環状イミド系除草剤の植物色素生合成に及ぼす影響

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Original Article

Effect of Cyclic Imide Herbicides on Pigment Formation in Plants*

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Effect of a certain cyclic imide class of herbicides on pigment formation in two species of plants, *Scenedesmus acutus* and *Nicotiana xanthi*, was investigated using cell culture techniques. All the cyclic imide class of compounds assayed exhibited bleaching activity (chlorophyll decrease) in both plant species. However, neither phytoene nor ζ-carotene was detected in the least when *Scenedesmus* was grown in the dark with the herbicides present, and carotenoid biosynthesis was little or not affected when *Nicotiana* was cultured in the light in the presence of the herbicides. These findings may indicate that most possibly, chlorophyll formation in both plant species is primarily affected by the herbicides, which in turn inhibit carotenoid build-up.

INTRODUCTION

As we previously reported,[1] cyclic imide herbicides exhibiting light-dependent herbicidal action, being characteristic of most of diphenyl ether herbicides, are considered as bleaching herbicides, because white or decolorized plant tissues have been observed after treatment to plants. Oxadiazon and other herbicides related to cyclic imide herbicides shown in Fig. 1 are now classified also as the cyclic imide class of herbicides, because they have light-dependent herbicidal action and other similar plant responses, including bleaching activity, although of different chemical structures.[2] According to our recent studies using the bleaching diphenyl ether herbicides, most of such herbicides can exert their bleaching action by either inhibiting biosynthesis of chlorophylls or carotenoids, or by causing peroxidative destruction of pigments already formed.[3] This observation has been confirmed through the experiments using green microalgae (*Scenedesmus acutus*), which are grown either autotrophically in the light or heterotrophically in the dark, and form photosynthetic pigments even in darkness. To confirm which course the cyclic imide class of herbicides primarily inhibit, we discuss, in this paper, the influence of certain cyclic imide herbicides on pigment formation in *Scenedesmus* and also in the higher plant, *Nicotiana xanthi*.

* Mechanism of Action Studies on Cyclic Imide Herbicides (Part 2). See Ref. 2) for Part 1.

MATERIALS AND METHODS

1. *Chemicals*

N-Substituted 3, 4, 5, 6-tetrahydrophthalimides were prepared by condensation reactions of 3, 4, 5, 6-tetrahydrophthalic anhydride and appropriate amines or ammonium salts in an acetic acid medium.[4] Oxadiazon, 5-*tert*-butyl-3-(2, 4-dichloro-5-isopropoxyphenyl)-1, 3, 4-oxa-

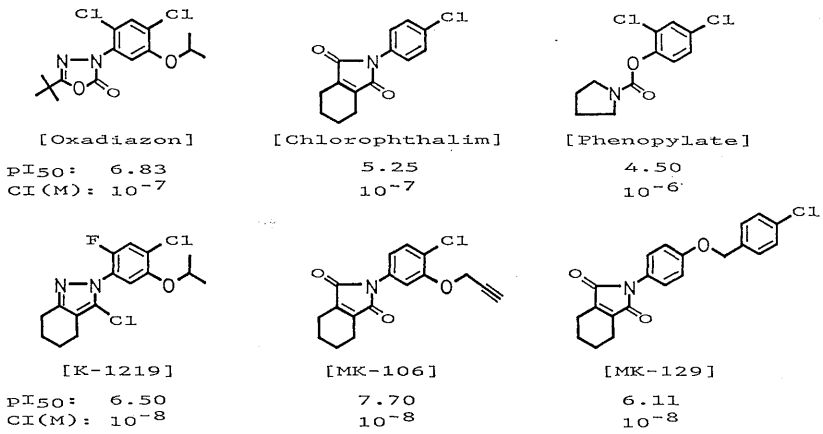

Fig. 1 Cyclic imide class of herbicides.

pI_{50}: Negative logarithm of the molar concentration of herbicides which produces a 50% inhibition of the root growth of sawa millet. This value shows a good correlation with herbicidal activity of cyclic imide class of herbicides.[1,4] CI (Chlorophyll Inhibition): Order of molar concentration of herbicides which causes chlorophyll inhibition in *Nicotiana xanthi*.

