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LIGHT-INDUCED CHANGES IN PHENOLIC INHIBITORS OF INDOLEACETIC ACID OXIDASE IN COTYLEDONS OF *PHARBITIS NIL**

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Abstract—*Pharbitis* cotyledons contain methanol-soluble substances which inhibit the oxidation of 3-indolylacetic acid (IAA) by peroxidase. Such substances are much more abundant in etiolated than in light-grown plants, and transfer of etiolated plants from dark to white light results in their prompt decrease. Surprisingly, transfer of a light-grown plant from white light to darkness also results in a marked decrease in the titer of such substances. Thus, it appears that there is a metabolic regulation such that the synthesis of IAA oxidase inhibitors proceeds best in darkness in dark-grown plants and best in light in light-grown plants.

Approximately twenty phenolic compounds can be detected in methanolic extracts of cotyledons after two-dimensional paper chromatography. Only five are active as inhibitors of IAA oxidation, and of these, three are increased dramatically in quantity by far-red light. Red light itself produces only small effects, but when given prior to far-red, it greatly diminishes the effectiveness of the far-red. The possible relevance of such changes in photomorphogenesis are considered.

INTRODUCTION

THE Japanese morning glory (*Pharbitis nil* Chois), variety Murasaki (Violet), is a very sensitive short-day plant.[1] It produces flower primordia after only one inductive dark period, but retains the vegetative habit for at least three months when subjected to continuous light conditions. An important and unusual distinction from other short-day plants such as cocklebur and soybean is the photoperiodic sensitivity of the cotyledons.[2] This characteristic permits studies to be made on very young plants, thus affording an unusual opportunity for a biochemical analysis of events accompanying floral induction in large numbers of plants. Such a detailed analysis has been made by Imamura and his colleagues at Kyoto in a long series of papers. It will be useful, before proceeding with a description of the present work, to summarize the distinctions between *Pharbitis* and other more familiar experimental short-day plants. (1) Far-red light (FR) given prior to a dark period inhibits floral induction in *Pharbitis*,[3,4,5] but promotes in cocklebur.[6] In both plants, red light (R) administered after FR reverses the effect of FR. (2) In cocklebur, the inhibitory effect of very low light intensity or of repeated very short photoperiod prior to the inductive long dark period can be counteracted

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by the administration of sucrose or of acids of the tricarboxylic acid cycle.[7] Although similar inhibitions of flowering can be detected in low-intensity irradiated *Pharbitis*,[3] the reversing effects of sucrose and organic acids cannot be detected.[8] (3) FR administered during the first 12 hr of the inductive dark period strongly inhibits flowering in *Pharbitis*,[9,10] but has little or no effect in cocklebur.[8,11] (4) R inhibits flowering in cocklebur when administered anywhere after the first 2 or 3 hr of the inductive dark period,[12] while in *Pharbitis*, the period of sensitivity is limited to 8–12 hr after the beginning of the dark period, irrespective of the length of the dark period.[9] (5) R-FR reversibility is readily detectable in cocklebur,[6,13] but cannot be easily noted in *Pharbitis*.[9,14] (6) Floral initiation in cocklebur is very sensitive to light conditions following the inductive dark period,[15,16,17] while *Pharbitis* is little influenced by post-inductive light conditions.[8,9,10]

These differences indicated to us the inadequacy of many generalized schemes invented to explain floral initiation, based mainly or exclusively upon one experimental organism, usually cocklebur. They also argued for further biochemical documentation of events occurring during induction in *Pharbitis*, with a view to their possible utility in any generalized biochemical explanation of flowering.

Given that one wishes to investigate biochemical events following photoinduction, how does one decide which of the numerous categories of substances in plants to choose? For a variety of reasons, summarized below, we have chosen phenolic substances. Tang and Bonner[18] reported the existence in etiolated peas of an enzyme system which destroys the plant growth hormone 3-indolylacetic acid (IAA). This enzyme, called IAA oxidase, is frequently accompanied in tissue by a water-soluble, low molecular weight inhibitor whose concentration is sensitive to light.[19,20] Later, Hillman and Galston[21] demonstrated that the synthesis of the inhibitor is controlled by a R-FR reversible photoreaction. The inhibitor has subsequently been analyzed and shown to contain flavonoid complexes, built around a kaempferol nucleus.[22,23] In green peas, the inhibitor, which is sensitive to photoperiod,[20] is probably a quercetin derivative.[24] In similar investigations, Konishi[25] with the long-day plant *Silene armeria*, and Watanabe and Stutz[26] with *Lupinus* found that the synthesis of IAA oxidase inhibitor is increased with increasing photoperiod. Also, Sági and Garay[27] found that phenols in the leaves of *Lupinus*, which act as inhibitors of IAA oxidase in *in-vitro* tests,

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rise in quantity following long-day treatment. Clearly, the possibility that changes in phenolics regularly accompany photoinduction must be further investigated.

