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THE ROLE OF XANTHOXIN IN THE INHIBITION OF PEA SEEDLING GROWTH BY RED LIGHT

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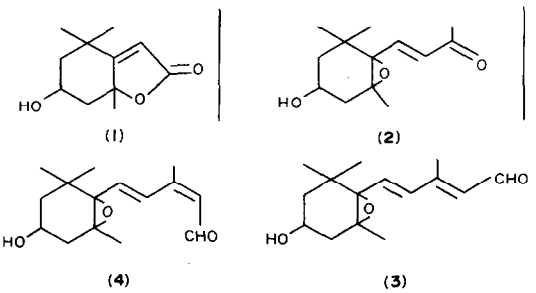
Key Word Index—*Pisum sativum*; Leguminosae; dwarf pea; growth inhibition; xanthoxin; carotenoids; peroxidase; lipoxygenase.

Abstract—Intermittent periods of red light illumination inhibit the growth of dwarf pea seedlings within 1 day and stop length increase completely in 3 days. *Cis,trans*-xanthoxin is synthesized within the plant during the 1st day of illumination and reaches a maximum level on the 3rd day. The red light treatment also causes an increase in the levels of violaxanthin, linoleic acid and peroxidase, lipoxygenase and carotene-bleaching activities in the plant. The possible control of xanthoxin production is discussed.

INTRODUCTION

In 1967, Taylor and Smith reported that on illumination of dried carotenoids extracted from *Urtica dioica* a substance was produced that inhibited the germination of cress seeds [1]. The inhibitor, which was non-acid, was derived from violaxanthin and neoxanthin and was evident after as little as 15 min illumination of dried violaxanthin [2]. Analysis of the degradation products showed four main components, termed loliolide (**1**), "butenone" (**2**), and *trans,trans*- (**3**) and *cis,trans*- (**4**) xanthoxin [3,4]. The *cis,trans*-xanthoxin is the most inhibitory component. The two xanthoxin isomers have been found to exist naturally [5,6].



Xanthoxin has been shown to be active in several bioassays for growth inhibition [7], but the only natural inhibition studied in relation to xanthoxin has been the red-light induced inhibition of elongation of various plants. In the case of dwarf pea seedlings, illumination by red light induced a 50-fold increase in the concentration of violaxanthin and a five- to eight-fold increase in the concentration of both xanthoxin isomers while the abscisic acid concentration remained constant [8].

Any naturally-occurring production of xanthoxin from violaxanthin might be expected to be caused by enzymes active in oxidizing such double-bond structures, e.g. lipoxygenase and peroxidase [9,10]. In fact Firn and Friend have shown that both xanthoxin isomers and "butenone" are produced by the action of soybean lipoxygenase on purified violaxanthin in the presence of linoleic acid [11].

The work described here was undertaken to investigate further the levels of xanthoxin in light- and dark-grown pea seedlings with special reference to the state of growth inhibition, together with the levels of the main precursor, violaxanthin, and the enzymes lipoxygenase and peroxidase and the substrate linoleic acid, which may be involved in the biological degradation process.

RESULTS

Inhibition of growth by intermittent red light

Dark-grown dwarf-pea seedlings increased in weight and length over the 9-day period studied

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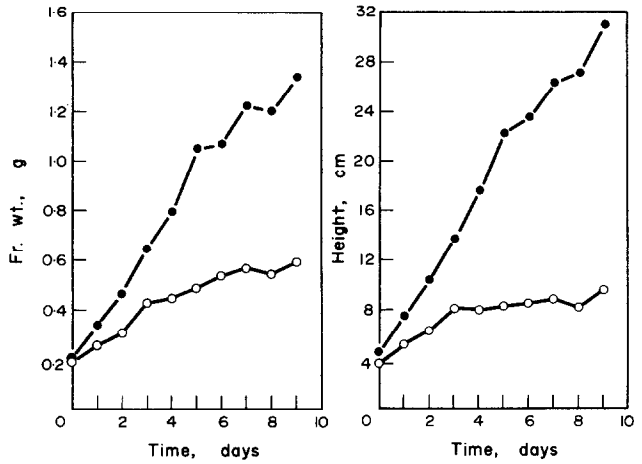

Fig. 1. Growth curves for pea seedlings in complete darkness and red light. The average (a) fr. wt and (b) height, per seedling grown in complete darkness (●) or in a light regime where the seedlings receive 1 min of red light illumination every 30 min (○). 20–30 Seedlings were measured in every case.

