PHOTOSYNTHETICA 37 (4): 553-558, 1999

Inhibition of pigment biosynthesis in cucumber cotyledons by isoxaflutole

S. KUSHWAHA and P. C. BHOWMIK

Department of Plant and Soil Sciences, University of Massachusetts, Amherst, MA 01003, U.S.A.

Abstract

Isoxaflutole [5-cyclopropyl-4-(2-methylsulphonyl-4-trifluromethylbenzoyl)isoxazole] is a new preemergence herbicide for broad-spectrum weed control in maize. The effect of isoxaflutole on chlorophyll (Chl) and carotenoid (Car) biosynthesis was investigated in cucumber (*Cucumis sativus* L.) cotyledons. Etiolated tissue was incubated with 5 mM isoxaflutole for 24 h and irradiated (60 μmol m^{-2} s^{-1}). The irradiation for 3 h did not reduce Chl *a*, Chl *b*, and Car contents, but after a 28-h irradiation the contents of Chl *a* and Car decreased by 35 and 15 %, respectively, and the content of Chl *b* increased by 24 %. Increasing the concentration of isoxaflutole beyond 5 mM resulted in reduction of Chl *a* (71 %), Chl *b* (20 %), and Car (31 %) contents. Similarly, increase in irradiance from 60 to 180 μmol m^{-2} s^{-1} resulted in larger reduction of Chl and Car contents. Exogenously supplied 5-aminolevulinic acid did not reverse the isoxaflutole-inhibited Chl synthesis, whereas an exogenously supplied homogentisic acid lactone reversed the inhibition of pigment synthesis due to isoxaflutole.

Additional key words: 5-aminolevulinic acid; *Cucumis sativus*; homogentisic acid; hydroxyphenyl pyruvate dioxygenase.

Introduction

Isoxaflutole (IFT) is a member of isoxazole class of herbicides (Cain *et al.* 1993), a systemic preemergence herbicide (Anonymous 1995). At low doses it controls broadleaf and grass weeds in maize (*Zea mays* L.) (Luscombe *et al.* 1995, Bhowmik *et al.* 1996a,b, Vrabel *et al.* 1996, Kushwaha and Bhowmik 1997) and sugarcane (*Saccharum officinarum* L.) (Luscombe *et al.* 1995). Under no-tillage system, IFT excellently controls annual broadleaf and grass weeds (Bhowmik *et al.* 1996a, Vrabel

Received 15 July 1999, *accepted* 18 October 1999.
Fax: (413)545-3958, e-mail: kushwaha@pssci.umass.edu
Abbreviations: ALA, 5-aminolevulinic acid; Car, carotenoid; Chl, chlorophyll; HGA, homogentisic acid; IFT, isoxaflutole.
Acknowledgments: Appreciation is extended to Rhone-Poulenc Company for supplying herbicide. We thank Dr. Zdzislaw K. Koszanski for his suggestions and comments, and to Dr. Kalidas Shetty for letting us to use partly his lab facility. Help from Randall G. Prostak, Andy Drohen, and Sowmya Mitra is acknowledged.

et al. 1996a,b). Maize is tolerant to IFT at recommended doses (Anonymous 1995, Bhowmik *et al.* 1996a,b, Vrabel *et al.* 1996, Kushwaha and Bhowmik 1997). Carotenoids (Car) are important for photosynthesis by protecting chlorophyll (Chl) against photooxidative destruction by singlet oxygen (Anderson and Robertson 1960). Car with at least nine conjugated double bonds prevent Chl photodestruction by quenching triplet state of Chl molecules and by removing oxygen from excited Chl-oxygen complex *via* the Car-epoxide cycle (Anderson and Robertson 1960). If a herbicide prevents Car formation, Chl - although formed - does not accumulate which results in bleached symptoms. Luscombe *et al.* (1995) report that IFT acts *via* disrupting pigment biosynthesis by inhibiting *p*-hydroxyphenylpyruvate dioxygenase, thereby preventing the formation of quinone required for Car biosynthesis in susceptible plant species. The objectives of our experiments were to: (*a*) study the effect of IFT concentration, irradiance, and time duration on the biosynthesis of Chl *a*, Chl *b*, and Car, and (*b*) examine the role of 5-aminolevulinic (ALA) and homogentisic acids (HGA) on the reversal of the Chl *a*, Chl *b*, and Car biosynthesis inhibition of a cucumber cotyledon system.