RESULTS

Distribution of IAA Oxidase Activity and Inhibitors in Pharbitis

Homogenates of cotyledons and hypocotyls of light- or dark-grown *Pharbitis* plants (tissue blended with an equal volume of pH 6·1 0·01 M potassium phosphate) have no IAA oxidase activity even after 48 hr dialysis against buffer or water to remove inhibitors. However, reconstituted acetone powders of 3-day-old cotyledons, when properly diluted, show some activity, which, depending on the source, is differently enhanced by the addition of Mn^{2+} and DCP.

Direct assay of the inhibitor content of the various organs was made by adding various dilutions of inhibitor extract to the horseradish root peroxidase (HRP) test system. The results (Table 1) show that roots contain little inhibitor but that aerial organs have considerable inhibitor.

TABLE 1. INHIBITORY ACTIVITY OF EXTRACTS OF 2-DAY-OLD *Pharbitis* PLANTS GROWN UNDER CONTINUOUS DARKNESS (D), CONTINUOUS LIGHT (L) OR LIGHT TERMINATED BY ONE INDUCTIVE 16-HR DARK PERIOD (16D × 1)
10 μg acetone powder of the organ added per ml reaction mixture

Extract added	Condition of plant from which extract was made	% inhibition of IAA destruction
0 (control)		0
Cotyledon	D	86·7
Cotyledon lamina	L	70·0
Cotyledon lamina	16D × 1	74·6
Petiole	16D × 1	77·3
Hypocotyl	16D × 1	70·9
Root	D	0
Root	16D × 1	ca. 1

Comparative Inhibitor Content of Induced and Non-induced Cotyledons

Pharbitis plants grown under continuous light for 2 days were subjected to one 16-hr dark period, while other plants were left in the light as a control. Certain plants of both treatments were allowed to remain under continuous light for 2 weeks, at which time it was ascertained that the light controls showed 0% flowering, and the dark-exposed plants showed 100% flowering. Immediately after the completion of the single dark period, other plants of both conditions were harvested, separated into lamina, petiole, hypocotyl and root,* and the IAA oxidase inhibitor activity of each fraction determined. Another batch was given a 16-hr dark period, followed by 2 hr of light prior to harvest and determination of inhibitor content. The results are shown in Fig. 1, in which the ordinate represents the magnitude of dilution of the extract at which 50% inhibition of the HRP test system is produced. Clearly, the cotyledons have much more inhibitor than all other organs, and etiolated tissue has a higher titer than does any tissue exposed to light.

* In the dark series, the cotyledon was too small to be separated into lamina and petiole. It was therefore harvested as a unit.

Kinetics of the Light-induced Decrease in Inhibitor Content of the Cotyledons

We next examined the time course of alteration of inhibitor content in light-grown plants following transfer from noninductive (continuous light) to inductive conditions. The latter consisted of exposure to one (16D × 1) or two (16D × 2) 16 hr dark periods. Seedlings were grown for 2–3 days in continuous light and were then subjected to one or two inductive 16-hr dark periods (4 p.m. to 8 a.m.) followed by a return to continuous light conditions. Other seedlings were kept under continuous light as vegetative controls. Cotyledons of all three experimental series were harvested at 4-hr intervals and extracted with boiling water for 9 min. Dilutions of this extract were then tested for inhibitory effect in the HRP assay. The data are shown in Fig. 2, in which the ordinate again represents relative inhibitory activity of the