diazol-2(3*H*)-one, was obtained by the cyclization of 1-(2,4-dichloro-5-isopropoxyphenyl)-2-pivaloylhydrazine which was prepared by acylation of the corresponding phenylhydrazine, with phosgene in toluene.[5] Phenopylate, 2,4-dichlorophenyl *N*,*N*-tetramethylenecarbamate, was prepared by treating 2,4-dichlorophenol with phosgene in toluene and condensing the resultant 2,4-dichlorophenyl chloroformate with pyrrolidine.[6] Farmaid, 3,5-dimethylphenyl 4-nitrophenyl ether, was prepared by the reaction of potassium 3,5-dimethylphenolate with *p*-chloronitrobenzene in dimethylformamide at 130–150°C.[7] All the reaction products were purified through recrystallization, distillation or/and column chromatography, and their structures were confirmed by IR-, NMR-spectroscopy and elementary analysis for C, H and N (also for halogen for some compounds). Physical properties for all the compounds prepared are listed in Tables 1 and 2.

Methoxyphenone, 3,3′-dimethyl-4-methoxybenzophenone, was kindly provided by Nippon Kayaku Co., Ltd.

2. *Determination of Pigment Formation in Scenedesmus acutus*

The unicellular green microalga, *Scenedesmus acutus*, was cultivated in a culture flask heterotrophically in the dark under sterile conditions, according to the method described by Sandmann and Böger.[8] Growth was determined based on the packed cell volume (pcv) in graduated microcentrifuge tubes of 80 μl capacity. Chlorophyll was determined after extracting with hot methanol (65°C, 15 min) according to the method of Mackinney.[9] Carotenoids were extracted from cells with hot methanol containing 6% KOH (w/v). After cooling, total carotenoids were transferred into petroleum ether (bp 60–80°C) including 10% (v/v) diethyl ether, and the extract was used for spectrophotometric determination of total carotenoids or further separation and analyses of carotenoid precursors, phytoene and ζ-carotene. Quantification by optical absorption and all other details of this procedure were carried out by the method previously described.[10] Herbicides were added using 10 mM stock solutions in methanol or methanol/dimethylformamide (1 : 1, v/v). Molar I_{50} values were determined from the plots of inhibitory activity against concentration on a logarithmic probability scale.

3. *Pigment Determination in Calli of Nicotiana xanthi*

Callus isolated from leaves of *Nicotiana xanthi* was induced on a Murashige-Skoog (MS)

agar medium containing NAA (1 ppm) and kinetin (0.3 ppm) and maintained on the same medium at 25±2°C in the dark. Test compounds were added to the MS agar medium [A] and MS broth medium [B], in a form of dimethylsulfoxide solution. The callus grown in the dark was transferred to the each medium and cultured at 25±2°C under illumination by a fluorescent lamp (2000 lux of light intensity) for 7 days. Then, the callus was immersed in acetone for 24 hr at 5°C and filtered using Whatman CF/A. Pigments, chlorophylls and carotenoids, in the acetone solution were determined by photospectrometric methods of Arnon[11] with some modification and of Davies,[12] respectively.

4. Inhibition of Root Growth by a Cyclic Imide Class of Herbicides

The inhibitory activity against sawa millet (*Echinochloa utilis*) was determined using the method previously reported.[4] The root length of seedlings grown in the media containing various concentrations of compounds and the fixed amount of Tween 20 was measured. The molar concentration required for 50% inhibition against root growth relative to the control was determined from the dose-response relationship by means of the probit analysis. The values were represented as pI_{50}, logarithm of a reciprocal of the molar concentration for 50% inhibition. A good correlation has been found to exist between the pI_{50} values and the herbicidal activity of the cyclic imide class of herbicides obtained in the pot tests, which were performed using crabgrass, common purslane and hairly galinsoga in upland conditions and using barnyard grass, toothcup, umbrella plant and monochoria in paddy conditions.[1,13] Herbicidal activity of the imide herbicides will be discussed focusing on their pI_{50} values.

