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Mechanism of action of sulcotrione, a 4-hydroxyphenylpyruvate dioxygenase inhibitor, in developed plant tissues

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Abstract

The herbicides diuron, fluridone, or sulcotrione differently reduced chlorophyll (Chl) and carotenoid (Car) contents. Four days after herbicide treatment, application of sulcotrione resulted in a Chl/Car ratio of 5.88, similar as in untreated controls; diuron resulted in ratio of 5.24, while fluridone induced a greater diminution in Car contents and yielded a final ratio of 7.02. Sulcotrione induced a more rapid decrease than fluridone did in the quantum yield of photosystem 2 (PS2) as monitored by Chl fluorescence. Measurements of DPIP reduction with isolated thylakoids indicated that sulcotrione was a more effective inhibitor of the Hill reaction in cucumber, a herbicide sensitive species, than in maize, a herbicide-insensitive species. These results are consistent with the view that inhibition of electron transport *via* reduction in plastoquinone contents in plants leads to the major herbicidal effect of sulcotrione in mature green tissues.

Additional key words: carotenoid biosynthesis; chlorophyll/carotenoid ratio; cucumber; *Cucumis sativa*; fluridone; maize; photosynthetic electron transport; *Zea mays*.

Introduction

Direct or indirect inhibition of carotenoid (Car) biosynthesis produces a bleaching symptom in plants (Böger 1996). The chemicals inhibiting directly Car biosynthesis are norflurazon, fluridone, and diflufenican that primarily attack phytoene desaturase (PD) (Kowalczyk-Schröder and Sandmann 1992, Böger 1996). When the content of coloured Cars is decreased by these herbicides (Böger 1996, Jung *et al.* 1999), protective functions of Cars in plants cannot be maintained. The direct consequences include an albino growth and a photobleaching (necrosis and desiccation) by a rapid destruction of photosynthetic membranes by excessive reactive oxygens (Markgraf and Oelmüller 1991, Böger 1996, Hess 2000). On the other hand, the chemicals inhibiting indirectly Car biosynthesis are triketone compounds whose primarily target is 4-hydroxyphenylpyruvate dioxygenase (HPPD, EC 1.14.2.2.) (Prisbylla *et al.* 1993, Lee *et al.* 1997). These HPPD inhibitors cause plants to bleach similarly as the PD inhibitors. Initial research on the action mechanism of triketone compounds reported a decrease in contents of carotenes and Chls with a concomitant accumulation of phytoene *in vivo* (Mayonado *et al.* 1989, Sandmann *et al.* 1990), suggesting a classical inhibition of phytoene desaturase. However, surprisingly, the site of action is not necessarily the PD (Sandmann *et al.* 1990, Prisbylla *et al.* 1993, Secor 1994).

The enzyme HPPD is associated with a step in the catabolic metabolism of phenylalanine and tyrosine in most organisms (Crouch *et al.* 1997). It catalyses the conversion of 4-hydroxyphenylpyruvate and oxygen molecule to homogentisate and carbon dioxide, the first committed step in the biosynthesis of plastoquinone (PQ) and tocopherol (Crouch *et al.* 1997). Sulcotrione belongs to a new class of herbicides, which inhibit HPPD (Secor 1994). This causes significant reduction of the PQ and tocopherol contents in treated plant tissues, which further leads to reduction of phytoene desaturase activity and Car contents in chloroplasts (Prisbylla *et al.* 1993). However, physiological actions due to the PQ deficiency would not be manifested only by a reduction of Cars. PQ participating in various physiological processes in plants is a redox component in photosynthesis (electron carriers), an

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Abbreviations: AI – active ingredients; Car – carotenoid; Chl – chlorophyll; DPIP – 2,6-dichlorophenolindophenol; F_m' – maximal fluorescence yield of an irradiated sample; F_t – steady-state fluorescence yield at any given time; HPPD – 4-hydroxyphenylpyruvate dioxygenase; PD – phytoene desaturase; PET – photosynthetic electron transport; PS2 – photosystem 2; PQ – plastoquinone; Q_B – secondary quinone electron acceptor; q_P – photochemical fluorescence quenching.