Materials and methods

Cucumber (*Cucumis sativus* L.) cv. Dasher II seeds were grown on blotting paper in polyethylene tray in complete darkness for 5 d at 26 °C. Distilled deionized water was added to tray as needed to maintain a constant moisture. Cotyledons of the 5-d-old etiolated cucumber seedlings were excised under green radiation (0.02 μmol m^{-2} s^{-1}) without their hypocotyl hooks. Groups of 10 cotyledons were placed in 3-cm petri dishes containing 4 cm^3 of distilled deionized water or IFT solutions (0.1, 0.5, 1.0, 5.0, and 10.0 mM). Petri dishes were placed in darkness at 26 °C for 24 h followed by irradiation (60 or 180 μmol m^{-2} s^{-1}) for 28 h, unless stated otherwise, provided by cool-white fluorescent bulbs. Relatively low irradiance was used to minimize photo-oxidation of Chl. IFT or HGA (*Fisher Scientific*) were applied as 100 μM in dark or light. Pigments were extracted by overnight placing the known quantity of cotyledons in 10 cm^3 of N,N-dimethylformamide. Absorbances at 480, 640, and 663 nm were determined using a *Hitachi* model *U-1100* spectrometer. Chl *a* and *b* amounts were calculated using the formula of Arnon *et al.* (1956) and Car using the formula of Hager and Meyer-Bertenrath (1966) with the exception that the 480 nm wavelength was used instead of 440 nm.

All experiments were repeated twice with four replications. Values were subjected to analysis of variance (ANOVA) and means were evaluated by Duncan's multiple range test at the 5 % level of significance.

Results and discussion

Time course: When etiolated cotyledons were incubated in 5 mM IFT solution in dark for 24 h followed by irradiation up to 24 h, the cotyledon fresh mass was not

affected by the herbicide (Table 1). Yet at the 48 h irradiation period, the cotyledon fresh mass was reduced by 39 %. IFT did not reduce Chl *a*, Chl *b*, and Car contents of the cotyledons during 24-h dark incubation and these pigments were not affected by the herbicide even after 3 h of irradiation. Hence the IFT did not interfere with the

Table 1. Effects of various irradiances on cotyledon fresh mass and chlorophyll (Chl) *a*, *b*, (*a*+*b*), and carotenoid (Car) contents. Cotyledons from 5-d-old cucumber seedlings were incubated in dark with 5 mM isoxaflutole for 24 h and irradiated for various duration. Fresh mass and pigment contents are presented as % of water treated control. Means within the column followed by the same letter are not significantly different as determined by Duncan's multiple range test at 5 % level.

Irradiation [h]	Fresh mass	Chl *a*	*b*	(*a*+*b*)	Car
0	100 a	100 a	100 ab	100 a	100 a
1	109 a	106 a	109 a	110 a	99 a
3	118 a	97 a	100 ab	99 a	96 a
6	111 a	82 a	90 ab	83 b	90 ab
12	108 a	72 b	84 b	69 c	86 bc
24	106 a	51 c	61 c	53 d	78 c
48	61 b	27 d	23 d	27 e	54 d

synthesis of pigment precursors during the 24-h dark incubation nor in their conversion into pigments during early phases of greening. Similar results have been reported with ICIA 0051 [2-(2-chloro-4-methylsulfonyl)benzoyl-1,3-cyclohexane-dione] (Nandihalli and Bhowmik 1992), amitrol (3-amino-*s*-triazole), dichloromate (3,4-dichlorobenzyl methylcarbamate), and pyrichlor (2,3,5-trichloro-4-pyridinol) (Burns *et al.* 1971). At 6 h, Chl *b* and Car contents were identical to the water treated control, and Chl *a* and Chl (*a*+*b*) were reduced by 18 and 17 %, respectively (Table 1). At 12 h, Chl *a*, Chl *b*, Chl (*a*+*b*), and Car were reduced by 28, 16, 31, and 14 %, respectively. By 48 h of irradiation a maximum reduction of 73, 77, 73, and 56 % was observed for these pigments, respectively. Cars prevent Chl from photodestruction (Anderson and Robertson 1960). Chl inhibition may be due to insufficient Car content (Ridley and Ridley 1979). Chl *a* may be photooxidized first followed by the reduction in Chl *b* content. Devlin *et al.* (1976) reported a 40 % reduction in Chl (*a*+*b*) content of maize and wheat due to 0.1 g m^{-3} norflurazon (4-chloro-5-(methyl-amino)-2-(0,0,0-trifluoro-*m*-tolyl)-3(2H)-pyridazinone) treatment. Dahlin and Timko (1994) found significant differences in Chl (29 and 14 %) and Car (66 and 5 %) contents of untreated and norflurazon treated pea (*Pisum sativum* L.) plants grown under weak red radiation. Inhibition of Car biosynthesis was the cause for bleaching in cucumber cotyledons treated with SC-0774 (Bhowmik and Nandihalli 1990) and ICIA-0051 (Nandihalli and Bhowmik 1992), in wheat after floridone and norflurazone application (Bartels and Watson 1978) and, in maize, wheat, and alfalfa (*Medicago sativa* L.) after norflurazon application (Devlin *et al.* 1976).

