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Callus Systems to Assay Herbicidal Peroxidation: Effects of Peroxidizing Herbicides on Habituated Tobacco, *Nicotiana glutinosa*, Green Cell Cultures

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The typical physiological parameters caused by the so-called peroxidizing herbicides, namely (1) growth inhibition, (2) chlorophyll decrease, (3) accumulation of protoporphyrin IX, (4) short-chain hydrocarbons formed through peroxidative destruction of membranes, and (5) alleviation of peroxidation by adding gabaculine (an inhibitor for the formation step of 5-aminolevulinate which is a precursor of protoporphyrin IX) and diuron (an inhibitor of photosynthetic electron transport), were obtained using habituated *Nicotiana glutinosa* (HNG) cells. The date correlated well with the physiological parameters assayed by unicellular microalgae, *Scenedesmus acutus*. Since the HNG cells are of a higher plant and its handling is rather easy for culture and phytotoxic assay of the herbicides, the assay system using the HNG cells is considered to give a convenient method for this sort of peroxidizing herbicides.

Introduction

Plant tissue cultures are often used to study the mode of action of herbicides, because they exhibit several advantages, *e.g.* providing a convenient assay of phytotoxic action in cell levels and so on, as compared with the intact plant[1,2]. However, doubts remain whether the mechanisms found in plant tissue cultures accurately reflect those of intact plant cells. In general, plant tissue cultures are carried out heterotrophically supplying sugar as the sole carbon source. Accordingly, in such heterotrophic cultures, a photosynthetic activity apparently lowers compared with that of intact plant leaves, and therefore the herbicides targetting photosynthesis usually indicate no response in the tissues cultured[3,4]. This paper reports the use of green callus systems of habituated tobacco cells grown as mixotrophic cultures in the light but used in suspension to quantitate herbicidal effects caused by peroxidizing herbicides.

Materials and Methods

1. *Chemicals*

Chlorophthalim, *N*-(4-chlorophenyl)-3, 4, 5, 6-tetrahydrophthalimide, was prepared by the condensation reaction of 3, 4, 5, 6-tetrahydrophthalic acid and 4-chloroaniline, according to our

method[5]. Oxyfluorfen, 2-chloro-4-trifluoromethylphenyl 3-ethoxy-4-nitrophenyl ether, was prepared by treating with ethanolic KOH 1, 3-*bis* 2-chloro-4-(trifluoromethyl) phenoxy-4-nitrobenzene, synthesized from resorcinol[6]. Both compounds were purified by recrystallization and their structures were confirmed by IR-, NMR-spectrography and elementary analysis for C, H, N and halogen.

Chemicals, analytical grade, for cell cultivation were purchased from Wako Chemical Corp., and fine chemicals including authentic protoporphyrin IX and buffers from Sigma, Münich.

2. *Cultivation of Nicotiana cells*

The HNG (habituated *Nicotiana glutinosa*) cells used in this experiment were the same as those described previously[7]. The HNG cells were obtained by selection from habituated *Nicotiana glutinosa*[8]. This strain has not required any organic additives, such as plant hormones, vitamines and others, except sucrose in the medium. HNG cells build up green chloroplasts when grown in the light in the presence of sugar. Chlorophyll content and photosynthetic O_2 evolution of HNG cells were 47.9 μg/g fresh weight and 7.23 μmoles/g fresh weight/hr, respectively. The agar medium (AΣM 80T) for proliferating HNG cells consisted of KH_2PO_4 4, KNO_3 12, KCl 4, $Mg(NO_3)_2 \cdot 6H_2O$ 24, $Ca(NO_3)_2 \cdot 4H_2O$ 4, NH_4NO_3 16, and $(NH_4)_2SO_4$ 16 meq per liter (wherein, $\Sigma M = K + Mg + Ca + NH_4 = 20 + 24 + 4 + 32 = 80$; $\Sigma A = NO_3 + H_2PO_4 + SO_4 + Cl = 56 + 4 + 16 + 4 = 80$ meq/l). Concentration and composition of the minor inorganic constituents were the same as those of Murashige-Skoog medium[9]. The medium included 30 g sucrose and 8 g agar per liter. The pH-value of the medium was adjusted to 6.0 with 0.1 N KOH solution. The HNG cells were grown on the above medium aseptically for 15 to 20 days at 25°C under continuous light of 6,000 lux.