enzyme cofactor or an antioxidant (Mayer *et al.* 1990, Hundall *et al.* 1995, Norris *et al.* 1995, Wanke *et al.* 2000). In a detailed observation of the herbicide-treated plants, the pattern of plant bleaching associated with inhibition of HPPD is distinctive and not identical to those tissues treated with herbicides such as norflurazon and fluridone which inhibit phytoene desaturase directly (Mayonado *et al.* 1989, Sandmann *et al.* 1990). In addition, our preliminary experiment showed that HPPD inhibitors induced a faster necrosis/desiccation in developed green tissues as well as a more apparent albinism in undeveloped tissues than the PD inhibitors. This difference might elucidate the action mechanism of HPPD inhibitors. With this hypothesis, Kim *et al.* (1999) proposed that the reduction of PQ contents by sulcotrione might have a larger effect on photosynthetic electron transport (PET) than phytoene desaturase, especially in mature green tissues.

The purpose of this report was to present additional evidence that sulcotrione has in mature green tissues an action mechanism different from that of PD inhibitors.

Materials and methods

Chemicals: Technical grade diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] was purchased from *Sigma Chemical Co.* (St. Louis, MO, USA). Fluridone [1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-pyridin-4(1*H*)-one] and sulcotrione [2-(2-chloro-4-methane-sulfonylbenzoyl) cyclohexane-1,3-dione] were a gift from *Dow Agrosciences* (Indiana, USA) and *Syngenta* (Surrey, UK), respectively.

Measurements of the *in planta* content of photosynthetic pigments and of the quantum yield of PET: Twenty cucumber seeds were planted in 350 cm^2-area pots and grown in a greenhouse for 8 d. Diuron, fluridone, and sulcotrione were sprayed upon cotyledons at 80 % of full growth at rates of 3, 10, and 50 mg(active ingredients, AI) m^{-2}, respectively. The application rates induced approximately equivalent visual injury (bleaching and desiccation of almost 90 %) at 6 d after treatment in a preliminary greenhouse experiment. Herbicides were dissolved in acetone and diluted with water including a non-ionic surfactant (*Tween 20*) and sprayed at 0.4 cm^3 m^{-2}. Final concentrations of acetone and of *Tween 20* in the solutions were 50.0 and 0.1 %, respectively. The treated plants were grown for 4 d in a controlled growth chamber at 25 °C with a photoperiod of 14 h and irradiance of 55 μmol m^{-2} s^{-1}. Photosystem 2 (PS2) quantum yield [$(F_m' - F_t)/F_m'$] was measured with a pulse amplitude modulation fluorometer (*PAM-2000*, *Walz*, Effeltrich, Germany) at 0.1, 1, 2, 3, and 4 d after herbicide treatment and calculated according to Genty *et al.* (1989). Contents of Chl and Cars in the cotyledon tissues (per 1 g fresh mass) were extracted 4 d after herbicide treatment with 12 cm^3 of acetone and quantitated by the method of Lichtenthaler (1987).

Herbicidal activity after foliar treatment: 350 cm^2-area pots were filled with sterilised, sandy loam soil (pH 6.0) including 1.0 % organic matter and appropriate amount of fertilisers. Cucumber (*Cucumis sativa* L.) and maize (*Zea mays* L.) were sown and kept in a greenhouse at 30/20 °C (day/night) with 14 h-photoperiod. Herbicides were dissolved in acetone and diluted with water including a non-ionic surfactant (*Tween 20*) and sprayed, 10 d after inoculation, at 0.4 cm^3 m^{-2}. Final concentrations of acetone and of *Tween-20* in the solutions were 50.0 and 0.1 %, respectively. The biological activity of herbicides was evaluated visually 6 d after treatment using a scale between 0 and 100 where '0' represented no herbicidal effect and '100' represented complete death.