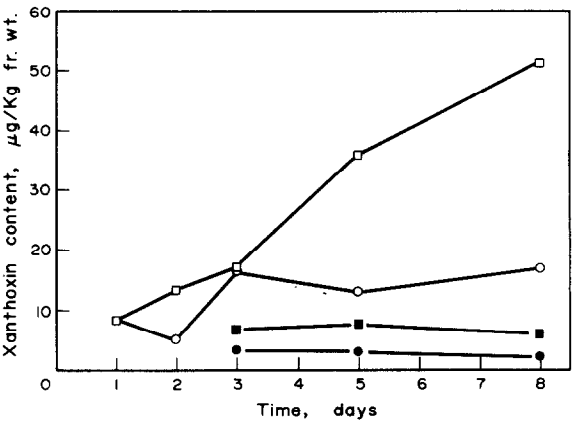

Fig. 2. Xanthoxin content of pea seedlings grown in complete darkness and red light. *Trans,trans* xanthoxin in light-grown (□) and dark-grown (■) seedlings, *Cis,trans* xanthoxin in light-grown (○) and dark-grown (●) seedlings. The time scale refers to days after the red-light illumination commenced.

(Fig. 1). The gain in dry weight was proportionally less than that for fresh weight from 3 days onward. Seedlings grown under the red light regime grew much less than dark-grown seedlings. Within one day (50 min illumination), the difference in length between light- and dark-grown seedlings was significant at an 0·5% level. After three days in the light, length increase virtually stopped although there were further steady increases in fresh and dry weight (Fig. 1).

Effect of light on xanthoxin content

Preliminary experiments on seedlings illuminated or kept in the dark for 8 days confirmed an approx five-fold increase in both isomers in the light [8]. In further experiments, pea seedlings were illuminated for 1, 2, 3, 5 and 8 days and analysed for their xanthoxin isomer content (Fig. 2). An increase in both isomers by day 1 was apparent. In the case of the *cis,trans* isomer, levels reached a maximum after 3 days and then remained steady. The level of the *trans,trans* isomer increased continually over the time course. Two additional peaks were often present on the chromatogram traces. One co-chromatographed with acetylated "butenone" and its levels behaved similarly to *trans,trans*-xanthoxin, increasing with time. The other peak was not due to acetylated loliolide. It was present in large quantities in 3-days illuminated seedlings but subsequently decreased to a lower steady level.

Effect of light on pigment content

After 8 days illumination with intermittent red light pea seedlings contained 63 times more β-carotene, 15 times more violaxanthin and 13 times more lutein + neoxanthin than did seedlings of the same age grown in complete darkness. All carotenoids analysed were present in both stem and leaf tissue in red light grown and dark-grown seedlings. After 3 days illumination each of the carotenoids had reached its maximum level in each seedling and since there was a subsequent increase in fresh weight, the carotenoid content decreased when expressed on a unit fresh basis (Fig. 3).

Effect of light on activity of peroxidase and lipoxygenase

Peroxidase, lipoxygenase and carotene-bleaching activities were measured at 3, 5 and 8 days and the activities of each enzyme on a per seedling basis were higher in the red light-treated than in the dark-grown plants (Table 1). Peroxidase activity increased over the course of the experiment with a faster rate of increase in the light-treated than in the dark-grown plants. Lipoxygenase activity more than doubled between 3 and 5 days in both light and dark-grown plants and then fell between days 5 and 8 by 12% in the light-treated

