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THE EFFECT OF ETIOLATION, LIGHT AND
LIGHT QUALITY ON THE GROWTH AND
ENDOGENOUS PLANT HORMONE LEVELS IN
SQUASH SEEDLINGS

by

LAWRENCE MARK SEGAL, B.Sc.

A thesis submitted to the Faculty
of Graduate Studies and Research
in partial fulfilment of the
requirements for the degree of
Master of Science

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ABSTRACT

The effects of darkness and of different light conditions on the growth rate and the endogenous levels of growth-regulating substances in the hypocotyls of squash seedlings (*Curcubita pepo* var. Golden Hubbard) has been examined.

Plants were grown in darkness or under 16h photoperiods, and were sampled at 2-day intervals up to 2 weeks after germination. After harvesting and extraction, growth substances were partitioned into neutral and acidic ether fractions which were then analysed by gas chromatography (GLC). In other experiments, the effects of light spectral quality and de-etiolation on hormone levels were studied.

Results indicate that the rapid growth of etiolated hypocotyls cannot be explained by enhancement of the endogenous levels of several growth-promoting substances as compared with light-grown material. Furthermore, no major differences in the levels of growth-inhibiting substances were found when etiolated and light grown tissues were examined. Red light treatment resulted in higher levels of both IAA and TOL than did blue light. In contrast, IPA levels were much higher in plants grown in blue light than in red. The de-etiolation of 10-day old seedlings with white light resulted in a rapid increase in the level of IPA in the hypocotyl and cotyledon tissue.

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INTRODUCTION

(A) LIGHT AS AN ENVIRONMENTAL FACTOR AFFECTING THE GROWTH AND DEVELOPMENT OF PLANTS

Light is one of the basic environmental stimuli affecting many physiological and biochemical processes in plants. Included among these effects are its photomorphogenic influences on etiolated seedlings.

The inhibition of stem growth by light in etiolated seedlings was observed by Blaauw (1915) and later work revealed that growth was most inhibited by red light at around 650 nm (Weintraub and McAlister, 1942). A complementary effect of light in reducing stem growth and enhancing leaf expansion was demonstrated by Parker *et al* (1949). Numerous other morphological effects have been observed when etiolated seedlings are exposed to light. Some of these include stimulation of epidermal hair growth, hypocotyl or epicotyl hook unbending, biosynthesis of pigments, and the enhancement of geotropism (Leopold and Kriedemann, 1975).

Photomorphogenesis in plants may be mediated via light-induced changes in key factors affecting cell growth, such as enzyme action, membrane permeability and the levels of endogenous growth-regulating substances. The possibility that phytohormones may be involved will be discussed in detail in this section.

(B) THE ROLE OF THE COTYLEDONS IN THE CONTROL OF HYPOCOTYL GROWTH

The cotyledons appear to play a major role in the effects of light on the growth of the hypocotyls. Katsumi *et al* (1965b) found that exogenous gibberellin did not greatly stimulate cucumber hypocotyl growth in the absence of the cotyledons, whereas auxin-promoted elongation was not influenced by their removal. It was suggested that a factor supplied

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by the cotyledons was required for a gibberellin-induced growth response. When the cotyledons of sunflower plants were removed, etiolated growth of the hypocotyl was reduced (Bruinsma *et al* 1975). However, the inhibitory effect of light was reduced when the cotyledons were covered in the light-grown plants. Goto (1977) obtained similar results with etiolated bean seedlings. He found that if the duration or intensity of light exposure was reduced, the inhibitory effect of the cotyledons on hypocotyl growth in the light was diminished. A morphological separation of spectral sensitivity in cucumber seedlings was demonstrated by Black and Shuttleworth (1974). They found that the extension growth of the hypocotyls was inhibited by both blue and red light. The major part of the inhibition due to red light was mediated through the cotyledons, while blue light acted directly on the hypocotyls. The authors suggested that auxin and gibberellin substances may be implicated in this process, but provided no experimental evidence to support this possibility.

