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THE EFFECT OF CHLOROPHYLL-BLEACHING HERBICIDES ON GROWTH, CAROTENOID AND DIOSGENIN LEVELS IN CELL SUSPENSION CULTURES OF *DIOSCOREA DELTOIDEA*

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Key Word Index—*Dioscorea deltoidea*; Dioscoreaceae; suspension cultures; diosgenin production; phenylpyridazinone herbicides; norflurazon; amitrole; difunon.

Abstract—Bleaching herbicides of the phenylpyridazinone group (norflurazon, metflurazon, SAN 9774 and SAN 9785) and difunon and amitrole were added to cell suspension cultures of *Dioscorea deltoidea* at 1 μM. Difunon inhibited growth and carotenoid biosynthesis and metflurazon inhibited growth. Norflurazon was the only herbicide tested that influenced diosgenin production. Norflurazon increased the rate of diosgenin biosynthesis so that 180 mg/l were obtained after 14 days of incubation as compared to 30 days for the control to reach the same level.

INTRODUCTION

Diosgenin is used in the commercial production of female steroid hormones and steroidal pharmaceuticals. The highest level of the steroidal sapogenin diosgenin, are found in cell suspension cultures of *Dioscorea deltoidea* A-51 when growth has ceased [1–3]. The early biosynthetic precursors of diosgenin are common to many other metabolites (phytol, carotenoids, chlorophyll, terpenes, etc.) [4] and we surmised that inhibiting the formation of one or more of these metabolites might increase the level of diosgenin.

Some herbicides of the phenylpyridazinone group (metflurazon and norflurazon) have been shown to inhibit carotenoid biosynthesis in higher plants [5–7], while others inhibit photosynthetic electron transport [7]. The primary action of norflurazon, metflurazon [6] and difunon [8] is to inhibit the enzyme phytoene synthetase [6]. Amitrole [9] and difunon also have bleaching effects. Amitrole has been shown to inhibit cyclisation of lycopene to α- and β-carotene [9]. The herbicides used are shown in Fig. 1.

RESULTS AND DISCUSSION

Effects of various herbicides on growth and carotenoid biosynthesis in D. deltoidea *suspension cultures*

Different herbicides were added to cell suspensions of *D. deltoidea* on the second day of growth (Fig. 2). Difunon and norflurazon inhibited growth four days after addition and upon further incubation there was a decrease in biomass (Fig. 2a). Amitrole did not inhibit growth. Norflurazon caused a total inhibition of carotenoid biosynthesis three days after addition and difunon five days after addition (Fig. 2b). Amitrole also inhibited carotenoid biosynthesis five days after addition and after that the carotene concentration decreased. These results show that the herbicides were able to influence both growth and carotenoid biosynthesis in cell suspension cultures of *D. deltoidea*.

Other phenylpyridazinones were added to cell suspensions of *D. deltoidea* on the second day of growth and their influence on growth was measured on the fourth and tenth day (Table 1). SAN 9758 did not influence growth.

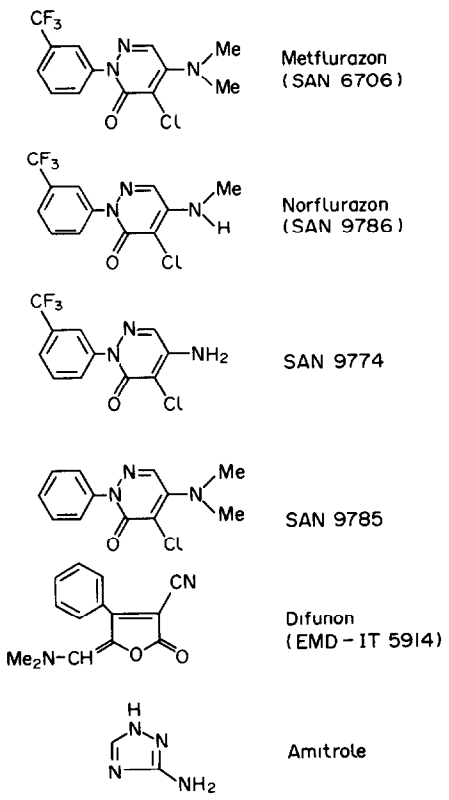

Fig. 1. Structures of herbicides added to suspension cultures of *D. deltoidea*.

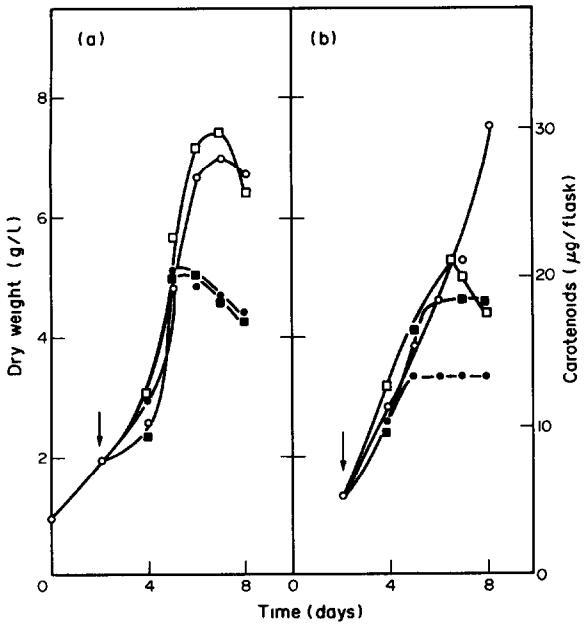

Fig. 2. The effect of three herbicides on growth (a) and carotenoid production (b) of cell suspension cultures of *D. deltoidea*. ○——○, Control; ●——●, 1 μM norflurazon, □——□, 1 μM amitrole; ■——■, 1 μM difunon.

