Phytochemistry, 1975, Vol 14 pp 2551-2554 Pergamon Press Printed in England

EFFECT OF DARKNESS ON ISOPEROXIDASES IN TOBACCO TISSUE CULTURES

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(*Revised received* 24 *April* 1975)

Key Word Index—*Nicotiana tabaccum*, Solanaceae, tobacco, isoperoxidase patterns

Abstract—In order to study the effect of light on the tobacco tissue culture WR-132, 5 passages (10 days' growth per passage) of these cells were grown in darkness, and 3 passages were separately grown in intense light (16000 lx). All other growth conditions were the same. The resulting isoperoxidase patterns present in these cells and in their growth media were analyzed at 2-day intervals during this period and then compared with the isoperoxidase patterns of cells grown under dim light conditions (10 lx). A new cathodic isoperoxidase (C_n) appeared in the medium within 2 days after the cells were placed in the dark. C_n was present in all media of WR-132 cell cultures analyzed throughout the 5 passages grown in darkness. The fifth passage in darkness produced total cessation of growth (apparent death). C_n increased and new anodic isoperoxidases A_a, A_b, A_d and A_e appeared in the media as the cells approached death in darkness.

INTRODUCTION

McCown and coworkers [1] have reported marked differences in the isoperoxidase ("peroxidase isoenzyme") patterns of *Dianthus* callus cultures grown under 4 combinations of environments: dark and light (fluorescent light of 2000 lx), warm and cold (0–5°). Of 4 enzymes studied, only peroxidases were recovered from the medium and they matched ones found in the callus and stem, but they were not influenced qualitatively by the growth conditions involving changes in temperature and/or light. De Jong *et al.* [2], on the other hand, found that the major isoperoxidases released into the medium by WR-132 tobacco suspension cells varied with culture temperature. The latter workers also found little correlation between the excreted isoperoxidases and the intracellular ones at 13°. They further reported that the major isoperoxidase released into the medium of the cells grown at 13° had a different electrophoretic mobility from that of any of the isoperoxidases present in the cells at that temperature. Parish [3] noted that synthesis of all isoperoxidases was stimulated in light-grown coleoptiles of wheat and inhibited in light-grown first leaves. Brulfert and Trippi [4] found that the number of isoperoxidases in leaves increased to a maximum more rapidly in plants undergoing a long photoperiod than in those undergoing a short photoperiod.

The present study was undertaken to learn more about the effect of darkness, low and intense light on the production of the isoperoxidases of WR-132 tobacco suspension cells.

RESULTS

Cells grown in darkness

Growth and color. Fresh weights of 2-day-old WR-132 cells grown in darkness (dark cells) were found to be slightly higher in the first 4 passages than 2-day-old cells grown under the usual low intensity light (10 lx cells). In 4-day cells of each, little difference was observed. From day 6–10,

Table 1. Isoperoxidases in the media of dark cells

	Relative intensity of staining*										
	Isoperoxidase										
	C_n	C_4	C_3	C_a	C_2	A_a	A_b	A_c	A_d	A_e	A_f
	Relative mobility†										
Age passage no.	1·0	0·88	0·73	0·5	0·27	0·25	0·4	0·5	0·61	0·81	1·2
10 day normal	—	H	W	—	H	—	—	H	—	—	—
2 day I dark	M	VW	VW	—	VW	—	—	M	—	—	—
10 day I dark	VW	H	W	—	H	—	—	VH	—	—	—
4 day II dark	M	VW	VW	—	VW	—	—	M	—	—	—
10 day II dark	W	H	W	—	M	—	—	VH	—	—	—
4 day III dark	VW	VW	VW	—	VW	—	—	M	—	—	VW
10 day III dark	VW	H	W	—	M	—	—	M	—	—	VW
4 day IV dark	M	VW	VW	—	VW	—	—	W	—	—	VW
2 day V dark	H	M	W	—	W	VW	VW	W	VW	VW	—
10 day V dark	H	VH	M	VW	W	W	W	W	VW	W	—

Cells were grown for 10 days in weak light (normal), and then grown for 5 passages (I–V) of 10 days each in the dark. Cells were harvested at intervals, and the peroxidases in the media were separated by starch gel electrophoresis.

* Relative intensities, using 3-amino-9-ethyl carbazole as substrate, are given as: VH = very heavy; H = heavy; M = moderate; W = weak; VW = very weak; and — = not detected.