(C) THE RELATIONSHIP BETWEEN LIGHT, LIGHT QUALITY AND ETIOLATION AND GROWTH REGULATING SUBSTANCES IN PLANTS

1. Gibberellins

The suggestion that gibberellins might play an important role in the etiolated growth of stems was first made by Lockhart (1956) who found that exogenously applied gibberellic acid (GA_3) reversed the inhibitory effect of light on stem growth. Later work indicated that applied GA_3 overcame the photoinhibition of stem growth in *Latucia sativa* (Frankland and Wareing, 1960), *Curcubita* sp., *Cucumis* sp. (Lockhart and Deal, 1958; Katsumi *et al*, 1965a) and *Phaseolus aureus* (Nwachuku and Lockhart, 1964). However, when Mohr and Appuhn (1962) looked for a similar relationship in *Sinapsis alba*, no interaction between light and gibberellic acid was

found. These workers strongly challenged Lockhart's original proposal when they found that the growth of *Sinapis* seedlings saturated with exogenous GA_3 was still controlled by the phytochrome system and that the endogenous growth of hypocotyls, and growth induced by applied GA_3 , were qualitatively different. This work was later repeated by Wakhloo (1975) who concluded that native GA's are not involved in the etiolated growth of shoots.

A number of workers have concentrated their studies on the effects of exogenous gibberellins and GA-biosynthesis inhibitors on the growth and biochemistry of excised hypocotyl segments under various environmental conditions. Nakamura *et al* (1966) found that GA_3 greatly stimulated the growth of excised plumular hooks from the third internodes of etiolated peas. Specifically, they found that GA treatment exerted its effects on growth of the sections by greatly increasing cell length. DNA synthesis in light-grown cucumber hypocotyl tissue was studied by Degani *et al* (1970) who examined 3H -thymidine incorporation into DNA when the tissues were incubated in water or in a solution of $GA_{7/4}$. They found a very strong correlation between the increase in length of gibberellin treated tissue and the rate of 3H -thymidine incorporation into the DNA of the tissue. The increase in the length of GA-treated sections was strikingly reduced when the DNA-synthesis inhibitor 5-fluorodeoxyuridine (FdUrd) was applied 12 h prior to hormone treatment. The authors suggested that this might indicate that at least some DNA synthesis is a prerequisite of hormone-induced elongation.

In other biochemical investigations, Katsumi (1976a) studied the effects of N,N'-dicyclohexylcarbodiimide (DCCD), an inhibitor of membrane bound ATPases, on the growth of cucumber hypocotyl sections. When ADP, ATP or GA_3 were supplied to the hypocotyl sections, the inhibitory effect of DCCD on growth was eliminated. This suggested that GA_3 and certain

nucleotides behave similarly with respect to membrane-bound ATPases. Recently, the action of several catecholamines in relation to GA-induced lettuce hypocotyl elongation was examined by Kamisaka (1979). He found that epinephrine, norepinephrine, dopamine and 3,4-dihydroxymandelic acid synergistically enhanced the growth of GA-treated hypocotyls. The presence of these catecholamines in higher plants suggest that these substances may act synergistically to enhance the action of endogenous growth regulators. When extracts from lettuce seedlings were analysed by TLC, zones of activity were found which corresponded well with authentic dopamine and epinephrine.

The role of turgor and cellular extensibility in relation to hypocotyl growth and gibberellin treatment has also received attention in the past. Stuart and Jones (1977) found that lettuce hypocotyl sections incubated in the light in the absence of GA_3 do not elongate in the presence of added salts. However, etiolated tissue elongates well in either GA_3 solutions or water, and growth is enhanced by the addition of KCl. Although these authors found that increased cell turgor is not the driving force for the growth of the hypocotyl sections, changes in the extensibility of the tissue incubated in the dark or in the light with GA correlate well with the increased growth rates. This is in agreement with older studies of etiolated and light-grown pea stems (Yoda and Ashida, 1960; Lockhart, 1961). However, GA_3 has not been shown to affect cell wall extensibility in excised cucumber hypocotyls (Cleland *et al*, 1968; Katsumi and Kazama, 1978).

Due to the difficulty of extraction and quantitative estimation, studies of changes in endogenous GA levels in relation to light treatment and etiolated growth were not frequently performed in the 1960's. However, when such studies were attempted it became clear that in most cases, extractable gibberellin levels in etiolated tissue were either lower or

similar to that found in tissue grown in the light.