RESULTS AND DISCUSSION

1. Effect of Cyclic Imide Herbicides on Pigment Formation in the Green Microalga, Scenedesmus acutus

Pigment formation was examined using heterotrophic (dark) cultures of *Scenedesmus acutus*, since the species can form photosynthetic pigments even under heterotrophic conditions in the dark and a possible photooxidative destruction of pigments by test chemicals can be excluded under such conditions. The effects of a certain cyclic imide class of compounds (**1**–**14**) and the two reference herbicides (exactly, farmaid and methoxyphenone) for comparison on the inhibition of chlorophylls (or bleaching activity) and the colored carotenoids in this alga, and the algal growth under these conditions are shown in the figures of pI_{50} in Table 1. Data checked concerning accumulation of the carotene precursors, phytoene and ζ-carotene, and herbicidal activity expressed as pI_{50} values are presented also in Table 1.

All the cyclic imide class of compounds (**1**–**14**) assayed exhibited the bleaching activity; however, neither phytoene nor ζ-carotene, both of which are important precursors in carotenoid biosynthesis, was detected in the least for these compounds. Bleaching activity of the cyclic imide class of compounds was quite different (according to the chlorophyll inhibition; see the column of chlorophyll inhibition in Table 1). Thus, compounds **5** (chlorophthalim), **6**, **9**, **10** and **11** (MK-129) were the most potent inhibitors, and followed by **2** (phenopylate), **7** and **8**. Compounds **1** (oxadiazon), **3** and **4** were moderately active. Little activity was observed with compounds **12**, **13** and **14**; the compound **14** being the less active.

There is a rather stronger chlorophyll decrease by almost all the compounds as compared to that of carotenoids, although we have some exceptions as observed in compounds **3** and **8**. A possible photooxidative degradation of pigments may be excluded here under the dark conditions, and it should be again remarked that no carotenoid precursors are found for these cyclic imide class of compounds. These facts may corroborate the hypothesis that most possibly, the chlorophyll biosynthesis is primarily affected by the cyclic imide class of herbicides, which in turn inhibit carotenoid build-up.

Although we can find a closer relationship between the chlorophyll inhibition and the growth inhibition by the compounds tested [pI_{50}(growth inhibition) = 0.937 pI_{50}(chlorophyll inhibition) + 0.301; $n = 13$, $r = 0.977$, $s = 0.281$], there is a small discrepancy between the pigment decrease and the herbicidal activity [pI_{50}(herbicidal activity) = 0.822 pI_{50}(chloro-

Table 1 Influence of cyclic imide class of herbicides on the formation of chlorophylls and carotenoids in hetrotrophic cultures of *Scenedesmus acutus* over 48 hr.