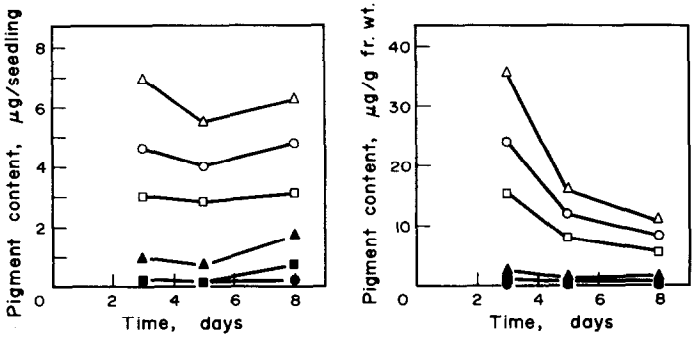

Fig. 3. Carotenoid pigment content of pea seedlings grown in complete darkness and red light. Pigment content (a) per seedling and (b) per g. fr. wt at various times after the red light illumination was begun. β-carotene (○) in light (●) in darkness; violaxanthin (□) in light (■) in darkness; lutein + neoxanthin (△) in light (▲) in darkness.

and 27% in the dark-grown plants. Carotene-destroying activity rose faster in the light-grown than in the dark-grown plants particularly between days 3 and 5. There was a relatively small rise between 5 and 8 days.

Effect of light on lipid content

In the dark linoleic acid made up nearly 50% of the total fatty acid content. There was further synthesis of linoleic acid in both dark- and light-treated plants but the massive synthesis of linolenic acid in the light-grown plants caused a decrease in linoleic acid content as a percentage component of the total fatty acids (Table 2).

DISCUSSION

Previous work on this system has shown that the levels of xanthoxin, but not abscisic acid, are raised after 8 days illumination of pea seedlings with intermittent red light. At this time the illuminated seedlings were much shorter than the etiolated seedlings [8]. The present investigation shows that stem elongation is inhibited within one day and stops altogether within 3 days. If the light treatment is actually stimulating the production of an inhibitor within the plant [12], the level of this inhibitor should be higher in the light grown tissue within 1 day and might be expected to reach a maximum in 3 days. These results show that *cis, trans*-xanthoxin (**4**), a known plant growth inhibitor, satisfies these criteria. The level of **4** is increased within one day and reaches a maximum level after 3 days. The levels of other known violaxanthin breakdown products **2** and **3**, continue to rise with time. The unknown acetylated compound may be a precursor of **2**, **3** and/or **4**.

The effect of light may be either to raise the level of the xanthoxin precursor or the activity of one or more enzymes required for the conversion [8].

Table 1. Peroxidase, lipoxygenase and carotene-bleaching activities in pea seedlings grown in darkness and in intermittent red light

Time (days)	Peroxidase activity U/g fr. wt	Peroxidase activity U/seedling	Lipoxygenase activity U/g fr. wt	Lipoxygenase activity U/seedling	Carotene-destroying activity U/g fr. wt	Carotene-destroying activity U/seedling
3 D	169	33	478	92	542	105
3 L	642	76	1738	206	883	105
5 D	273	102	567	211	384	143
5 L	1521	396	1665	433	664	173
8 D	337	223	231	153	227	150
8 L	2474	1163	818	384	417	196

n Days in the light implies 48 *n* min of illumination.
One unit (U) of peroxidase activity produces an increase in A460 of 0·1 A units in 1 min.
One unit (U) of lipoxygenase activity represents an uptake of 10 nmol O_2/min.
One unit (U) of carotene-bleaching activity represents a bleaching of 1 nmol in 1 min.

Table 2. Total fatty acid content of pea seedlings grown in the dark or in flashing red light

	Age of seedling and environmental conditions									
	3 Days				5 Days		8 Days			
	Dark		Light		Light		Dark		Light	
Fatty acid	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
16:0	0·15	28·3	0·25	23·2	0·32	27·1	0·33	17·7	0·40	18·1
18:0	0·02	3·8	0·04	3·7	0·09	7·6	0·04	2·1	0·06	2·7
18:1	0·01	1·9	0·03	2·8	0·04	3·4	0·04	2·1	0·02	0·9
18:2	0·25	47·3	0·43	39·8	0·35	29·6	0·94	50·3	0·61	27·4
18:3	0·10	18·8	0·33	30·5	0·38	32·3	0·52	27·8	1·12	50·8

(a) = mg/g fr. wt. (b) = wt%.