Herbicide concentration and irradiance: The inhibition of the synthesis of various pigments in cucumber cotyledons depended upon herbicide concentration and

irradiance (Fig. 1). At each concentration, pigment content was lower at irradiance of 180 than 60 μmol m^{-2} s^{-1}. An increase in Chl *a* content was observed at 0.1 to 1.0 mM IFT concentrations under 60 μmol m^{-2} s^{-1}. Similarly, Chl *b*, Chl (*a*+*b*), and Car

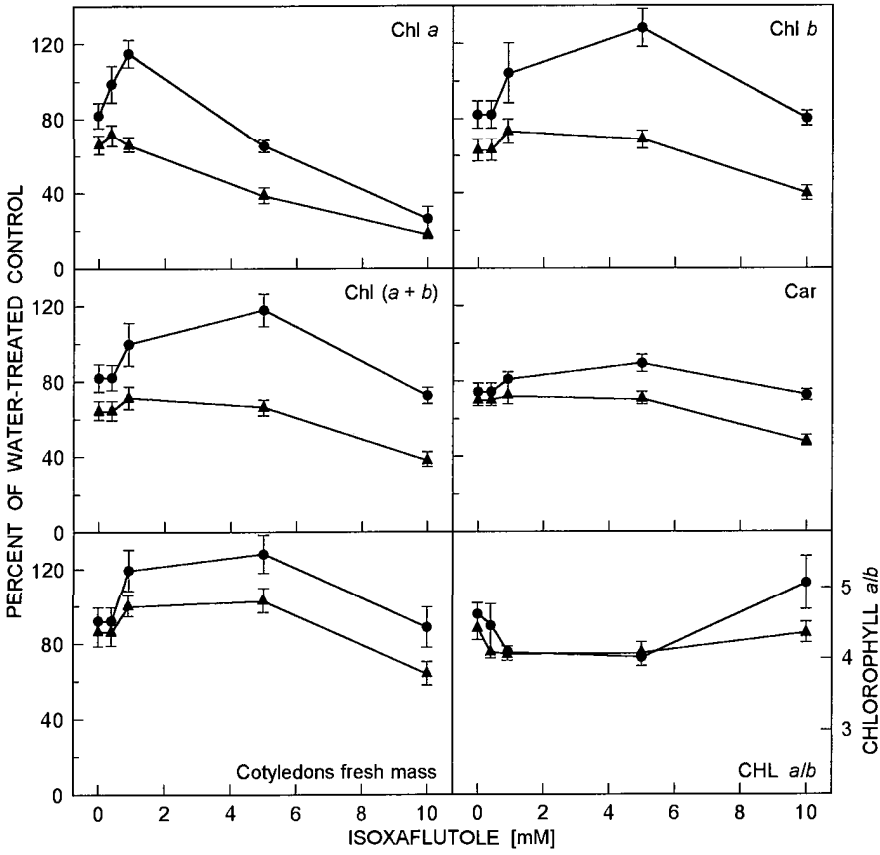

Fig. 1. The influence of irradiance and isoxaflutole on fresh mass, chlorophyll (Chl) *a*, Chl *b*, Chl (*a*+*b*), and carotenoid (Car) contents in 5-d-old cucumber cotyledons incubated in various isoxaflutole concentrations in dark for 24 h and exposed to irradiance of 60 (●) or 180 (▲) μmol m^{-2} s^{-1} for 28 h. Vertical bars represent standard error of means.