Inhibition of the Hill reaction: 2,6-dichlorophenolindiphenol (DPIP) reduction assays were conducted according to the modified method of Anderson *et al.* (1994). Cucumber or maize leaves (10 g fresh mass) were ground using a sample mixer for 15 s at high speed in 100 cm^3 of extraction buffer containing 100 mM tricine at pH 7.8, 400 mM sorbitol, 3 mM $MgCl_2$, and 2 mM sodium ascorbate. The extract was filtered twice through *Miracloth* and centrifuged at 1 100×*g* for 5 min. The supernatant was discarded and the pellet was gently re-suspended in washing buffer consisting of 100 mM Tricine at pH 7.8, 10 mM NaCl, and 6.8 mM $MgCl_2$. The solution was re-centrifuged at 2 000×*g* for 5 min, and the pellet was re-suspended to provide a concentration of 0.4 mg Chl per 1 cm^3 of suspension medium consisting of 10 mM Tricine at pH 7.8, 100 mM sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$. The assay was initiated by adding 1 cm^3 of thylakoid membrane suspension, 8.5 cm^3 assay buffer, and 0.5 cm^3 herbicide. The assay medium consisted of 10 mM Tricine at pH 7.8, 100 mM sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl, 220 mg m^{-3} of gramicidin A, 95 μM DPIP, and herbicides. DPIP reduction was calculated from the change of absorbance before and after irradiation (640 μmol m^{-1} s^{-1}) for 2 min.

Results and discussion

Herbicide-induced changes in the contents of photosynthetic pigments and in the quantum yield of PET: In order to better understand the mode of action of sulcotrione, its effects on pigment contents and photosynthetic quantum yield in the matured cucumber cotyledons were compared with those of other herbicides that induce visually similar symptoms such as fluridone, a PD inhibitor, and diuron, an electron transport inhibitor in PS2. Experiments were carried out under low irradiance to better clarify the causes of photobleaching. Both Chl and Car contents were decreased in different extent by all herbicide treatments (Table 1). Four days after treatment, sulcotrione resulted in a Chl/Car ratio of 5.88, similar as in untreated controls, while diuron resulted in 5.24, and fluridone resulted in a greater lowering of Car content and yielded a final ratio of 7.02. This suggested that the action mechanism of sulcotrione could be more 'diuron-like' than 'fluridone-like'.

Table 1. Changes in contents of chlorophylls (Chls) and carotenoids (Cars) [g m^{-3}] of cucumber cotyledons by diuron, fluridone, and sulcotrione. Cucumber cotyledons at 80 % of full growth were treated with three different herbicides at rates to give approximately equivalent herbicidal effect: 3 mg(AI) m^{-2} of diuron, 10 mg(AI) m^{-2} of fluridone, and 50 mg(AI) m^{-2} of sulcotrione. Chls and Cars were quantified 4 d after herbicide treatment. Means ± SE of three replicates.

	Chls	Cars	Chls/Cars
Control	115.8±5.9	19.7±1.1	5.88
Diuron	81.7±1.6	15.6±0.4	5.24
Fluridone	89.9±2.2	12.8±0.2	7.02
Sulcotrione	96.8±4.5	16.5±0.8	5.88

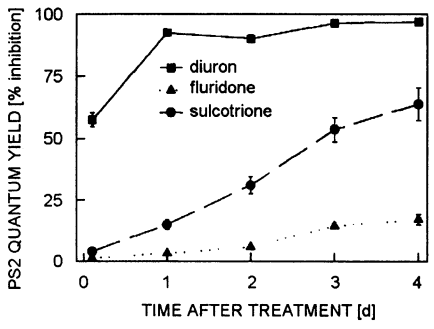

Fig. 1. Inhibition of PS2 quantum yield determined by chlorophyll fluorescence in cucumber cotyledons treated with diuron (3 mg m^{-2}), fluridone (10 mg m^{-2}), and sulcotrione (50 mg m^{-2}).