Wheeler (1960) examined the GA-like activity in leaf discs grown in the light and dark, and found that the biological activity peaked after 4 days of incubation in the light. He was unable to detect any activity in cultures incubated in the dark. Ogawa (1964) found that the GA-like activity of *Pharbitis nil* was highest in the cotyledons, and that the hypocotyls and roots contained progressively lower amounts of the hormone. TLC and bioassay were used to estimate the changes in the GA-like activity of these organs during the first 7 days after germination. Although no major differences were detected between the etiolated and red-light grown material, GA-like activity increased rapidly in the cotyledons of plants exposed to red light. The activity was maximal on the sixth day, when 70 ng GA₃ equivalents/cotyledon were present in the light-grown material. Activity in etiolated cotyledons did not exceed 30 ng GA₃ equivalents/organ. A similar experiment was performed by Kende and Lang (1964), this time with *Pisum sativum*. Two distinct zones of activity were found on the TLC system they employed; one which had a similar R_f value to that of GA₅, and the other which co-chromatographed with GA₁. However, they also found no significant differences between the levels of gibberellin activity in etiolated and red-light treated stem tissues.

The effect of white light, as compared with darkness, has been examined in *Phaseolus multiflorus* (Crozier and Audus, 1968). Two zones of GA-like activity were found, but no provisional identifications were made by co-chromatography on TLC. Although the GA-contents of plants from the two light regimes were similar in total, differences were detected in the distribution of activity between the two zones. Estimates of the total activity present were made by bioassay, and ranged from 0.27 to 3.6 ng GA₃ equivalents per seedling. An interesting conclusion

drawn from this work was its support for the suggestion that light initiates *de novo* GA synthesis. GA-like activity was found only in the roots of light-grown material, and since roots had been previously suggested by Paleg (1965) as the primary site of GA biosynthesis, the idea gained credibility.

Changes in both free and bound GA's were studied in *Pharbitis nil* during a period of 18 days after germination (Barendse and Lang, 1972). Although the levels of free GA's remained fairly constant in etiolated and light-grown seedlings throughout the experiment, GA's were present in higher levels in the light-grown material. Furthermore, $^3\text{H-GA}_1$ applied to seedlings grown under both conditions was metabolised in the same way. This strongly suggested that any difference in total GA levels would likely be due to differential rates of synthesis. Since no major differences were detected in free GA levels, the authors suggested that light may exert its effect by changing the plant's sensitivity to GA, such that GA is required in higher amounts in the light to elicit a growth response equal to that in the dark. A similar conclusion was made by Acton and Murray (1974) who found that light-grown hypocotyls of *Lupinus alba* had considerably higher levels of GA-like activity than etiolated material (75 pmol vs 5 pmol) of the same physiological age.

A number of studies concerning the relationship between phytochrome and GA levels were undertaken after Reid *et al* (1968) found that a red light stimulus induced a rapid rise in GA-like activity in excised, etiolated barley leaves. The GA activity increased greatly after only 15 min of incubation following the red light treatment, and declined when the incubation period was increased. Evidence from inhibitor studies indicated that the rise in GA activity was due to *de novo* synthesis rather than conversion from bound forms. Loveys and Wareing (1971) performed a similar experiment with excised wheat leaves, and found that

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the red light stimulus enhanced the amount of free GA in the tissue. Bound-GA was more abundant in the etiolated tissue than that grown in the light. It has been recently suggested that the red light-induced increase is due to a release of GA from the etioplasts of leaves, rather than to *de novo* GA biosynthesis (Graebe and Roper, 1978). When a red light stimulus was followed by a far-red treatment of etiolated pea seedlings, no reversal of the red light treatment on GA levels was observed (Neskovic and Konjevic, 1974). This suggests that it is unlikely that GA synthesis is under phytochrome control.

All of the studies mentioned thus far involved the use of simple chromatographic techniques, such as paper and thin-layer chromatography for analysing plant extracts, followed by determination of the GA levels by bioassay. Few investigations to date have utilized modern separatory methods such as gas chromatography or high performance liquid chromatography (HPLC). In the early 1970's the identification of several gibberellins in plant extracts by GLC and GC-MS was first achieved. The gibberellins of etiolated and light-grown *Phaseolus coccineus* seedlings were studied by Crozier *et al* (1971) and by Bowen *et al* (1973). These workers were able to establish conclusively the presence of GA₁, GA₄, and GA₁₉ in the etiolated tissue, while light grown seedlings of the same age contained GA₁, GA₄, GA₅, and GA₂₀. Although the total level of GA's was determined to be similar under both conditions, GA₄ levels were significantly lower in the light-grown tissue. It was suggested that light may have some effect on the biosynthesis or further metabolism of this GA.

2. Inhibitor Substances

Once the presence of naturally-occurring growth inhibitor substances in plants had been established in the mid-1960's, several

groups of workers focused their attention on the mechanism of light-inhibition of stem growth in relation to these substances.