Clearly the possible precursors, violaxanthin and neoxanthin, are synthesised very rapidly on illumination [13]. Both peroxidase and lipoxygenase were active in the dark and their activities were increased by the red light treatment used in these experiments. It is interesting to note that lipoxygenase activity does not correlate with carotene destroying activity. The former goes up from days 3 to 5 and then declines between days 5 and 8 whereas the latter is going up over the whole period of the experiment. In the case of each enzyme activity the values for the light-treated plants are higher than those for the dark-treated ones. Presumably the difference between the two activities can be explained on the basis that not all the lipoxygenase isoenzymes are equally efficient in carotene-destruction [14]. The substrate of lipoxygenase, linoleic acid, is present in lipids in high quantities throughout the course of the experiment. The intermittent red-light treatment appears to differ little from continuous light in its effects on fatty acid synthesis [15].

Since the levels of the xanthoxin precursors and the activities of the possible enzyme are all increased by the red light treatment, it is difficult to state whether it is the level of one of these components which is controlling xanthoxin production. The one which responds most dramatically to the light treatment is violaxanthin which on a weight basis is 15-fold higher in the light than in the dark. Compared with our *in vitro* experiments [11], it might well be that the concentration of violaxanthin in dark-grown peas is just at the threshold limit. However when the concentration is increased 15-fold there is only a 2-fold increase in xanthoxin concentration presumably this increase takes it well above the threshold. Since both pea peroxidase [16] and lipoxygenase [17,18] exist in a variety of isoenzymatic forms, it may therefore be that a particular form is rapidly stimulated by the red light and that its cellular environment is close to that of the xanthophyll substrate, and, in the case of lipoxygenase, an unsaturated lipid. On the other hand the whole cellular environment may alter, since it is now known that lipoxygenase has a particulate subcellular localization.* These conditions may favour the conversion of violaxanthin to xanthoxin. An alternative possibility is that light, which stimulates xanthophyll synthesis, may also trigger a synthetic route to xanthoxin from lower molecular weight precursors.

* Gardner, D. C. J. and Friend, J. (1974) unpublished; Galliard, T., Fishwick, M. J. and Wardale, D. A. (1974) personal communication.

EXPERIMENTAL

Treatment of plant material. Dwarf pea (*Pisum sativum* var. Meteor) seeds were planted in moist Vermiculite and germinated in complete darkness at 21°. When the seedlings were 2–4 cm tall some were transferred to a light box providing 1 min red light every 30 min [8]. In all experiments humidity was kept high by use of a humidifier and open dishes of water. For the assay of growth, 20 light-grown and 20 dark-grown seedlings were excised above the cotyledons. Their lengths were measured to the base of the terminal leaf, they were weighed and placed in a 100° oven. Dry weight was determined when a constant wt had been reached.

Xanthoxin extraction and assay. At various times seedlings were excised above their cotyledons, weighed and frozen at −20° overnight. Xanthoxin was extracted with ether and purified by the method of Firn *et al.* [6]. After the purified ether extract had been taken to dryness the residue was redissolved in 80% methanol and extracted at least twice with the same vol. of petrol (60–80). H_2O was then added to give a 40% methanol soln which was extracted twice with the same vol. of petrol (40–60). This ensured a considerably greater removal of neoxanthin [19], which can be photolytically converted to xanthoxin [2].

All other extraction and purification procedures were as described [6], except that the first thin-layer purification step was omitted. The acetylated compounds were separated by GLC on a 3% OV-17 column [6,11].

Pigment analysis. Pigments were extracted in acetone, transferred to ether, washed and dried. The ethereal extract was evaporated to a small volume and chromatographed on 0·5 mm magnesia-Kieselgur GTLC plates with acetone–petrol (b.p. 40–60)(1:1) as solvent [20]. The pigments separated as follows; β-carotene (R_f 0·86); violaxanthin (R_f 0·62); lutein + neoxanthin (R_f 0·30); chlorophylls (R_f 0–0·1). The β-carotene was eluted with acetone, taken to dryness under N_2 and assayed spectrophotometrically in hexane. The xanthophylls were eluted with acetone containing up to 10% ethanol, evaporated under N_2 and assayed spectrophotometrically in ethanol. The lutein and neoxanthin could be separated by further TLC on silica gel G with ether as solvent [20].