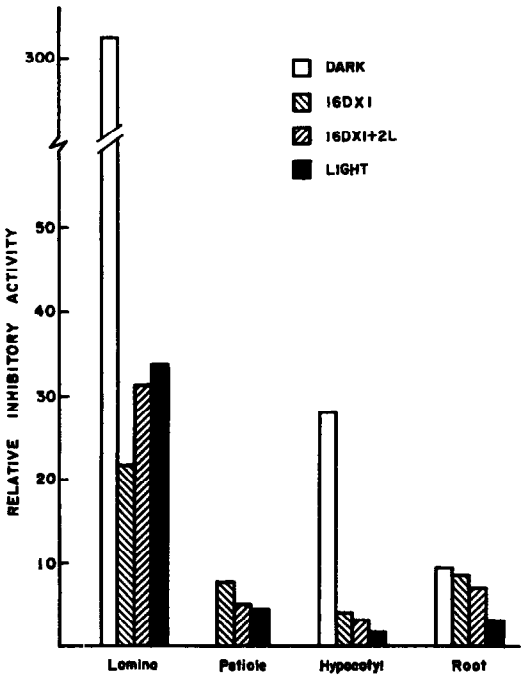

FIG 1. INHIBITOR CONTENT OF VARIOUS ORGANS UNDER SEVERAL LIGHT CONDITIONS.

extract. The inhibitor content of plants kept in continuous light remained at a constant level. Transfer of such plants from light to dark resulted, surprisingly, in a fall in inhibitor level, reaching ca. one-fortieth of the dark level by the end of the 16-hr inductive period. Return of the plants to light resulted in a prompt (within the next 4 hr assay sample) reversal of the decline in inhibitory activity. In both 16D × 1 and 16D × 2, there are indications of a repetition of this oscillation, which for the 16D × 1 series must be considered to be a perpetuated rhythm. The major decrease in inhibitory activity in both series occurred approximately 48 hr after the beginning of the first dark period. The additional oscillation in the 16D × 1 curve and its absence in the 16D × 2 curve 24 hr after the start of the first dark period (at 8.20 p.m. on 21 January) is difficult to explain, unless the second dark period, introduced in the 16D × 2 series only, is out of phase with and thus damps out the oscillation induced by the first dark period.

To determine whether the decrease in inhibitor level upon transfer from light to dark is correlated with floral induction, another experiment was performed, in which plants growing in continuous light were transferred to darkness for 8 (8D × 1) or 16 (16D × 1) hr. Only the

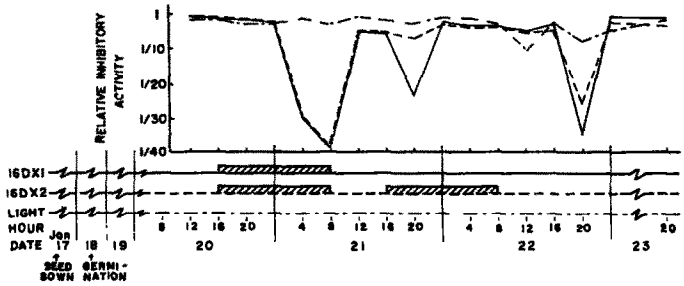

FIG. 2. THE EFFECT OF 16-HR DARK PERIODS (SHADED BARS) ON INHIBITOR LEVEL OF COTYLEDONS.

latter series responded by floral induction when selected plants of the batch were permitted to develop in continuous light for another 2 weeks. Cotyledons were excised as before at 4-hr intervals, starting at the end of the first 8 hr of the dark period. As shown in Fig. 3, the maximal decrease in inhibitor was manifested in both series by the twelfth hour after the start of the dark period, whether or not the plant was returned to light at the 8-hr point. This fact shows

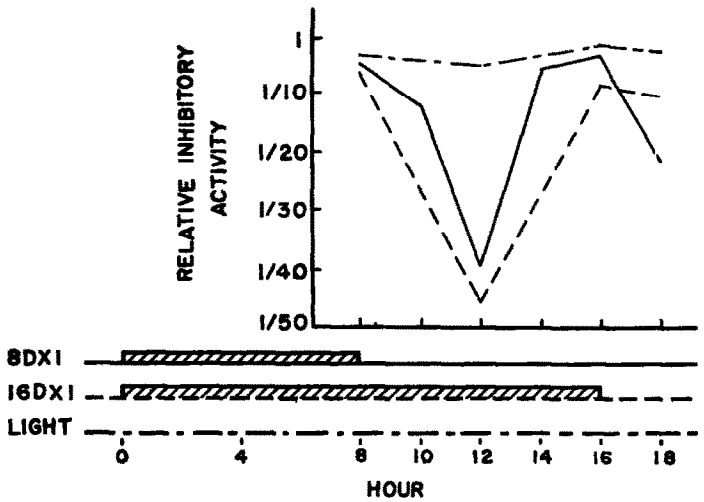

FIG. 3. THE EFFECT OF 8- AND 16-HR DARK PERIODS ON INHIBITOR LEVELS.

that the change in inhibitor level is not compulsorily linked to floral induction, and also reinforces the view that the oscillations, once initiated by a light–dark transition, may be self-sustained.