contents and cotyledon fresh mass increased at 0.5 to 5.0 mM concentrations of IFT under 60 μmol m^{-2} s^{-1} and at 0.5 to 1.0 mM concentrations under 180 μmol m^{-2} s^{-1}. Chl *a* content was influenced (increase up to 1 mM followed by decrease beyond 1.0 mM concentration) by IFT first and to greater extent as compared to Chl *b* at both irradiances, suggesting that the low Car content reduces the Chl *a* content first. Low Car content resulted in photooxidation of Chl *a* and thereby in reduced Chl *a/b* ratio (Fig. 1). Nandihalli and Bhowmik (1992) reported an increase in Chl *a/b* ratio due to ICIA-0051 in cucumber cotyledons. The inhibition of Chl *a* synthesis by IFT may be due to either an insufficient chlorophyllide *a* pool or to herbicide interference with

the biosynthetic steps leading to Chl *b*. Increased reduction of Car content as compared to the reduction of Chl (*a*+*b*) content suggests that IFT primarily inhibits Car biosynthesis. Similarly, fresh mass of cotyledon increased at 1 and 5 mM IFT at both irradiances. However, IFT beyond 5 mM resulted in reduced cotyledon fresh mass.

ALA and HGA treatment: Pretreatment of cotyledons with 100 μM ALA (treatment pairs 1-2, 4-5, and 7-8) did not reverse the Chl inhibition (Table 2). Yet 100 μM HGA (treatment pairs 1-3, 4-6, and 7-9) resulted in reversal of inhibition of Chl

Table 2. Effects of 100 μM 5-aminolevulinic acid (ALA), and 100 μM homogentisic acid lactone (HGA) in reversal of the inhibitory effects of 5 mM isoxaflutole (IFT) on chlorophyll (Chl) and carotenoid (Car) biosyntheses in 5-d-old cucumber cotyledons (values presented as % of water treated control). Means within the same column followed by the same letter are not significantly different as determined by Duncan's multiple range test at 5 % level.

Treatment no.	Dark	Light	Fresh mass	Chl *a*	*b*	(*a*+*b*)	Car
1	IFT	H_2O	76 cd	65 d	59 de	64 cd	54 efg
2	IFT	ALA	77 cd	55 d	50 e	54 d	50 efg
3	IFT	HGA	91 bc	101 b	95 bc	100 b	77 bc
4	H_2O	IFT	98 b	79 bcd	70 cde	77 bcd	56 def
5	ALA	IFT	99 b	95 bc	86 bcd	93 bc	62 de
6	HGA	IFT	123 a	147 a	143 a	146 a	83 b
7	IFT	IFT	44 e	56 d	52 e	55 d	40 g
8	IFT+ALA	IFT+ALA	68 d	51 d	43 e	49 d	46 fg
9	IFT+HGA	IFT+HGA	89 bcd	94 b	86 bcd	93 b	69 cd
10	H_2O	H_2O	100 b	100 b	100 b	100 b	100 a

biosynthesis. ALA is the precursor for tetrapyrrole in Chl biosynthesis (Granick and Sassa 1971). Conversion of *p*-hydroxyphenylpyruvate to homogentisate is the key step in plastoquinone biosynthetic pathway (Yasunobu *et al.* 1954). Therefore, this reversal of inhibition of Chl biosynthesis suggests that IFT does not inhibit Chl biosynthesis directly. Our results support the findings of Luscombe *et al.* (1995) that IFT acts *via* disruption of Car by inhibiting hydroxyphenylpyruvate dioxygenase activity required for the conversion of *p*-hydroxyphenyl-pyruvate to plastoquinone, a cofactor in Car biosynthesis pathway. 100 μM ALA reverses the inhibition of Chl synthesis induced by 100 g m^{-3} ICIA-0051, a herbicide inhibiting Car synthesis (Nandihalli and Bhowmik 1992). Similarly, Wilkinson (1993) reported naphthalic anhydride [5 mg kg^{-1}(sand)] to significantly reverse Chl *a* and *b* contents decreased by 500 μg kg^{-1} norflurazon concentration and also to induce significant reversal of carotenogenesis inhibition by norflurazon.

In summary, our findings suggest that IFT induces foliar bleaching that is primarily due to inhibition of Car synthesis. Low Car content results in photooxidation of Chl. Chl *a* was reduced more than Chl *b* resulting in reduction in Chl *a*/*b* ratio.

Isoxaflutole inhibits Car biosynthesis first, followed by Chl *a* and Chl *b*. The higher the irradiance the greater is the reduction in Chl and Car contents.