Zeevaart (1971) found that when spinach seedlings were transferred from a short-day to a long-day environment, the ABA-like inhibitory activity in the seedlings increased 2½-fold in the first day. He also found that ABA levels were higher at the end of the light period than at the beginning. However, since the species used in these experiments had a long-day flowering requirement, and endogenous ABA levels increased under this condition, the author concluded that ABA may not function as an endogenous growth inhibitor. Later studies (Zeevaart, 1974) indicated that although ABA levels in spinach changed as a result of a shift in the duration of the photoperiod, no such alteration was observed in the levels of xanthoxin.

The levels of inhibitor substances in etiolated and light-grown peas were studied by Higgins and Bonner (1974). They found two distinct zones of inhibitory activity on the thin layer chromatograms of extracts of the seedlings. One of these was subsequently identified by GLC as ABA and the other was suspected to be xanthoxin but not clearly identified as such. The ABA levels in the light-grown shoots were estimated at approximately 7.5 ng/g fresh weight, with about 4 ng/g fresh weight present in the etiolated tissue. However, none of the second inhibitor substance, which the authors called "Y", was found in etiolated shoots in large enough quantities to obtain an estimate of its concentration. In contrast, light-grown shoots contained between 3-6 ng/g fresh weight of this substance. These findings suggested that ABA is probably not involved in the light-inhibition of pea stem growth, but that perhaps another inhibitor might be. Further studies were conducted by Anstis *et al* (1975) with pea seedlings grown under intermittent red light, or in darkness, over an 8-day period. Xanthoxin levels were determined in

tissue extracts by GLC and were consistently higher in the plants given the light treatment. They also found that xanthoxin precursor substances were synthesized rapidly on illumination of the seedlings. Xanthoxin-like activity was also found to be higher in the hypocotyls of light-grown sunflower seedlings than in the etiolated tissues. (Thompson and Bruinsma, 1977). Analysis of the substances in extracts of these plants was carried out by HPLC, and the fraction displaying the maximal growth inhibitory effect in bioassay was eluted from the column at a retention time similar to that of authentic xanthoxin. Positive identification of the inhibitory substance in the plant sample itself, however, was not made.

Differences in the levels of inhibitors in relation to light treatment have also been studied in tall and dwarf bean (Goto, 1978a; 1978b) and radish seedlings (Hasegawa and Miyamoto, 1978). Although very little difference in the total ABA-like activity was found when dwarf and tall strains of bean were compared, the activity in light-grown cotyledons of the dwarf strain was considerably higher than that observed in cotyledons of comparable etiolated plants. Differences in the levels of xanthoxin-like activity were considerably less pronounced. When diffusible inhibitor levels in the upper portion of the shoot were examined after a 24 h light stimulus, no inhibition was observed using a bioassay. This indicated that photo-inhibition of hypocotyl growth could not be based directly upon the amounts of xanthoxin and ABA-like inhibitors produced in the cotyledons, since it was shown that no transport of these substances out of the cotyledons occurred. In radish seedlings, higher levels of both neutral and acidic inhibitors were observed after 3-day old seedlings were exposed to white light for 24 h. However, no attempt was made to estimate the amount of diffusion of these inhibitors from the cotyledons to the stems.

The interaction of phytochrome with light-stimulation of ABA synthesis was examined by Tucker (1977). In tomato plants grown under far-red light, bud lengthening was inhibited and the ABA-like activity increased (compared to white-light treatment) in the stems and mature leaves of plants given this treatment. However, when Loveys (1979) grew tomato plants under red and far-red light, the ABA levels, measured by GLC, were higher under red-light conditions, while the extension growth was three times greater under far-red light. An important observation made in this work was that although the growth rate changed very rapidly in response to a change in light quality, endogenous ABA levels were much slower to change. This would seem to preclude ABA as an endogenous regulator of the initial growth response.

3. Auxin Substances

Much of the work done in previous years dealing with the interactions of light treatment and etiolated plant growth with auxins, has focused on the effects of exogenous application of these substances under a variety of environmental conditions.