	Cyclic imide herbicides or *N*-substituted phenyl-3,4,5,6-tetrahydrophthalimides	mp (°C)	Herbicidal activity (pI_{50})	Growth inhibition (I_{50})	Chlorophyll inhibition (I_{50})	Carotenoid inhibition (I_{50})	Accumulation[a] Phytoene [mg/ml pcv]	Accumulation[a] ζ-Carotene [mg/ml pcv]
(**1**)	Oxadiazon	90–91	6.83	—	1×10^{-6} (27%)	1×10^{-6} (5%)[d]	0	—
(**2**)	Phenopylate	Liquid[b]	4.50	2×10^{-6}	2.5×10^{-6}	5×10^{-6}	0	0
(**3**)	*N*-(2-Chlorophenyl)-3,4,5,6-tetrahydrophthalimide	91–92	3.49	1×10^{-5}	2×10^{-5}	2×10^{-5}	0	0
(**4**)	*N*-(3-Chlorophenyl)-	86	3.93	8×10^{-6}	1×10^{-5}	—	0	0
(**5**)	*N*-(4-Chlorophenyl)- [Chlorophthalim; MK-616]	166–167	5.25	2×10^{-7}	2×10^{-7}	5×10^{-7}	0	0
(**6**)	*N*-(2-Methylphenyl)-	136–137	4.64	8×10^{-7}	8×10^{-7}	1×10^{-6}	0	0
(**7**)	*N*-(3-Methylphenyl)-	106–107	3.78	7×10^{-6}	2.5×10^{-6}	5×10^{-6} (30%)	0	0
(**8**)	*N*-(4-Methylphenyl)-	124–125	4.34	3×10^{-6}	1×10^{-6}	1×10^{-6}	0	0
(**9**)	*N*-(4-Methoxyphenyl)-	100–101	4.90	8×10^{-7}	5×10^{-7}	1×10^{-6}	0	0
(**10**)	*N*-(4-Nitrophenyl)-	175–177	3.76	5×10^{-7}	5×10^{-7}	5×10^{-7} (29%)	0	0
(**11**)	*N*-[4-(4-Chlorobenzyloxy)phenyl]-; [MK-129]	163–164	6.11	5×10^{-9}	4×10^{-9} (35%)	8×10^{-9} (35%)	0	0
(**12**)	*N*-Methyl-	52–53	3.01	2.5×10^{-4}	$\sim1\times10^{-4}$	$\sim1\times10^{-4}$	0	0
(**13**)	*N*-Cyclohexyl-	77–78	2.87	1×10^{-4}	1×10^{-4}	$>10^{-4}$	0	0
(**14**)	*N*-Benzyl-	Liquid[c]	4.07	1×10^{-4}	3×10^{-4}	1×10^{-4}	0	0
(**15**)	Farmaid[e]	80–81	4.05	$>10^{-4}$	$\sim1\times10^{-4}$	$>10^{-4}$	0	[†]
(**16**)	Methoxyphenone[e]	62–63	—	1×10^{-6}	7×10^{-6}	2×10^{-6}	—	[†]

a) Accumulation of carotenoid precursors was checked at the I_{50} concentration of each compound for carotenoid inhibition; [†]: detected, 0: not detected.
b) bp 150–153°C/3 mmHg.
c) n_D^{23} 1.5620.
d) Percent inhibition at the indicated inhibitor concentration.
e) The reference herbicide.

phyll inhibition)—0.490; $n=12$, $r=0.931$, $s=0.360$, by deleting compounds **1** and **14**]; *e.g.* compounds **1** and **14** are more herbicidal than deduced from the pigment inhibition in *Scenedesmus*, while the small contrary holds for the compound **10**. This may point to additional effects by these compounds besides the pigment inhibition or existence of a detoxifying metabolism in plants.

The herbicide methoxyphenone (compound **16**) causing carotenoid inhibition[14] was used as a reference chemical in this experiment, and confirmed also in *Scenedesmus* that the herbicide more inhibited carotenoid formation than chlorophyll. Compound **15** (farmaid), a diphenyl ether herbicide with non light-dependent action[7] was examined for a comparison with the cyclic imide class of herbicides. The result showed that this compound was not a good bleacher even at 10^{-4} M of concentration. However, this herbicide yielded a small amount of ζ-carotene. Such a fact is somewhat different from other diphenyl ether herbicides which inhibit carotenoid biosynthesis by accumulating phytoene.[3] Further investigation is necessary before we discuss a relation between this fact and the light-independent herbicidal action of this herbicide.

2. *Effect of a Cyclic Imide Class of Herbicides on Pigment Formation in the Tobacco Plant, Nicotiana xanthi*

We have previously observed inhibition of chlorophyll formation by the cyclic imide class of herbicides (compounds **5** and **11**) in higher plants, such as sawa millet and rice plants, in which both herbicides have shown 17–67% inhibition of chlorophyll formation at 10^{-6} M compared with the control.[1] However, it is still difficult to obtain the precise data on the inhibition for further discussion, due to the complexity of experiments and analyses. Therefore, to confirm also in higher plants that the cyclic imide class of herbicides primarily inhibit chlorophyll biosynthesis, as observed in the green microalga (*Scenedesmus*), we examined the influence of certain cyclic imide herbicides on pigment formation in the tobacco plant, *Nicotiana xanthi*, using cell culture techniques. As shown in Table 2, all the cyclic imide herbicides assayed exhibited strong inhibition of chlorophyll formation (or bleaching activity) at a very low concentration (about 10^{-8} M), whereas carotenoid biosynthesis was little or not affected. Chlorophyll inhibition by the cyclic imide herbicides in *Nicotiana* was a little stronger than in *Scenedesmus* (*cf.* Table 1).