Environmental stresses, herbicidal compounds, and other factors may change the Chl/Car ratio. When an inhibitor of Car biosynthesis is applied to etiolated cucumber cotyledons, it usually induces a greater decrease in Cars than Chls under low irradiance. In contrast, a Chl biosynthesis inhibitor induces a greater decrease in Chl than Car contents (Kim *et al.* 1990, 2000). It is expected that herbicides inhibiting electron flows in PS2, even if they do not target a photosynthetic pigment biosynthesis, would change the Chl/Car ratios. When the electron flow is blocked by herbicide binding in the Q_B pocket of the D1 protein, the charge pair formed decays by a charge recombination pathway that involves formation of a Chl triplet in the heart of the reaction centre. This triplet is thus able to react with triplet oxygen to give singlet oxygen. The singlet oxygen formed in the reaction centre damages adjacent Chl-bearing proteins, leading to the disconnection of the Chls from their energy transfer system. These disconnected Chls will then be involved in the photogeneration of more singlet oxygen, and if excessive, the Chls can be self-decomposed (Hess 2000, Rutherford and Krieger-Liszkay 2001). Thus the diminution of Chl contents rather than Car contents seems to be more sensitive to diuron. Considering that sulcotrione inhibits PQ biosynthesis and PQ acts as electron carrier in PET, it is plausible that the change of Chl/Car ratio in sulcotrione-treated tissues would be closer to that of diuron. In this study, such a tendency was shown, and sulcotrione treatment resulted in a Chl/Car ratio of 5.88 and diuron treatment in a ratio of 5.24 (Table 1). Here the ratio was not completely in accord with both chemical treatments, probably because of Car reduction through PQ deficiency induced by sulcotrione.

Under the same conditions as the experiment on Chl/Car ratio, an inhibition of PS2 quantum yield was investigated by the pulse amplitude modulation fluorometer in a time course after treatment with the three herbicides. Chl *a* fluorescence is a good indicator for monitoring an initial response of herbicides associated with PET because it shows, non-destructively and sensitively, the degree of PS2 damage in plants (Gleiter and Renger 1993, Schreiber *et al.* 1996).

Analysis of herbicide-induced changes in Chl *a* fluorescence indicated that the fastest inhibition of PS2 quantum yield was recorded when using diuron. This is probably because diuron inhibits the quantum yield of PS2 through direct binding at Q_B site of PS2 but the other herbicides through inhibition of metabolite biosynthesis. Of the metabolite biosynthesis inhibitors, sulcotrione exerted a much more rapid inhibitory effect on the quantum yield of PS2 than fluridone did (Fig. 1). The inhibition of quantum yield by the three herbicides paralleled the extent to which they reduced the Chl content relative to Cars (Table 1). Thus, although Car contents were less diminished 4 d after treatment with sulcotrione than fluridone, PS2 quantum yield was inhibited more rapidly in the sulcotrione treatment. Indirect inhibition of PS2 by fluridone can be related to Car deficiency induced by inhibition of phytoene desaturase (Sandmann *et al.* 1996, Trebst and Depka 1997). If sulcotrione-mediated inhibi-

tion of PS2 were induced by a similar mechanism, sulcotrione should inhibit PS2 less than fluridone. However, we observed an opposite effect. Kim *et al.* (1999) reported that Chl *a* fluorescence in sulcotrione-treated cucumber cotyledons was closer to that induced by diuron than by norflurazon. That is, diuron decreased quantum yield of electron transport and photochemical fluorescence quenching (q_P) immediately but increased the steady-state fluorescence yield (F_t) greatly in the light, with a decreased magnitude upon dark adaptation. The profile of changes of q_P and F_t in sulcotrione-treated tissues was similar to that in diuron-treated tissues, however, the decline in quantum yield was faster in the diuron-treated tissues. Norflurazon also decreased quantum yield as sulcotrione did, but induced a slight increase in F_t and a prominent increase in non-photochemical fluorescence quenching (q_N) in contrast to those of diuron and sulcotrione (Kim *et al.* 1999).