Katsumi *et al* (1965) found that applied GA's and auxins produce gross morphological changes in light-grown cucumber hypocotyls that are indistinguishable, but that both are required for normal seedling growth. When IAA was applied alone, or with GA, to plumular hook sections of etiolated pea seedlings, no growth promotion was observed (Nakamura and Takahashi, 1968). However, when the auxin inhibitor, 4-chlorophenoxyisobutyric acid (PCIB), was applied to GA-treated sections, the growth promoting activity of GA was inhibited. Since the inhibition was completely overcome by treatment with IAA, the results suggested that auxin is necessary for the growth of GA-treated hook sections, but that

auxin and gibberellin act differently on the growth of this tissue.

DCCD, an inhibitor of membrane-bound ATPases, was shown to inhibit severely the IAA-induced elongation of light-grown cucumber hypocotyl sections (Katsumi, 1976b). However, this inhibition was partially reduced when the concentration of exogenously applied IAA was increased, indicating a close association of auxin action with membrane-bound ATPase. When GA_3 was applied simultaneously with DCCD, no inhibitory effect on IAA-induced growth was observed, indicating an interaction between these substances in the growth of the tissue. No difference between the optimal concentrations of IAA required for hypocotyl elongation in light and darkness was found in cucumber hypocotyl segments (Kazama and Katsumi, 1978). Dei and Tsuji (1978) found that when etiolated cucumber cotyledons were given a short pulse of red light, chlorophyll synthesis was enhanced during a subsequent period of continuous illumination. Exogenously applied IAA was shown to interact with the red light treatment (unlike GA_3) and to increase further chlorophyll synthesis, thus implicating the growth regulator in this process.

Early studies on the effects of light on the endogenous levels of IAA in plant organs indicated that light reduced the auxin concentration. Fletcher and Zalik (1964) studied the relationship between light quality and IAA levels in bean seedlings. They found that the amounts of auxin in plants grown under blue, red, yellow and far-red light were lower than in etiolated plants. A relationship between the length of the hypocotyl under each of the light conditions and the amount of IAA was also observed. When Briggs (1963) exposed corn coleoptiles to red light, he also found that light reduced the amount of diffusible auxin in the tissue, compared to dark-grown controls. However, when Kang and Ray (1969) examined the endogenous auxin level in bean hypocotyl hooks given red light treatment, no decline in the level of extractable auxin was

detected, as compared with etiolated controls. These workers suggested that no relationship exists between red-light exposure and the yield of diffusible auxin. Since all three studies mentioned above involved rather crude methods for the estimation of endogenous auxin levels, it is difficult to decide if the results of one study are more reliable than another.

Acton and Murray (1974) found low, but measurable levels of auxin activity in the etiolated hypocotyls of lupin. In contrast, plants grown in the light had higher amounts of IAA-like activity in the coleoptile bioassay. When exogenous IAA was applied to the seedlings grown in the light, the inhibition of hypocotyl growth was reduced in proportion to the amount of IAA applied. In contrast, the increase in hypocotyl length of etiolated plants was reduced as the concentration of applied IAA increased. The authors suggested that the IAA-induced inhibition of growth in the dark was due to differential sensitivity of the hypocotyl tissue, such that auxin levels which are 'sub-optimal' in light-grown tissue are 'supra-optimal' in etiolated plants. In another study, the IAA content of sunflower hypocotyls was examined after different light treatments (Bruinsma *et al.*, 1975). Diffusible IAA was measured spectrofluorometrically after 6 h of light exposure. These workers found no difference in the levels of IAA in the hypocotyls of shaded and light exposed plants. They also found no difference in levels of IAA in the shaded and light-exposed halves of phototropically bending hypocotyls, both halves containing about 60 ng IAA/g fresh weight. This result strongly contradicted the validity of the Cholodny-Went theory of differential IAA levels in light-exposed and shaded tissue as the mechanism responsible for phototropic bending.

4. Ethylene

The relationship between the levels of ethylene produced by plant tissues and the light stimulus given to the plant has received little attention. However, studies have shown that ethylene production is stimulated by exogenously applied IAA and kinetin, but it is unaffected by GA_3 (Fuchs and Lieberman, 1968; Imaseki *et al.*, 1975; Kondo *et al.*, 1975). It has also been demonstrated that IAA levels are diminished in etiolated pea-stem tissue when incubated with ethylene (Lieberman and Knecht, 1977).

Kang and Ray (1969) studied the changes in ethylene production in etiolated bean hypocotyl hook sections given a red-light stimulus, and found that this treatment caused a decrease in the ethylene production in the tissue. This reduction in ethylene production occurred even when the levels had been substantially increased by immersion of the tissue in auxin solution prior to red light treatment. It was concluded that ethylene plays a role in the light-stimulated hook opening response in this seedling. Recently, acetylcholine has been demonstrated to mimic the red-light effect on ethylene production (Parups, 1976), suggesting that it may also play a role in the hook opening response.