References

Anderson, T.C., Robertson, D.S.: Role of carotenoids in protecting chlorophyll from photodestruction. - Plant Physiol. **35**: 334-339, 1960.

Anonymous: RPA 201772 Technical Bulletin. A New Class of Corn Weed Control. - Rhone-Poulenc Company, Raleigh 1995.

Arnon, D.I., Allen, M.B., Whatley, F.: Photosynthesis by isolated chloroplast. - Biochim. biophys. Acta **20**: 449-461, 1956.

Bartels, P.G., Watson, C.W.: Inhibition of carotenoid synthesis by fluridone and norflurazon. - Weed Sci. **26**: 198-203, 1978.

Bhowmik, P.C., Nandihalli, U.B.: Influence of SC-0774 on chlorophyll and carotenoid biosynthesis. - Proc. Northeast. Weed Sci. Soc. **44**: 52-56, 1990.

Bhowmik, P.C., Prostak, R.G, Drohen, J.A.: Performance of early pre-plant treatments of EXP 31130A in annual weed control in no-tillage corn. - Proc. Northeast. Weed Sci. Soc. **50**: 149, 1996a.

Bhowmik, P.C., Prostak, R.G., Kushwaha, S.: Preemergence activity of EXP 31130A in annual weed control in corn. - Proc. Northeast. Weed Sci. Soc. **50**: 150, 1996b.

Burns, E.R., Buchanan, G.A., Carter, M.C.: Inhibition of carotenoid synthesis as a mechanism of action of amitrol, dichlormate, and pyriclor. - Plant Physiol. **47**: 144-148, 1971.

Cain, P.A, Cramp, S.M., Little, G.M., Luscombe, B.M.: EP Patent 0527036, 1993.

Dahlin, C., Timko, M.P.: Integration of nuclear-encoded proteins into pea thylakoids with different pigment contents. - Physiol. Plant. **91**: 212-218, 1994.

Devlin, R.M., Kisiel, M.J., Karczmarczyk, S.J.: Chlorophyll production and chloroplast development in norflurazon-treated plants. - Weed Res. **16**: 125-129, 1976.

Granick, S., Sassa, S.: Δ-aminolevulinic acid synthetase and the control of heme and chlorophyll synthesis. - In: Vogel, H.J. (ed.): Metabolic Regulation. Vol. V. Pp. 77-141. Academic Press, New York 1971.

Hager, A., Meyer-Bertenrath, T.: Die Isolierung und quantitative Bestimmung der Carotenoide und Chlorophylle von Blättern, Algen und isolierten Chloroplasten mit Hilfe dünnschichtchromatographischer Methoden. - Planta **69**: 198-217, 1966.

Kushwaha, S., Bhowmik, P.C.: Effects of EXP 31130A and FOE 5043 on weed control and corn yield. - Proc. Northeast. Weed Sci. Soc. **51**: 33, 1997.

Luscombe, B.M., Pallet, K.E., Loubiere, P., Millet, J.C., Melgraejo, J., Vrabel, T.E.: RPA 201772: A novel herbicide for broadleaf and grass weed control in maize and sugarcane. - Proceedings 1995 Brighton Crop Protection Conference Weeds. Vol. 1. Pp. 35-42. 1995.

Nandihalli, U.B., Bhowmik, P.C.: Inhibition of chlorophyll and carotenoid biosynthesis by the herbicide ICIA-0051. - Photosynthetica **26**: 355-362, 1992.

Ridley, S.M., Ridley, J.: Interaction of chloroplasts with inhibitors: Location of carotenoid synthesis and inhibition during chloroplast development. - Plant Physiol. **63**: 392-398, 1979.

Vrabel, T.E., Cartier, J.P., Mosier, D.G., Duckworth, W.: A new preemergence herbicide for conventional tillage corn. - Proc. Northeast. Weed Sci. Soc. **50**: 153, 1996a.

Vrabel, T.E., Cartier, J.P., Striegel, W.G., Duckworth, W.: EXP 31130A: A new preemergence herbicide for no-tillage corn. - Proc. Northeast. Weed Sci. Soc. **50**: 154, 1996b.

Wilkinson, R.E.: Naphthalic anhydride partial reversal of carotenogenesis inhibition by norflurazon. - Pestic. Biochem. Physiol. **47**: 81-86, 1993.

Yasunobu, K., Tanaka, T., Knox, W.E., Mason, H.S.: Fed. Proc. Fed. amer. Soc. exp. Biol. **17**: 340, 1954.