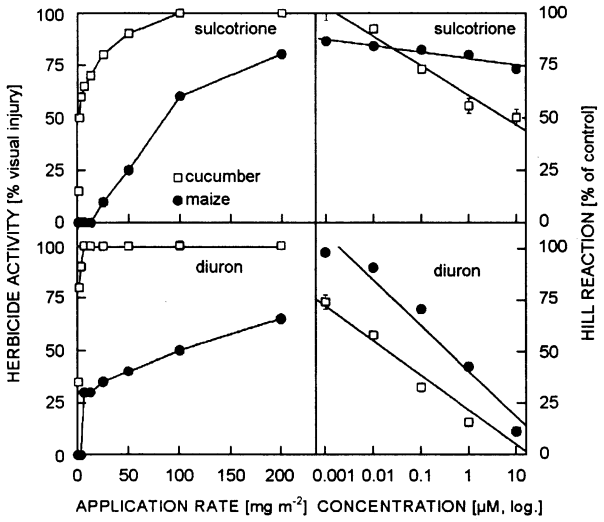

Fig. 2. Effects of sulcotrione and diuron on the herbicidal activity in a greenhouse and on the Hill reaction in thylakoid membranes isolated from cucumber and maize leaves. Herbicidal effect was determined 6 d after application.

Relation between herbicidal activity *in vivo* and inhibition of the Hill reaction: Effects of sulcotrione on the herbicidal activity under greenhouse conditions and on the Hill reaction were compared. Consistent with the result of whole plant response in each species, the Hill reaction following treatment with sulcotrione was more strongly inhibited in the isolated thylakoids of cucumber than of maize (Fig. 2). This is consistent with the major effect of sulcotrione in mature tissues. Considering that PQ is a mediator of PET as well as an essential cofactor for phytoene desaturase (Mayer *et al.* 1990, Norris *et al.* 1995), it could be expected that sulcotrione should indirectly affect both processes. By the way, sulcotrione should probably exert a relatively greater effect on PET in mature green tissues, since rates of *de novo* Car biosynthesis in mature green tissues are relatively low but rates of photosynthesis remain fairly high (Hess 2000). Wanke *et al.* (2000) demonstrated that the turnover rate of PQ in spinach cells was high with a half-life of about 15 h. These results are consistent with the typical bleaching (whitening) symptoms induced by sulcotrione, which are caused by a Car deficiency-induced degradation of Chl (Sandmann *et al.* 1990, Böger 1996). The symptoms are observed in the developing tissues rather than in the mature tissues of treated plants.

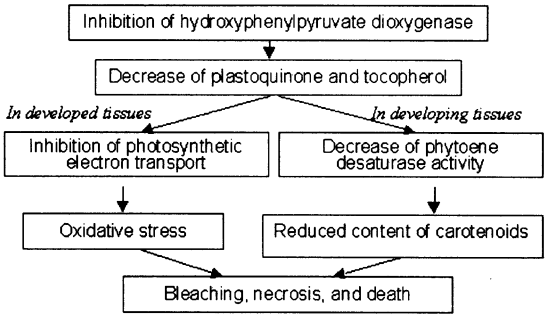

Fig. 3. Possible action mechanism of sulcotrione in different developmental stages of plants.

In conclusion, we propose that the major herbicidal consequences of sulcotrione differ between mature and immature leaf tissues. In developing tissues such as new leaves, the major effect is inhibition of Car biosynthesis, whereas in older leaves and other mature tissues the major effect is inhibition of PET (Fig. 3) through a deficiency of PQ pools. However, as a minor effect, PQ deficiency may also lead to a decrease of Car contents induced by PD inhibition, and the low content of Cars contributes a little to the herbicidal efficacy in mature green tissues.

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