3. *Determination of phytotoxic activity by herbicides*

Herbicides as indicated were added to 0.1 g of the proliferating cells placed into gas-tight 10-ml vials suspended in 2 ml of liquid culture medium (AΣM 80T medium except agar). Two ml cell suspension was shaken at 25°C in the light (10,000 lux). After a 48 hr incubation treatment, cell growth, chlorophyll content and short-chain hydrocarbon formation were determined. Measurement of short-chain hydrocarbons in vials was performed according to the Nicolaus[10]. The cells from each vial were collected individually into a Buchner funnel, after which it was rinsed by suction with 10 to 20 ml of deionized water. Then, each sample was wrapped in aluminum foil, and weighed as fresh weight. After measurment of fresh weight, the cell samples were employed for determination of chlorophyll content. The chlorophyll content was determined by the method accrding to Arnon[11]. The I_{50} and pI_{50} values for growth inhibition, chlorophyll decrease and short-chain hydrocarbon formation were caluculated by the method described elsewhere[12]. For the determination of protoporphyrin IX (Proto IX), the herbicides were treated at the concentration of 10^{-5} M to 0.2 g of the proliferating cells into gas-tight 10-ml vials including 4-ml liquid culture medium (AΣM 80T medium except agar). Four ml cell suspension was incubated at 25°C in the light (10,000 lux). After the period of treatement indicated, the cells were washed with deionized water and the extraction of Proto IX was carried out according to Ho *et al.*[13], with some modification. Proto IX contents were determined after HPLC separation with a fluorecent detector (Shimadzu LC-9A system).

Results and Discussion

The effects of peroxidizing herbicides, chlorophthalim and oxyfluorfen, on growth chlorophyll content and short-chain hydrocarbon (ethane, entylene and propane in an approximate ratio of 7 : 2 : 1) formation in HNG cells were investigated after a 48 hr incubation treatment (see **Table 1**).

The growth of HNG cells was inhibited by peroxidizing herbicides. Decreased chlorophyll content and short-chain hydrocarbon formation were observed in the presence of both herbicides. *p*-Nitrodiphenyl ethers and cyclic imides induce destruction of plant membranes, chlorophyll and cell constituents. Particularly, the unsaturated fatty acids of the acyl lipids of membranes are affected. This destruction is accompanied by short-chain hydrocarbon formation arising from lipid peroxidation. Short-chain hydrocarbon formation is a useful index of herbicide-induced peroxidation. Short-chain hydrocarbon formation in HNG cells may have arisen from lipid peroxidation by peroxidizing herbicides.

Table 1 also shows the influence of gabaculine (3-amino-2, 3-dihydrobenzoic acid) or diuron (3-(3, 4-dichlorophenyl)-1, 1 dimethylurea) on the peroxidizing effects by the both herbicides.

Table 1. Effects of gabaculine and diuron in the peroxidizing effects by peroxidizing herbicides in habituated *Nicotiana glutinosa* cells after a 48 hr incubation treatment.

Culture condition	Growth (mg fresh weight)	Chlorophyll content (μg/g fresh weight)	Short-chain hydrocarbon formation (nM/g fresh weight)
Control	81. 8	39. 3	1. 27
(+) Chlorophthalim (10^{-5} M)	30. 7	4. 7	5. 85
(+) Chlorophthalim Gabaculine (10^{-3} M)	69. 1	38. 1	1. 15
(+) Chlorophthalim Diuron (10^{-5} M)	70. 8	34. 7	1. 47

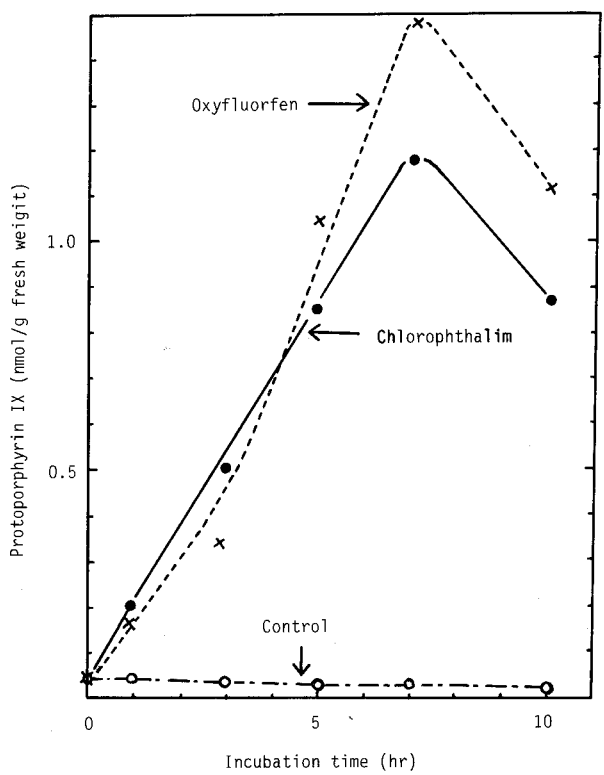

Fig. 1 Time-course accumulation of protoporphyrin IX (Proto IX) in the presence of chlorophthalim and oxyfluorfen (10^{-5} M) in habitated *Nicotiana glutinosa* (HNG) cells.