The herbicidal activity of compound **17** (MK-106) was extremely high; being more than 10-fold stronger than other cyclic imide herbicides (**1**, **5** and **11** in Table 2), although the chlorophyll inhibition by this compound was on a level with others. This fact may indicate that the compound **17** has a subjoined biochemical mechanism of action (maybe peroxidative action in the light; details of which will be discussed in our next paper) in addition to the chlorophyll inhibition, which may contribute to the stronger herbicidal effect. The biochemical data resulted from the experiments using *Nicotiana* also indicate that the cyclic imide class of herbicides exhibit a strong bleaching action by inhibiting chlorophyll biosynthesis with little effect on carotenoid levels.

The findings obtained from the present biochemical study using *Scenedesmus* and *Nicotiana* may prove that the cyclic imide class of herbicides decrease chlorophyll more than carotenoids by primarily affecting chlorophyll biosynthesis, which in turn inhibit carotenoid formation. And the stronger effect of the compounds on chlorophyll formation, leaving some decrease of carotenoids as a secondary response, may indicate that the chlorophyll-biosynthetic

Table 2 Influence of cyclic imide class of herbicides on pigment formation in cell cultures of *Nicotiana xanthi* (culture of callus on agar [A], and suspension culture [B]) over 7 days.

	Cyclic imide herbicides	mp (°C)	Concentration (μM)	Chlorophyll formation [μg/g (dry weight)]		Carotenoid formation[a] (%)		Herbicidal activity (pI_{50})
				[A]	[B]	[A]	[B]	
	Control	—	—	93.3	90.3	100	100	—
(**1**)	Oxadiazon	90–91	0.01	41.6	48.2	94	100	6.83
			0.10	37.9	—	83	—	
(**5**)	Chlorophthalim	166–167	0.01	84.1	—	102	—	5.25
			0.05	67.6	59.0	100	100	
			0.50	49.1	—	100	—	
(**17**)	*N*-(4-Chloro-3-propargyloxy)phenyl-3,4,5,6-tetrahydrophthalimide	146–147	0.01	49.4	59.1	88	100	7.70
			0.10	26.6	—	78	—	
	[MK-106]		1.00	8.6	—	36	—	
(**11**)	*N*-[4-(4-Chlorobenzyloxy)phenyl]-3,4,5,6-tetrahydrophthalimide	163–164	0.01	54.1	66.5	103	100	6.11
			0.10	14.2	—	73	—	
	[MK-129]		1.00	5.9	—	39	—	

[a] Data concerning carotenoid formation given as % inhibitions were calculated basing upon the datum relative to the control ([A]: 7.1 μg/g (dry weight) and [B]: 6.0 μg/g (dry weight), respectively).

pathway is the specific target of the compounds tested, if we exclude the possible additional activity concerning photoperoxidation. The chlorophyll-biosynthetic pathway remains to be investigated further to determine the precise inhibitory site by the cyclic imide class of herbicides.

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要　　約

環状イミド系除草剤の植物色素生合成に及ぼす影響*

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若林　攻

Oxadiazon, chlorophthalim, phenopylate などを含む広義の環状イミド系除草剤の植物色素生合成に及ぼす影響を，細胞培養系を用いて，2種の植物 (*Scenedesmus acutus* および *Nicotiana xanthi*) について検討した．得られたデータは，上記除草剤が両植物の chlorophyll 生合成を著しく阻害し，次いで carotenoid 生合成を2次的に阻害することを示した．この事実は，少なくとも実験に供された範囲の環状イミド系除草剤の作用点が chlorophyll 生合成行程に関わることを示唆しているようである．

* 環状イミド系除草剤の作用機構に関する研究（第2報）