Extraction and assay of peroxidase, lipoxygenase and carotene-bleaching. The enzymes were assayed in solubilized acetone powders prepared as described previously [18]. Peroxidase was solubilized in 0·02 M phosphate, pH 5·7 and assayed in the same buffer using guaiacol as substrate [21]. Lipoxygenase, solubilized in 0·05 M phosphate, pH 7 was assayed polarographically [18,22], and carotene-bleaching was measured by the method of Ben-Aziz *et al.* [9].

Extraction and separation of fatty acids. Total lipids were extracted in $CHCl_3$-MeOH (1:2) and washed with 0·2 vol. 0·73% NaCl [23]. Transmethylation was carried out in Teflon-sealed flasks using BF_3 in MeOH [24]. After incubation the methyl esters were washed with water, dissolved in hexane, and separated by GLC on a DEGS column, oven temperature 175°, nitrogen flow rate 3·0, and quantitated using a Perkin–Elmer F-11 with reference to standards.

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REFERENCES

1. Taylor, H. F. and Smith, T. A. (1967) *Nature (Lond.)* **215,** 1513.
2. Taylor, H. F. (1968) Plant Growth Regulators. (Society of Chemical Industry) Monograph **31,** p. 22.
3. Taylor H. F. and Burden, R. S. (1970) *Phytochemistry* **9,** 2217.
4. Burden, R. S. and Taylor, H. F. (1970) *Tetrahedron Lett.* 4071.
5. Taylor, H. F. and Burden, R. S. (1970) *Nature (Lond.)* **227,** 302.
6. Firn, R. D., Burden, R. S. and Taylor, H. F. (1972) *Planta (Berl.)* **102,** 115.
7. Taylor, H. F. and Burden, R. S. (1972) *Proc. R. Soc. Lond.* B. **180,** 317.
8. Burden, R. S., Firn, R. D., Hiron, R. W. P., Taylor, H. F. and Wright, S. T. C. (1971) *Nature (Lond.) New Biology* **234,** 95.
9. Ben Aziz, A., Grossman, S., Ascarelli, I. and Budowski, P. (1971) *Phytochemistry* **10,** 1445.
10. Dicks, J. W. and Friend, J. (1967) *Phytochemistry* **6,** 1193.
11. Firn, R. D. and Friend, J. (1972) *Planta (Berl.)* **103,** 263.
12. Simpson, G. M. and Wain, R. L. (1961) *J. Exp. Bot.* **12,** 207.
13. Henshall, J. D. and Goodwin, T. W. (1964) *Photochem. Photobiol.* **3,** 243.
14. Arens, D., Seilmeier, W., Weber, F., Kloos, G. and Grosch, W. (1973) *Biochim. Biophys. Acta.*, **327,** 295.
15. Trémolières, A. and Lepage, M. (1971) *Plant Physiol.* **47,** 329.
16. Siegel, B. Z. and Galston, A. W. (1967) *Plant Physiol.* **42,** 221.
17. Anstis, P. J. P. and Friend, J. (1974) *Planta (Berl.)* **115,** 329.
18. Anstis, P. J. P. and Friend, J. (1974) *Phytochemistry*, **13,** 567.
19. Krinsky, N. I. (1963) *Anal. Biochem.* **6,** 293.
20. Britton, G. and Goodwin, T. W. (1971) in *Methods in Enzymology* (McCormick, D. B. and Wright, L. D., eds.) **18c** p. 654, Academic Press, New York.
21. Dicks, J. W. and Friend, J. (1968) *Phytochemistry* **7,** 1933.
22. Galliard, T. and Phillips, D. R. (1971) *Biochem. J.* **124,** 431.
23. Roughan, P. G. and Batt, R. D. (1968) *Anal. Biochem.* **22,** 74.
24. Morrison, W. R. and Smith, L. M. (1964) *J. Lipid Res.* **5,** 600.