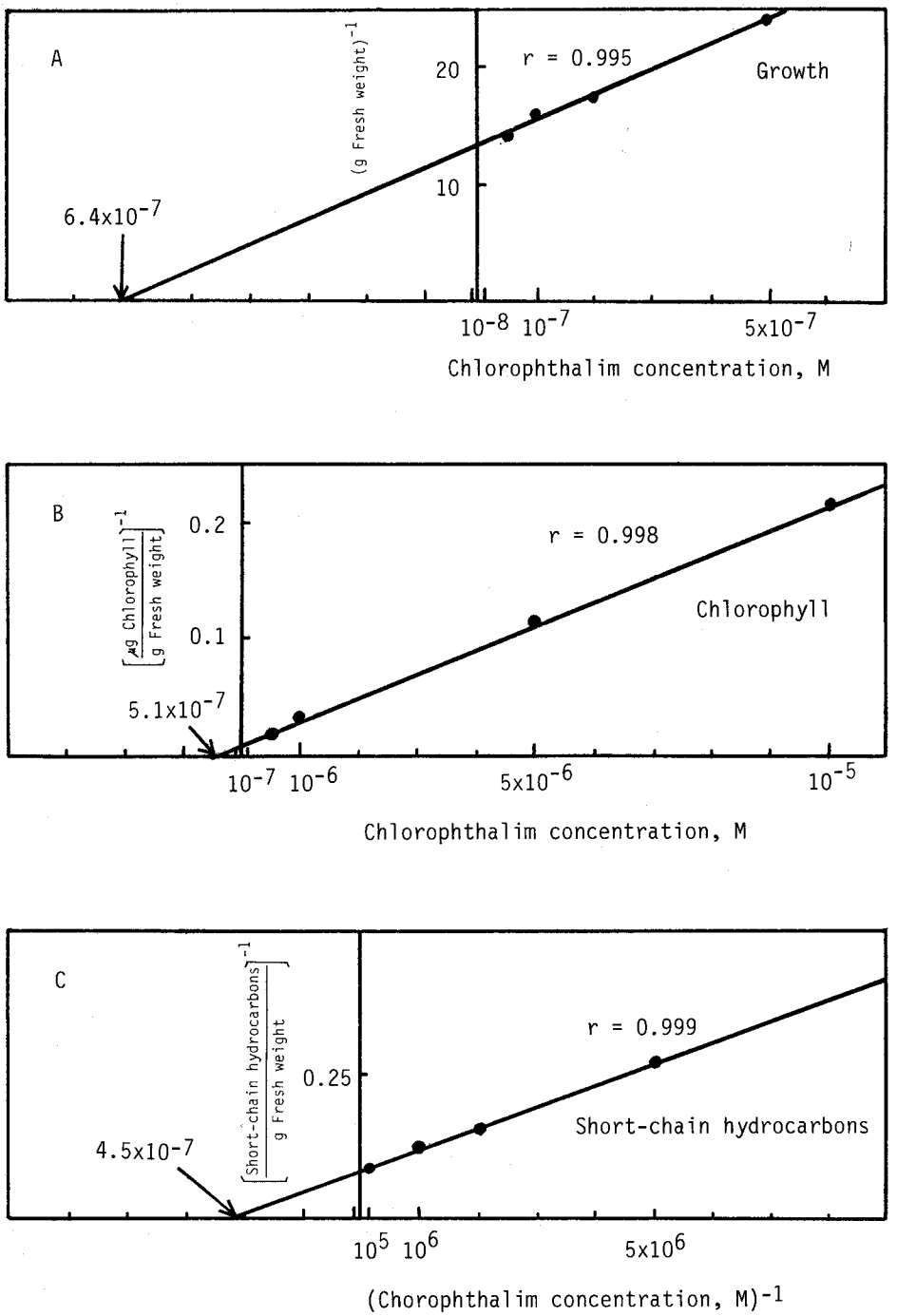

Fig. 2 Determination of I_{50}-values for growth inhibition, chlorophyll decrease and short-chain hydrocarbon formation in habituated *Nicotiana glutinosa* (HNG) cells.