The Effect of Brief Light Flashes on Dark-grown Plants

The previous experiments have demonstrated that IAA oxidase inhibitors in *Pharbitis* cotyledons decline if (*a*) dark-grown plants are exposed to light (Fig. 1) or (*b*) light-grown plants are transferred to darkness (Figs. 2, 3). We next investigated the effects of varying

quantity and quality of light energy on inhibitor level in etiolated plants. Two- or 3-day-old plants, grown in complete darkness, were given white light of the growth room for 1 min, 1 hr or 8 hr. Cotyledons were harvested immediately after the end of the irradiation and again after 4 and 8 hr. They were extracted with 50% methanol and assayed for inhibition in the HRP test. The results (Table 2) showed clearly that even 1 min of light given to etiolated plants results in an immediate depression of inhibitor level in the cotyledons, and that this altered level persists for at least 8 hr but disappears by 24 hr after the light.

TABLE 2. EFFECT OF WHITE LIGHT ON THE IAA OXIDASE INHIBITOR ACTIVITY OF 3-DAY-OLD PREVIOUSLY DARK-GROWN *Pharbitis* COTYLEDONS

	λ methanolic extract/10 ml required for 50% inhibition of IAA oxidation			
Light exposure	Immediately before treatment	Immediately after treatment	After 8 hr	After 24 hr
Dark	0·06		0·06	0·16
1 min		0·15	0·15	0·12
1 hr		0·15	0·12	0·28
8 hr		0·21		0·15

In the next experiments, the light flashes consisted of (*a*) 1 min red light (ca. 500 ergs/cm^2 per sec), from a red fluorescent tube covered with red cellophane, (*b*) 30 sec far-red light (ca. 750 ergs/cm^2 per sec) from an incandescent source filtered through red and blue cellophane, (*c*) red followed by far-red and (*d*) 1 min bright white light of the growth room. Plants kept in total darkness served as controls. Cotyledons of the 2 to 3-day-old etiolated plants used in these experiments were harvested 0, 4, 8, 12 and 24 hr after treatment, were extracted with methanol and the extract assayed for inhibitor content. The data of Table 3 show that as

TABLE 3. THE EFFECT OF WEAK RED AND FAR-RED LIGHT AND OF BRIGHT WHITE LIGHT ON THE METHANOL-SOLUBLE INHIBITOR ACTIVITY OF *Pharbitis* COTYLEDONS PREVIOUSLY GROWN IN DARKNESS

	λ/10 ml required for 50% inhibition of IAA oxidation			
Illumination	0 hr	4 hr	8 hr	12 hr
Dark	0·24	0·28	0·15	0·15
1 min red light	0·15	0·14	0·38	0·11
30 sec far-red light	0·03	0·03	0·03	0·10
1 min red and 30 sec far-red light	0·11	0·15	0·13	
1 min bright white light	0·37	0·21	0·14	0·08

before, high intensity white light produces an immediate depression in inhibitor content, which is slowly reversed in darkness. However, low irradiances with both red and far-red light are effective in *increasing* the inhibitor content, far-red being by far the most effective. These photoinduced changes persist for at least 4 hr in darkness. The results after 8 and 12 hr are more difficult to assess, since the dark controls themselves show large changes, possibly due to endogenous oscillations.

Chromatographic Analysis of the Inhibitor Fraction

Two-dimensional paper chromatography revealed about 20 fluorescent spots in the methanol-soluble extract (the number varied somewhat with the light treatment) and 8–10 in the aqueous extracts (Fig. 4). Fractions W-1 and M-1 through M-5 (shaded on the chromatogram) show IAA oxidase inhibitor activity at a level of ca. 30λ per 10 ml of reaction mixture.