Metflurazon gave only 50% of the biomass and SAN 9774 showed a decrease in biomass after four days but after ten days this effect was lost. SAN 9774 is quite active on inhibiting plastid electron transport but only partially inhibits carotenoid synthesis at high concentrations [7]. Thus these results are not unexpected.

The effect of various herbicides on diosgenin production

Various herbicides (1 μM) were added to cell suspensions of *D. deltoidea* and the diosgenin concentration was measured after varying durations of incubation (Table 1). Metflurazon inhibited diosgenin production at all times while SAN 9774 and SAN 9785 inhibited diosgenin production when the cells were assayed on the fourth but not on the tenth day. Difunon slightly inhibited diosgenin production whereas amitrole had no effect. Norflurazon was the only herbicide that had a stimulating effect on diosgenin biosynthesis.

Norflurazon (1 μM) was added on day 7 and day 11 of growth (Fig. 3). Addition of norflurazon on day 7 inhibited growth and an increased rate in diosgenin biosynthesis was observed. A 115% increase in the concentration of diosgenin was obtained in 18 days as compared to 100% in the control after 30 days. Addition on day 11 gave a faster decrease in biomass but the production rate of diosgenin was not altered.

The results obtained with metflurazon were particularly surprising. This compound is thought to be directly metabolized to norflurazon yet their effects in *D. deltoidea* are different. It is possible that different species metabolize this compound in different ways. SAN 9774 and SAN 9785 were used as controls. They are known to act at different sites; presumably on photosynthetic electron transport [7, 8]. Therefore it was not surprising that they did not stimulate diosgenin synthesis. The reasons for the transient inhibitions of growth by metflurazon and SAN 9774 are less clear. These cells are not green since they are cultured in the dark. Herbicides acting solely on photosynthesis whether at the level of carotenoid biosynthesis

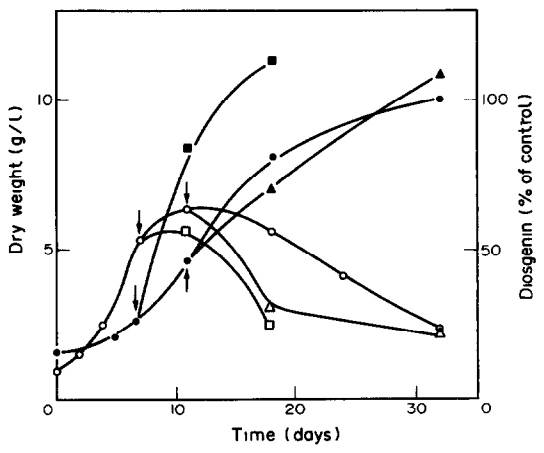

Fig. 3. The effect of norflurazon (1 μM) on growth (open symbols) and diosgenin production (full symbols) by cell suspension cultures of *D. deltoidea*. ○ and ●, Control; □ and ■, addition of norflurazon on day 7; △ and ▲, addition of norflurazon on day 11.

Table 1. Effect of various herbicides on growth and diosgenin production by *D. deltoidea* suspension cultures

Compound	Day of addition	Biomass (g cell dry wt/l)* Day of harvest 4	10	Diosgenin (mg/l) Day of harvest 4	10	14
Control	—	2 7	6.0	22	87	132
Metflurazon	2	1.2	2 6	16 (72)	18 (21)	—
Norflurazon	2	—	—	35 (159)	105 (120)	180 (136)
SAN 9774	2	1 3	6.0	4 (18)	70 (80)	—
SAN 9785	2	2.5	5 5	8 (36)	81 (93)	—
Difunon	5	—	—	—	92 (105)	109 (82)
Amitrole	5	—	—	—	95 (109)	130 (98)

*Biomass on day 2 was 1.9 g cell dry wt/l

†Diosgenin content on day 2 was 1.5 mg/l. Numbers in parentheses are % of control.

or electron transport should not affect growth. The inhibition of growth during the exponential growth phase can be explained in at least two ways: (1) norflurazon acts at more than one site in the cell, (2) certain compounds from the carotenoid pathway are needed for continued growth and division. This inhibition limits the use of norflurazon to judiciously chosen time of application when it will not affect growth as much but will divert metabolism towards the steroid pathways.

EXPERIMENTAL

Cultures of *D. deltoidea* cells were maintained and grown in the dark as described previously [1]. All experiments were performed in MS medium containing 0.1 mg/l 2,4-dichlorophenoxyacetic acid and 15 g/l sucrose. Dry wt determinations and HPLC assay of diosgenin were performed as described previously [1, 12].

Carotenoids were measured at 466 nm (Gilford, Oberlin, Ohio) after $CHCl_3$ extraction of harvested and lyophilized cells. All manipulations were done in the dark. β-Carotene was used as a standard.

Norflurazon, metflurazon, SAN 9774 and SAN 9785 were kindly provided by Dr. H. P. Schelling, Sandoz AG, Basel, Switzerland. Difunon was kindly provided by Dr. R. Mengel, Celamerck, Ingelheim, F.R.G. β-Carotene and amitrole were purchased from Sigma, St. Louis, Mo., U.S.A.

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