5. Cytokinins

Few experiments have been performed to determine the effect of etiolation, or light quality, on the endogenous levels of cytokinins. However, several studies have shown that when seeds are exposed to red light, a rapid rise in cytokinin activity occurs (Letham, 1978). It has not yet been conclusively demonstrated whether the red-light stimulation is reversible by exposure to far-red light. Cytokinin levels in the detached leaves and whole seedlings of poplar were also observed to

increase when exposed to red light immediately after the dark period (Hewett and Wareing, 1973). Unfortunately, a review of the literature yielded no information concerning the levels of cytokinins in etiolated and light-grown seedlings during the early stages of growth.

(D) AIMS OF THE PRESENT STUDY

It appears that the relationship between etiolation and light growth of hypocotyls and the endogenous levels of growth-regulating substances is a complex one. The many previous studies outlined in this Introduction serve to illustrate the difficulty in comparing and interpreting the data presented due to differences in the species used, age of the tissue and the type of stimulus provided. Much of the work offers only circumstantial evidence for the role of growth-regulating substances in etiolated growth. Furthermore, only a very small fraction of the research performed thus far has utilized the modern chromatographic techniques of GLC, HPLC, or GC-MS for the identification and quantitative estimation of the growth regulators present in the plant tissues employed.

Accordingly, the current study was undertaken to determine the changes in the levels of several endogenous growth regulators in both etiolated and light-grown hypocotyls of squash plants over a 2 week period, using the modern analytical method of gas chromatography. Studies were also performed to examine the effects of light quality and de-etiolation on the levels of growth regulators in the hypocotyl tissue of the plants. In each case, the changes in the levels of plant hormones were compared to the growth rates in order to determine whether any correlation exists between etiolation or photoinhibition and the endogenous levels of these substances.

MATERIALS AND METHODS

(A) SELECTION OF PLANT MATERIAL USED IN THE EXPERIMENTS

The initial selection of the plant material used in the experiments was made by comparing the hypocotyl growth shown by 8 varieties of squash (*Curcubita pepo*) after 14 days growth in either complete darkness or under 16h photoperiods at 23°C. Seeds of each variety were sown in vermiculite:perlite (50/50) mixture and watered at regular intervals during the following two week period. The average length of at least 25 hypocotyls randomly selected from the etiolated and light grown seedlings were compared for each variety, and the one displaying the greatest relative difference in growth under the two treatment conditions (var. Golden Hubbard) was selected for experimental use.

(B) GROWTH MEASUREMENTS AND ENVIRONMENTAL CONDITIONS

1. Etiolation/Light Growth Experiment

The growth of Golden Hubbard squash was examined during the first 14 day period following germination. All plants were initially germinated in the dark and at the 5-day stage, half of the plants were transferred to another growth chamber programmed to provide 16h photoperiods of white light. The average length and fresh weight of the hypocotyls from plants grown under these two environmental conditions was determined from samples of at least 25 plants after 5, 6, 8, 10, 12 and 14 days of growth. Dry weight measurements were also made at each stage of growth by placing samples of at least 20 hypocotyls in an oven at 95°C for 24h. After

cooling, each sample was weighed on a Mettler H20 analytical balance. The temperature in the controlled environment growth chambers was maintained at 23°C and the radiant energy level during the light period was 5.2×10^4 erg/cm²sec.

2. Light Quality Experiment

To examine the effects of light quality on phytohormone levels, squash seedlings were planted in the vermiculite:perlite mixture and placed in wooden boxes, fitted with coloured filters (Cinemoid) transmitting either blue or red light. Other plants were exposed to the full visible spectrum or to no light at all. A radiometer (YSI-Kettering Model 65) was used to ensure that the radiant energy to which plants were exposed was the same for all treatments (1.45×10^4 erg/cm²sec). Plants were grown for 10 days at 23°C under each of the light conditions, at which time the hypocotyls were harvested and the average length, fresh weight and dry weight of each sample was determined.