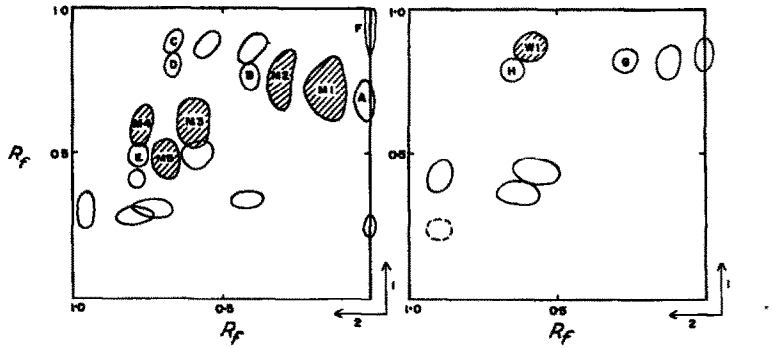

FIG. 4. CHROMATOGRAPHIC SEPARATION OF INHIBITORS (SHADED) AND OTHER PHENOLS.
Left, methanol-soluble ; right, water-soluble. Solvent 1—butanol : acetic acid : water, 4 : 1 : 2·2 (v/v). Solvent 2—5% acetic acid.

The spots labelled A and F appear in greatest quantity in dark-grown cotyledons. They decrease markedly, frequently to zero, upon illumination of the plant. A is especially sensitive to far-red light. B, C, D, and E, on the contrary, do not exist in quantity in dark-grown cotyledons, but appear after exposure to light. The appearance of B, C, and D is favoured by far-red, and E by red light. These light-induced changes, unlike those cited above for the inhibitory compounds, are constant with time after irradiation.

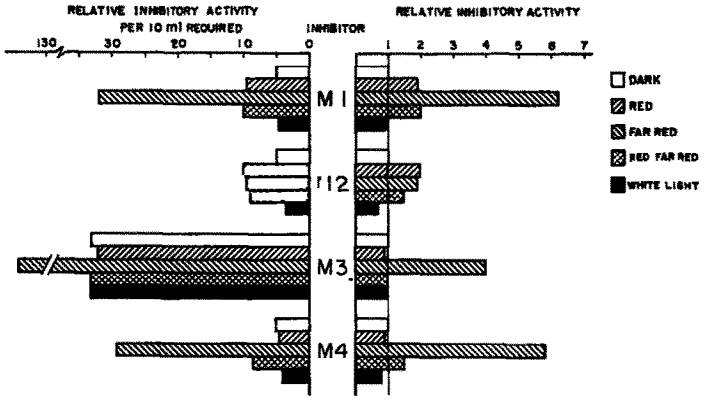

FIG. 5. EFFECT OF LIGHT ON SPECIFIC INHIBITORS.
Left, relative activity; right, all fractions in dark = 1·0.

The relative quantities of the individual methanol-soluble IAA oxidase inhibitors in the various conditions of irradiation are shown in Fig. 5. On the left are shown the relative inhibitory activity of the various methanol-soluble fractions, and their variation with light treatment. On the right, the relative quantities of each inhibitor are internally compared, with the dark condition assigned a value of unity. It can be seen that M_1, M_3 and M_4 are present in greatest quantity, and that all three are *increased* dramatically by far-red light.

Red light, on the other hand, produces only small effects and then only on M_1 and M_2. Significantly, red administered *prior* to far-red prevents FR from exerting its promotive effects on the synthesis of these compounds.

The R_f values, u.v. absorption maxima, response to phenolic spray reagents and fluorescence characteristics of each of the IAA oxidase inhibitors are detailed in Table 4. With the

TABLE 4. CHARACTERISTICS OF THE IAA-OXIDASE INHIBITORS FROM *Pharbitis* COTYLEDONS

Inhibitor	R_f (BuOH–acetic acid–H_2O, 4:1:2·2)	R_f (5% acetic acid)	u.v. absorption λ (mμ) maxima (H_2O) [EtOH]	u.v. absorption λ (mμ) minimum	Color of fluorescence in u.v. light
M_1	0·75	0·15	323, 295, 242 [327, 298, 247	262 266]	Blue white
M_2	0·75	0·29	322, 295, (242) [328, 298, 247	263 267]	Blue white
M_3	0·67	0·57	322, 295, 242 [327, 297, 245	263 260]	Blue white
M_4	0·60	0·69	322, 280, (243)	262	Blue white
M_5	0·52	0·60	228, 287, 278, 262	(247)	Greenish white
W_1	0·71	0·65	322, 282	245	Blue

All turned a dark-grey with ferric chloride: with the exception of M_5, all gave a blue color with ferric chloride–ferricyanide, a violet color with ammoniacal silver nitrate, and a yellow color with Na_2CO_3 solution.

exception of M_5, which lacks an absorption peak in the 322 mμ region and reacts only weakly to the ferric chloride–ferricyanide reagent, all have typical properties which mark them as phenolic. This view is reinforced by the effects of pH on the absorption spectra, especially in the region below 260 mμ. At pH 3, the absorbance in this region is greatly depressed, while at pH 9, it is greatly elevated.