† Relative mobility of each peroxidase was calculated by dividing the distance from the origin traveled by isoperoxidase C_n into the distance traveled from the origin by the isoperoxidase named.

however, the fresh weights of cells in all 4 passages in darkness were less than in 10 lx cells. In each of the first 4 passages, growth of cells increased from day 2 to 10. In the 5th passage of the dark cells, growth ceased. No further growth could be observed when the cells were placed under 10 lx, both with new or old medium.

Cells grown under 10 lx were bright yellow, while the medium was greenish-yellow in color. For dark cells, these same colors were observed for the first 3 passages. However, beginning with the 4th passage in darkness, differences appeared in the color both of the cells and of the medium. The color of the dark cells changed to brown, and the medium turned to light brown after day 8 of the 4th passage. During the 5th passage in darkness, the cells remained brown.

Isoperoxidase patterns of media. After the usual growth conditions in 10 lx, the culture medium showed only 1 anodic isoperoxidase zone (called A_c) and 3 cathodics (C_2, C_3, and C_4) (Table 1). In the medium in which the dark cells were grown, a new cathodic isoperoxidase band, designated as C_n, appeared in addition to the 3 cathodic bands usually found in the 10 lx cell medium. Changes in the amounts of this and other isoperoxidases with age, as estimated from staining intensities, are indicated in Table 1. The only anodic isoperoxidase (A_c) found in the media from 10 lx cells was also found in media from all passages of dark cells. In dark cells for 3 or more consecutive passages, additional anodic isoperoxidases appeared in the medium. A weakly staining anodic band, called A_f, appeared in the 3rd and 4th passages of dark cells, but did not appear in the non-growing 5th passage. Two other weak bands A_d and A_e could be seen in the 4th passage 8-day and 10-day media, as well as in media of the 5th passage cells. An additional 2 anodic bands, A_a and A_b, appeared in the 5th passage media. Their concentration increased with time in that final passage.

Isoperoxidase pattern of extracts of cells. No qualitative differences in the isoperoxidase pattern could be found in the extracts of cells of days 2, 4, 6, 8, or 10, when grown under 10 lx, or in the extracts of dark cells through 3 passages when compared with the extracts of 10 lx cells. Isoperoxidases C_1, C_2, C_3 and C_4 were found in all these cell extracts. An additional cathodic band (called C_a) which appeared between C_2 and C_3 was also found in the extracts (Table 2). The new

Table 2 Isoperoxidases in extracts of dark cells

	Relative intensity of staining*								
	Isoperoxidase								
	C_n	C_4	C_3	C_a	C_2	C_1	A_a	A_b	A_c
	Relative mobility†								
Age–passage no	1 0	0 88	0 73	0 5	0 27	0 17	0 25	0 4	0 81
10 day normal	—	M	W	VW	VW	W	—	—	—
10 day I dark	—	M	W	VW	VW	W	—	—	—
10 day II dark	—	M	W	VW	VW	W	—	—	—
10 day III dark	—	M	W	VW	VW	M	—	—	—
4 day IV dark	VW	M	W	VW	VW	M	VW	VW	VW
10 day IV dark	M	VH	W	W	VW	M	M	M	M
10 day V dark	W	VH	W	W	W	H	M	M	M

Cells were grown for 10 days in weak light (normal), and then grown for 5 passages (I–V) of 10 days each in the dark Cells were harvested at intervals, and the peroxidases of the cell extracts were separated by starch gel electrophoresis

* Relative intensities, using 3-amino-9-ethyl carbazole as substrate, are given as VH = very heavy, H = heavy, M = moderate, W = weak, VW = very weak, and — = not detected

† Relative mobility of each peroxidase was calculated by dividing the distance from the origin traveled by isoperoxidase C_n into the distance traveled from the origin by the isoperoxidase named

cathodic band, C_n, which had been found in the medium, as indicated earlier, could be demonstrated in the cell extracts of the 4th and 5th passages of the dark cells. Changes in the concentrations of these isoperoxidases with time are indicated in Table 2.

Although no anodics appeared in the dark cell extracts from the first 3 passages, three anodic peroxidases, A_a, A_b and A_c, appeared in the 4th and 5th passages (Table 2). These anodic isoperoxidases had the same mobilities as the A_a, A_b, and A_c found in the medium.