3. De-etiolation Experiment

Plants used in these experiments were initially grown in complete darkness at 23°C. Control plants were left in the dark for 10 days, at which time the hypocotyls and cotyledons were harvested. The experimental plants were exposed to white light at a radiant energy level of 5.2×10^4 erg/cm²sec for either 6 or 24 h prior to sampling. The fresh weight of the hypocotyls and cotyledons was determined prior to extraction of the growth substances from the tissues.

(C) SAMPLING PROCEDURE

1. Etiolation/Light Growth Experiment

Hypocotyls of plants grown for 5 days in the dark constituted the 'zero-time' material for this experiment. The hypocotyls of plants that were transferred at the 5-day stage to a growth chamber providing 16h photoperiods, along with a sample of those left in darkness were harvested on the next day (6 days after planting). Following this, plant material grown under the two experimental conditions was harvested at 2-day intervals up to the 14th day after planting. The amount of each sample varied from 80 to 150 g fresh weight of hypocotyl tissue, depending on the age of the plants.

2. Light Quality Experiment

Plants were grown under 16h photoperiods, exposed to either blue, red, white light or grown in complete darkness. On the 10th day after planting, the hypocotyls of each group were harvested. In most cases, 100 g fresh weight was obtained for each sample.

3. De-etiolation Experiment

Plants were grown in complete darkness for 9 days, at which time some were transferred to a growth cabinet and subjected to continuous white light for the next 24 h. Early on the 10th day, another group of dark-grown plants was transferred from the dark cabinet and exposed to the light for the next 6h only. At the end of the 10th day, the hypocotyls and cotyledons of plants exposed to light for either 24 or 6 h were harvested and at the same time, hypocotyls and cotyledons from the dark-grown control plants of the same age were also collected. Approximately 100 g fresh weight was

obtained for each of the hypocotyl samples, while the cotyledons yielded 50-60 g fresh weight per sample.

(D) PROCEDURES FOR THE EXTRACTION AND FRACTIONATION OF ENDOGENOUS GROWTH REGULATING SUBSTANCES

The procedure used for the extraction and solvent fractionation of the endogenous growth regulating substances is shown in figure 1.

After the hypocotyls to be studied had been harvested and their total fresh weight determined, each sample of tissue was finely macerated in a glass Waring blender containing approximately 200 ml of 80% acetone. The ground tissue extract was then transferred to a 500 ml erlenmeyer flask, sealed with aluminum foil and stored overnight in the dark at 2°C. This extraction period ensured the complete extraction of all soluble substances into the organic phase. The cellular debris was filtered off using a buchner funnel with Whatman No. 1 discs, and the flask was rinsed several times with acetone. The organic filtrate was then concentrated on a rotary evaporator, yielding an aqueous phase after all the acetone had distilled off. This aqueous phase was filtered under suction through a layer of celite (analytical filter aid) to remove precipitated pigments, and the residue in the evaporator flask was washed twice with small volumes of hot water and also subjected to filtration. The collected filtrate was then adjusted to pH 8.5 with 2N KOH and extracted 3 times with equal volumes of diethyl ether to yield the neutral ether fraction. This fraction should contain all neutral compounds that are ether soluble, such as the inhibitor xanthoxin and the auxin precursor, tryptophol. The aqueous extract was then re-adjusted to pH 2.5 with 2N phosphoric acid, and again extracted

Figure 1. Procedure for the extraction and solvent-partitioning
of hypocotyl and cotyledonary tissues of squash seedlings.

PROCEDURE FOR THE EXTRACTION AND FRACTIONATION OF GROWTH REGULATING
SUBSTANCES OCCURRING IN SQUASH

3 times with equal volumes of re-distilled ether. The extraction yielded the acidic ether fraction, which should contain all of the free acidic compounds that are soluble in ether, such as the indole acids, the free gibberellins and abscisic acid.

Both of these ether fractions were concentrated to a small volume on a rotary evaporator, which was then subjected to filtration through Whatman phase-separation paper to remove traces of water, which would contain impurities. The concentrated ether extracts were transferred to standard size test tubes and taken to complete dryness using a Buchler "Evapomix" evaporator. The tubes were sealed with parafilm and aluminium foil and then placed in a deep freeze at -20°C until required for derivatization and gas chromatographic (GLC) analysis.

(E) DERIVATIZATION OF ETHER-PARTITIONED FRACTIONS

1. Acidic Ether Fraction

The method chosen to derivatise the acidic compounds present in this ether fraction was methylation, as this procedure has been used successfully by previous workers in Dr. Wightman's laboratory and is a relatively simple and rapid procedure.