The absorption spectra of M_1, M_2, M_3 and M_4 are quite similar. The spectrum for M_1 in methanol, shown in Fig. 6, closely resembles that of 4-amino-2-hydroxybenzoic acid and its methyl ester.

DISCUSSION

Our data support the view that the phenolic substances of *Pharbitis*, like those of other plants,[20-27] vary qualitatively and quantitatively on exposure to light. The relevance of the observed changes to photomorphogenesis remain, however, obscure. While the sensitivity of substances M_1, M_3 and M_4 (Fig. 5) to red and far-red light suggest a close connection between the control of their synthesis and the photomorphogenic control of growth, the lack of correlation between effects of length of dark period on flowering and on IAA oxidase inhibitors (Fig. 3) speaks against a close connection.

The evidence, especially from Fig. 5, would indicate a multiplicity of photoreactions. Not only does red administered prior to far-red appear to reverse the effect of the latter, but bright white light acts differently from either red or far-red. Thus, a high-intensity light reaction, or a blue-sensitive reaction, known to influence anthocyanin synthesis[28] might be involved here. We hope to investigate this point further.

[28] S. B. HENDRICKS, *Science* **141**, 21 (1963).

The relation between the total extracted inhibitors of IAA oxidase and the individual phenols separated by chromatography is also somewhat unclear. Based on the extensive investigations of Goldacre *et al.*[29] the inhibitors are probably *o*-diphenols. Cofactors of IAA oxidase, which are usually monophenols, also exist in nature,[20] and the total inhibitor activity of an extract may be dependent on the ratio of these two types of substances. Thus, changes in the inhibitor activity as a result of illumination may be the result of promoted

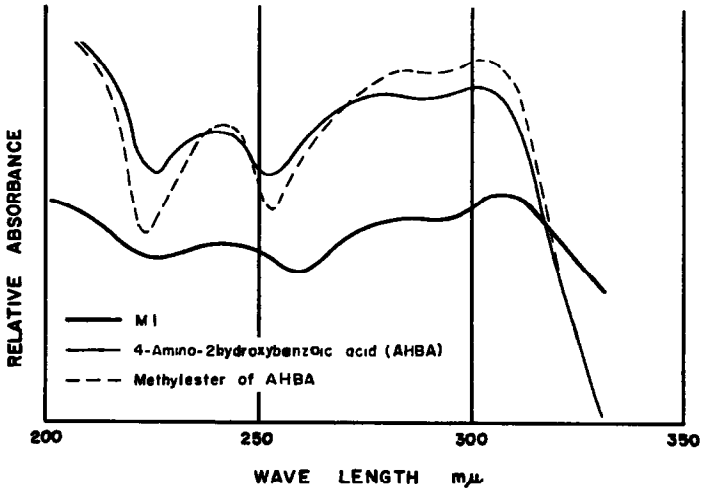

FIG. 6

synthesis of some substances, decreased levels of others, or both. This indicates the necessity, in future work, of pursuing effects on the individual compounds isolated by chromatography, rather than on the unfractionated extract.

EXPERIMENTAL

Plants

Seeds, obtained from Professor S. Imamura of Kyoto, were soaked in concentrated H_2SO_4 for 1–1½ hr with occasional stirring, then rinsed in running tap-water overnight and sown in wet, pre-washed "Mica Gro" vermiculite in a petri dish. At the time of germination the following day, selected seedling were transplanted in vermiculite to perforated polyethylene containers 28 × 33 × 15 cm. They were then exposed to continuous illumination of ca. 1500 ft-candles of mixed fluorescent and incandescent light (9:1 energy ratio) at a temperature of 23° ± 1° and humidity of 50–65%. The containers were automatically sub-irrigated twice daily with a solution of Hyponex (120 gm/100 l. tap water). After 2 days, the cotyledons had expanded; on day 3, the photoperiodic treatments were administered, after which the plants were returned to continuous light conditions. Floral initiation was determined after 2–3 weeks by dissection of the terminal bud under a binocular dissecting microscope, utilizing the criteria of Imamura and Takimoto.[30]

Extraction, Chromatography and Detection of Compounds

Tissues were weighed after harvest and wrapped in aluminium foil to prevent further light effects. They were then homogenized in a Waring blender with 50 vol. of chilled 50% aq.