Gabaculine and diuron are inhibitors for the formation step of 5-aminolevulinate which is a precursor of Proto IX, and photosynthetic electron transport inhibitor, respectively. The decreased chlorophyll content and short-chain hydrocarbon formation were alleviated with the simultaneous addition of gabaculine or diuron. These two compounds decreased peroxidation. In this experiment, the peroxidizing effects of two herbicides have concerned chlorophyll biosynthesis and photosynthesis. Accumulation of Proto IX in the presence of chlorophthalim and oxyfluorfen

were observed in HNG cells (see **Fig. 1**). The Proto IX contents in HNG cells increased with the lapse of time for 5 hr, thenceforth the Proto IX decreased. Proto IX levels in control HNG cells remained constant at low levels. Matringe and Scalla[14] have reported that diphenyl ether herbicides such as acifluorfen caused the accumulation of high levels of Proto IX by inhibiting protoporphyrinogen oxidase. The Proto IX accumulation as typical peroxidative parameter was observed in HNG cells.

As mentioned above, HNG cells showed the same response to peroxidizing herbicides as was documented and explained in detail with soybean cells[15] and unicellular microalgae[10].

Moreover, **Fig. 2** shows the proportional linearity between values of growth inhibition, chlorophyll decrease and short-chain hydrocarbon formation, and the concentration of peroxidizing herbicides. A linearity between growth inhibition and chlorophthalim concentration was established within a concentration of 5×10^{-8} to 5×10^{-7} M (see **Fig. 2-A**), and the I_{50}-value of the chemical for growth inhibition was determined. As shown in **Table 2**, pI_{50}-values ($=-\log I_{50}$-values), growth inhibition of HNG cells, for chlorophthalim and oxyfluorfen were 6. 19 and 6. 61, respectively. These pI_{50}-values were larger than the pI_{50}-values of the herbicides against sawa millet, but were smaller than pI_{50}-values aginst unicellular microalgae. The sensitivity of HNG cells to the peroxidizing herbicides was considered to be intermediate between that of sawa millet and unicellular microalgae.

The linearity between chlorophyll decrease and chlorophthalim concentration of 5×10^{-7} to 10^{-5} M was also found in HNG cells (see **Fig. 2-B**). The pI_{50}-values for chlorophyll decrease were caluculated as 6. 29 for chlorophthalim, and as 6. 88 for oxyfluorfen, respectively (see the pI_{50}-values in **Table 2**). Also in chlorophyll decrease, a slightly lower sensitivity in HNG cells was observed compared with unicellular microalgae. This fact may depend upon difference in productivity of chlorophyll between these plant species.

The linearity for short-chain hydrocarbon formation was found for chlorophthalim in a concentration of 10^{-5} to 10^{-6} M (see **Fig. 2-C**). The pI_{50}-values for short-chain hydrocarbon formation were 6. 35 for chlorophthalim, and 6. 69 for oxyfluorfen, respectively (see the pI_{50}-values in **Table 2**). The pI_{50}-values of oxyfluorfen in HNG cells and unicellular microalgae were 6. 69 and 7. 00, respectively, although the values of chlorophthalim in HNG cells and unicellular microalgae were 6. 35 and 6. 04, respectively. Experiments are under way to explain the discrepancy observed in chlorophthalim in short-chain hydrocarbon formation. Phytotoxic activities of peroxidizing herbicides can be conveniently assayed by using HNG cells.

Since HNG cells are of a higher plant and their handling is rather easy for culture and phytotoxic

Table 2. Phytotoxic activities of chlorophthalim and oxyfluorfen against some plant species.

Plant species	Chlorophthalim			Oxyfluorfen		
	pI_{50}			pI_{50}		
	Growth	Chlorophyll	Short-chain hydrocarbons	Growth	Chlorophyll	Short-chain hydrocarbons
Habituated tobacco cells (*Nicotiana glutinosa*)	6. 19	6. 29	6. 35	6. 61	6. 88	6. 69
Unicellular microalgae (*Scenedesmus acutus*)	7. 00	7. 10	6. 04	8. 00	8. 15	7. 00
Sawa millet (*Echinochloa utilis*)	5. 25	—	—	5. 76	—	—

— : not determined

assay of the herbicides, the assay system using the HNG cells is considerd to give a convenient method for this sort of peroxidizing herbicides.

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《和文要約》

Peroxidizing 除草剤の検索系：タバコ馴化緑色培養細胞に及ぼす peroxidizing 除草剤の影響

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タバコ馴化緑色培養細胞（HNG）は，peroxidizing 除草剤処理により，生長阻害，クロロフィルの減少，プロトポルフィリン IX の蓄積，膜破壊による短鎖の炭化水素の発生，およびクロロフィル生合成阻害剤（ギャバクリン）や光合成阻害剤（ジウロン）の同時処理による過酸化作用の軽減など，単細胞緑藻と同様な過酸化作用特有な生理現象を示した．高等植物の培養細胞は，その培養や除草剤の植物毒性試験のための操作が容易であることから，HNG 細胞を用いた peroxidizing 除草剤の検索系は非常に便利な実験系を提供してくれることが考えられた．