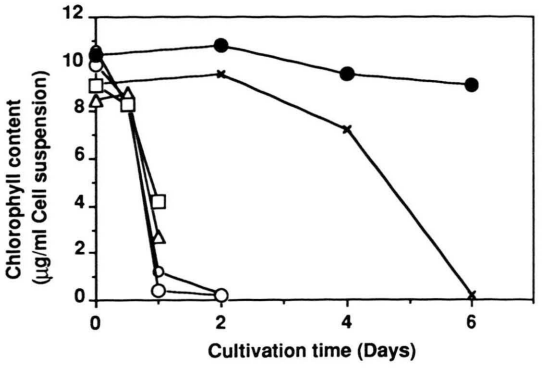

Fig. 2. Effect of AFM, oxyfluorfen and chlorophthalim on chlorophyll content of cultured cells in light and darkness. Mature cells (x−x, control) were cultured with 15 μM AFM in the light for 6 days. Immature cells were cultured with 6 μM AFM (○), 15 μM AFM (○−○), 15 μM oxyfluorfen (△−△) and 20 μM chlorophthalim (□−□) in light and with 15 μM AFM (●−●) in darkness.

Accumulation of tetrapyrroles in herbicide-treated cells

The immature and mature *M. polymorpha* cells were incubated for 6 days in fresh medium containing 15 μM of AFM and oxyfluorfen and 20 μM chlorophthalim in the light or dark. Fig. 3 shows that these peroxidizing herbicides induced a high PPIX accumulation in the immature cells during a 24 h light or dark incubation. However, only a trace amount of PPIX was detected in mature cells after 3 days light incubation. The levels of PPIX in the cells started to increase after 3 days in the light and attained a substantial accumulation of PPIX after 4 days.

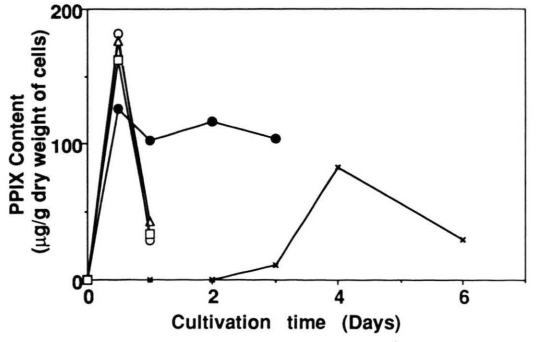

Fig. 3. Effect of AFM, oxyfluorfen and chlorophthalim on PPIX content of cultured cells in the light and dark. The mature cells (x−x) were cultured with 15 μM AFM for 6 days in the light. The immature cells were cultured with 15 μM AFM (○−○), 15 μM oxyfluorfen (△−△) and 20 μM chlorophthalim (□−□) in light and with 15 μM AFM (●−●) in darkness.

Fig. 4 demonstrates the appearance of a new fluorescent compound in the cells after a cultivation period of 24 h with AFM in the dark. The new fluorescent compound rose slowly after a stationary level of PPIX was attained. Upon exposure of dark-incubated cells for 72 h to light, the peroxidizing activity appeared rapidly compared with that in cells exposed to light and AFM simultaneously. At same time, all tetrapyrroles disappeared.

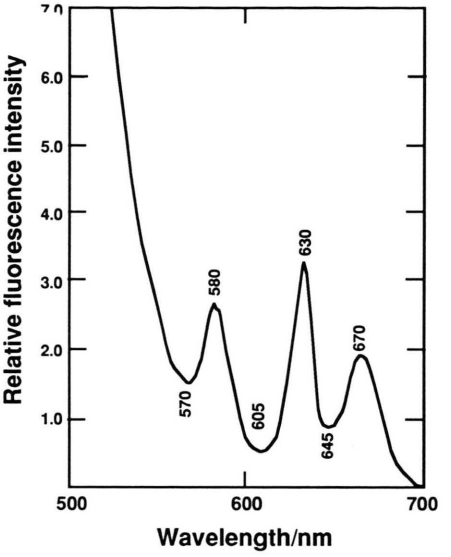

Fig. 4. Fluorescence emission spectra (excited at 420 nm) of the extract of dark-cultured cells with AFM present. Fluorescent pigments was isolated from immature cells incubated for 48 h in darkness (see Methods).

Effects of glutathione in herbicide-treated cells

GSH contents of immature cells increased rapidly after exposure to AFM both in the light and dark. The rate of GSH accumulation was much more faster than that of PPIX in the cells exposed to AFM under light (Fig. 5A). The amount of GSH in the treated cells was about 4-fold more than that in untreated immature cells. GSH production of AFM-treated cells reached a peak after 8 h and decreased rapidly during the following 9 h. As shown in Fig. 5A, with 15 μM AFM, the PPIX level rose rapidly after decline of GSH, reaching a

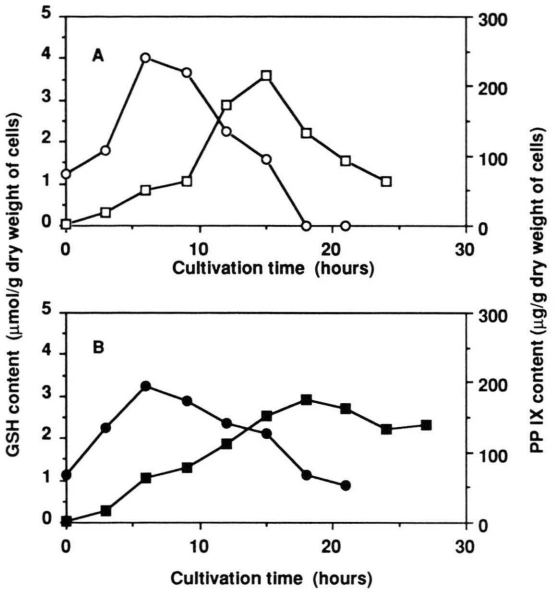

Fig. 5. Effects of AFM on protoporphyrin IX (PPIX) and glutathione (GSH) contents of cultured cells in the light and dark. The immature cells were cultured with 15 μM AFM in the light and dark for 24 h. The levels of GSH (○−○) and PPIX (□−□) in light and the levels of GSH (●−●) and PPIX (■−■) in dark were estimated.

peak after 15 h, leading subsequently to bleaching effects. In fact, addition of reduced GSH to the culture blocked the herbicidal activity of AFM in the cells (Fig. 6).