[29] P. L. GOLDACRE, A. W. GALSTON and R. L. WEINTRAUB, *Arch. Biochem. Biophys.* **43**, 358 (1953).
[30] S. IMAMURA and A. TAKIMOTO, *Botan. Mag. Tokyo* **68**, 235 (1955).

acetone. The resulting slurry was filtered by suction through a Buchner funnel, and the residue washed several times with additional charges of acetone. The acetone powder was then air-dried and was stored under vacuum in a desiccator at 2°. This powder was used as a source of IAA oxidase by dissolving it in pH 6·1 0·01 M phosphate buffer, centrifuging for 10 min at 10,000 *g* in the cold and retaining only the clear supernatant fluid, which could be stored several days in the frozen state.

Initially, inhibitors were extracted by immersion of the fresh tissue in 10 vol. boiling distilled H_2O for 8–10 min, followed by filtration. Later studies showed that additional quantities could be extracted with methanol, leading to the following standard procedure. Tissue was weighed, immersed in 20 vol. chilled 50% methanol and extracted for 24 hr at 2° in the dark, with two renewals of the solvent. The methanolic extracts were pooled and evaporated to dryness in a rotary flash evaporator at reduced pressure and at temperatures below 40°. The dried material was washed twice with petroleum ether to remove inactive, troublesome lipids, and then extracted thrice with absolute methanol in the cold, for a total extraction time of 24 hr. The methanol extract was again evaporated to dryness and taken up in distilled water, the volume in milliliters being equal to the original fresh wt. in grammes of the tissue from which the extract had been made. The methanol-insoluble residue was also taken up in the same volume of distilled water as above to serve as the aqueous extract. The aqueous solutions were stored in the frozen state.

Further fractionation of methanolic and aqueous extracts was accomplished by descending paper chromatography, using *n*-butanol:acetic acid:water (4:1:2·2, v/v) and/or 5% aq. acetic acid. The active substances were localized by fluorescence in short and long wavelength u.v., and by the reaction with the following phenol-detecting sprays: 2% $FeCl_3$; 3% aqueous $FeCl_3$–$K_3Fe(CN)_6$; 0·1 N ammoniacal $AgNO_3$ and 2 N sodium carbonate. Ultra-violet absorption spectra of active materials eluted from filter paper were obtained on a Model 3000 Spectracord recording spectrophotometer.

Enzymatic Determinations

The reaction mixture for IAA oxidase determinations contained 1 ml enzyme (prepared from fresh tissue or from reconstituted acetone powder), 1 ml 10^{-3} M IAA, 1 ml 10^{-2} M potassium phosphate, pH 6·1, ± cofactors [1 ml each of 10^{-3} M $MnCl_2$, 5×10^{-4} M 2,4-dichlorophenol (DCP)] and distilled water to a total volume of 10 ml. All ingredients except enzyme were preincubated for 10 min with shaking at 30°, after which time the enzyme was added, with vigorous stirring. One-ml aliquots were removed at 0, 30, 60, 90 and 120 min for determination of residual IAA by the Salkowski colorimetric technique[18] in a Klett colorimeter equipped with a green filter. Inhibitors were assayed by measuring the degree of inhibition of the destruction of IAA mediated by 5×10^{-7} M crystalline horseradish root peroxidase (HRP). This crystalline enzyme was used for convenience, and was shown to have the same response to inhibitors as *Pharbitis* root extracts. Generally 1 ml of a suitably diluted inhibitor extract was added per 10 ml reaction mixture so as to yield an insignificant lag period but a depressed steady state of IAA disappearance. The relative activities of various inhibitor fractions were expressed as the dilution which yielded 50% inhibition of the standard HRP-mediated reaction. In all such calculations, a concentration of 1·0 refers to the original aqueous extract, consisting of the inhibitor dissolved in the same volume of water as the tissue from which it had been extracted.