Cells grown under intense light

In the media of cells grown under 16000 lx, the isoperoxidase patterns contained the same bands C_2, C_3 and C_4 as had been found in control (10 lx) media. A cathodic band with the same mobility as C_n found in the media from dark cells could not be found in media from 16000 lx cells until the day 8 of the 2nd passage, and then only in traces from that time until day 10 of the 3rd passage. Isoperoxidase A_c, present in the media from all controls and in the media from all dark cells, was also present in the media from all 16000 lx cells. No significant differences could be seen in the isoperoxidase patterns of cell extracts from 10 lx cells and from all 3 passages of 16000 lx cells

DISCUSSION

Our findings demonstrate that the amount of illumination has a definite influence not only on the appearance of certain isoperoxidases both in the media and in the cells, but also on the release of isoperoxidases into the media by the cells. Most striking is the appearance of a new cathodic isoperoxidase C_n within 2 days after cells are transferred from 10 lx to dark. The amount of C_n in the medium was found to increase substantially during the 5th passage in darkness However, C_n could not be detected in cell extracts of dark cells until the 4th day of the 4th passage, but then increased slightly and persisted thereafter. A very small amount of C_n was also found, after the 8th day of the 2nd passage, in the media of 16000 lx cells. None, however, was detected in the media of the 10 lx cells. In the 4th and 5th passages, 3 anodic isoperoxidases, A_a, A_b and A_c appeared in the cell extracts of the dark cells. These 3 anodic isoperoxidases had the same mobilities as the A_a, A_b and A_c found in the media from 4th and 5th passage cells.

The cessation of growth during the 5th passage of dark cells indicates that some light is necessary for the continued growth of the cells. It appears, however, that even a weak light (*ca* 10 lx) is adequate to maintain growth over many passages for several years. The major, sustained increase in C_n and the appearance of A_a, A_b, and A_c in the media from WR-132 cell cultures as the cells approached death in darkness suggest the need for studying a possible relationship of these isoperoxidases to processes taking place as death of the cells approaches.

EXPERIMENTAL

Growth of tobacco cultures. Tobacco tissue WR-132 (*Nicotiana tabacum* L., var. Xanthi) obtained from Dr. A. C. Olson of USDA, Albany, California, was grown in suspension on the medium of Murashige and Skoog [5]. IAA was replaced by 2,4-D and kinetin was omitted. These cells have been reproducibly subcultured in this laboratory for several years. Wet cells of stock culture (2 g), used as an inoculum, were transferred aseptically to 40 ml medium in a 125 ml conical flask and grown on a reciprocal shaker (70 rpm). After a 10-day growth period on the shaker at 21° under weak (*ca* 10 lx) continuous illumination (10 lx cells), cells sampled for peroxidase patterns were collected by suction filtration. These cells were sampled for peroxidase patterns and part of the remainder grown in complete darkness at 21° in a growth chamber. Cells were sampled every 2 days for several passages (10-day growth per passage). Other 10 lx cells were also transferred to culture under continuous intense light (16000 lx) with all other conditions being the same as for the tissue grown in darkness. Cells were sampled every 2 days for 3 passages after being changed to 16000 lx.

Harvest and preparation of the enzyme. Cells were collected by filtration, using a preweighed glass tube containing a sintered glass filter. The fr. wt of the cells, and the vol and the pH of the medium were recorded, although these values are not given here. Combined filtrates from at least 4 flasks were stored at 4° for peroxidase assays. Cells from at least 4 flasks were washed with H_2O (10 × the vol of the original filtrate). 2 ml of 50 mM Pi buffer (pH 7), 1 g of glass beads and 0·7 g of washed, hydrated Polyclar AT were added per g of fr. tissue which was then homogenized at 5000 rpm for 10 min in a blender. Resulting homogenate was centrifuged for 15 min at 20000 *g* at 0–5°. The supernatant was retained for further enzymic assays.

Starch gel electrophoresis. Starch gel electrophoresis using a modified Smithies apparatus [6] was used for determining total isoperoxidase pattern. 53 g of Electrostarch were heated with 500 ml of 5 mM histidine buffer soln (pH 7) and degassed. Electrophoresis was effected at 400 V for 4·5 hr at 2°, using 0·41 M Na citrate buffer (pH 7) as bridge buffer. The isoperoxidase pattern was visualized by staining the gel in a mixture of 3-amino-9-ethyl carbazole, *N,N*-dimethyl formamide, 50 mM NaOAc (pH 5) and H_2O_2 [7] for 18 hr. The bands were reddish-brown in color.

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