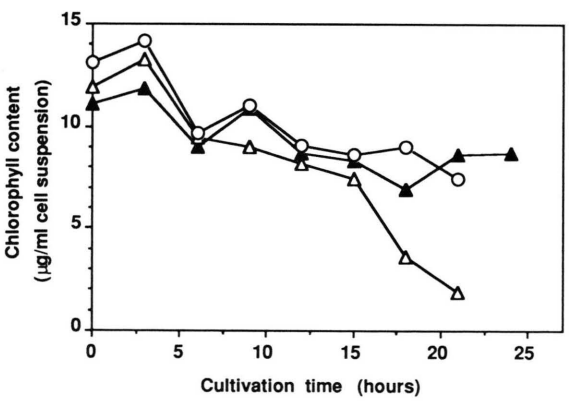

Fig. 6. Effect of exogenous glutathione on AFM treated cells. The immature cells (○−○) were cultured in a medium with 1 mM GSH and 15 μM AFM for 24 h at 25 °C in the light. Furthermore, immature cells were cultured with 15 μM AFM for 24 h in the light (△−△) and in the dark (▲−▲).

In the dark, AFM also caused the increase of the reduced form of GSH in cells (Fig. 5 B). At start of GSH decrease in the cell, the accumulation of PPIX for cell disruptions was sufficient and, after a transitory decrease, the level of PPIX content in the cells was maintained.

Discussion

Protoporphyrin IX is accumulated under the influence of peroxidizing herbicides in the light as was shown with intact cucumber [2, 17 18] or *Lemna paucicostata* [19]. Nicolaus *et al.* [20] could show that light is mandatory under autotrophic conditions, and the light effect is less pronounced unter heterotrophic or mixotrophic conditions in the cell. Cyclic imide herbicides caused PPIX accumulation in the light in the tolerant alga *Bumilleriopsis,* but not in the highly sensitive *Scenedesmus* [21]. The PPIX level in *Scenedesmus* is stabilized in the dark due to absence of photooxidation. Watanabe *et al.* [22] could quantitatively correlate herbicide-induced PPIX formation with typical peroxidative cell parameters like ethane formation or chlorophyll degradation. Such a study can be reliably performed with liquid cultures of *Scenedesmus*. Thus, to investigate the mode of action of bleaching herbicides, the choice of the biological system is important.

Ohta *et al.* have reported the establishment of a green suspension culture of a liverwort, *M. polymorpha,* in a chemically defined medium [8]. The cells can be maintained for long time under stable culture conditions. We found that the biological system using the liverwort cell culture is advantageous in the study on mode of action of peroxidizing herbicides. In immature cells chlorophyll biosynthesis proceeds more rapidly than in mature cells which leads to quick PPIX accumulation in the first case. PPIX accumulates as a photosensitizing agent by the herbicide treatment and again a positive correlation between the herbicide-induced PPIX accumulation and phytotoxic effects could be shown with liverwort cells.

The increase of GSH preceded the large increase of PPIX both in dark and light. Protection mechanism against the herbicide-induced peroxidation have been demonstrated in plants and algae [23, 24]. Schmidt and Kunert [11] have also observed that GSH and ascorbate increased by treatment of

bean leaves with oxyfluorfen and that these compounds play a protective role in cellular metabolism by acting as antioxidants against toxic peroxidation. In our experiments AFM caused a nearly 4-fold accumulation of GSH *vs.* untreated cells during the first 8 h of treatment, followed by a subsequent decline. A significant decrease of chlorophyll content in cultured cells was not observed as long as this high level of GSH was maintained. The decline of the GSH content in the cells led to accumulation of PPIX, reaching a peak after a 15 h cultivation with AFM followed by a rapid decrease of PPIX during the next 9 h (Fig. 5). Start of herbicide-induced cell disruption and photooxidative loss of PPIX occurred concomitantly. These observations suggest that the disappearance of GSH, a probable protective agent against oxidative damages, may allow oxidative processes by PPIX accumulation that affect the membrane lipids. In the light, the amount of GSH soon reaches a constant level which may represent the maximum capacity of the cells to supply the protective reductant. The PPIX level, however, continues to increase beyond this point. This extreme accumulation of PPIX eventually overtaxes the self-protecting capacity of the cells inducing disruption of chloroplast membranes.

In green cells of *M. polymorpha* we found the accumulation of a new fluorescent pigment during the cell cultivation with AFM in the dark. In the dark, AFM also caused the accumulation of GSH and PPIX, followed by a decline in GSH content during a 20 h incubation. The level of PPIX was found constant after the transitory decrease and a new fluorescent pigment showed up. In the dark, PPIX accumulated after the decline of GSH in cells, and is further metabolized to a PPIX-related fluorescent pigment. Apparently, these closely related PPIX compounds cause the rapid and light-dependent disruption of the chloroplast membrane.

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