Dry extracts were first re-dissolved in 10% MeOH in ether (all solvents being anhydrous) and were then methylated using the diazomethane procedure first described by Schlenk and Gellerman (1960). Nitrogen gas was bubbled through a large test tube half-filled with dry ether. The ether saturated N_2 gas was then channelled into another large test tube containing 10 ml of 10N KOH, 7 ml of dry diethyl ether and 7 ml of diethylene glycol monoethyl ether (carbitol). When ready to begin methylation of the extracts, a scoop ($\sim 1\text{g}$) of Diazald (N-methyl-N-nitroso-p-toluene sulfonamide) was added to the reaction mixture. The

resulting diazomethane gas produced was allowed to bubble through the sample vial for a period of approximately 3 minutes to ensure that all reactive groups were methylated. The sample, which usually had a yellow tinge after methylation, was then allowed to evaporate to dryness in a fume hood, after which it was stored in a dessicator over phosphorus pentoxide until required for GLC analysis.

2. Neutral Ether Extract

No derivatization was required for analysis of the neutral ether fraction, as the growth-regulating substances present in this fraction were sufficiently volatile in the gas chromatograph without prior esterification. Although silylation and acetylation have been used in previous investigations as methods for esterifying compounds in this fraction, good chromatography can be obtained with underivatized neutral growth regulators, such as Xanthoxin (Dorfling, 1979).

(F) PREPARATIVE GAS CHROMATOGRAPHY PROCEDURE

1. Calibration of the Column With Authentic Growth Regulators

Preparative gas chromatography was used as a preliminary purification procedure for both the acidic and neutral ether plant extracts in all experiments. The preparative column employed was a glass column (2.1 m long, 9 mm bore) packed with 3% OV-17 coated on Chromosorb W (acid washed) of 80/100 mesh. The gas chromatography apparatus was a Pye-Unicam model 104, which was equipped with dual heated flame-ionizing detectors and a temperature controlled preparative collection port. By means of a splitter apparatus, diagrammatically illustrated in fig 2, the effluent from the preparative column was separated into two streams, such

Figure 2. Diagram of the splitter apparatus used during preparative gas chromatographic analysis.

Table 1. Calibration of the preparative GLC splitter with ^{14}C - labelled plant growth regulators.

(A) ^{14}C - 3-Indoleacetic acid

Counts (Cpm)	Control (Cpm)	% RECOVERY
14651	21539	68.8
15077	21859	68.9
14164	22466	63.0
13245	21928	60.4
15756	21088	74.7
14900	21095	70.6
Average		67.8%

(B) ^{14}C - Absciscic acid

Counts (Cpm)	Control (Cpm)	% RECOVERY
17345	18331	94.6
17570	17921	98.0
18280	18518	98.7
6307	6620	95.3
2335	2693	86.7
Average		94.7%

(C) ^{14}C - Gibberellic acid 3

Counts (Cpm)	Control (Cpm)	% RECOVERY
4618	5589	82.6
4715	5554	84.9
3856	4683	82.3
8622	10117	85.2
7972	9891	80.6
Average		83.1%

that approximately 95% of the gas flowed out through the collection port and the remaining 5% moved into the flame-ionization detector. A standard analytical glass column (2.1 m long, 4 mm bore), also packed with 3% OV-17, was connected to the reference detector. Listed below are the GLC conditions used while determinations of the elution temperatures of a range of authentic growth substances were made:

Nitrogen Flow:	Preparative Column - 150 ml/min
	Reference Column - 32 ml/min
Hydrogen Pressure:	20 psi
Air Pressure:	7.5 psi
Attenuation Setting:	1×10^2
Chart Speed:	0.2"/min

Since only a 5% proportion of the sample injected in the preparative column entered the detector, relatively large amounts (normally more than 1 μ g) of each of the authentic growth substances were used to determine their elution temperatures. The temperature program employed to separate the compounds expected to be present in the acidic ether fraction started at 100°C and increased linearly at 3°C per minute until a final oven temperature of 315°C was reached. The program used for the neutral compounds similarly began at 100°C and increased at 3° per minute up to, 250°C. By chromatographing authentic standard compounds several times, reproducible elution temperatures were determined for indoleacetic acid (IAA), 4-Cl-indoleacetic acid (4-Cl-IAA), indolepropionic acid (IPA), abscisic acid (ABA), gibberellin A₇ (GA₇), gibberellin A₃ (GA₃), Xanthoxin, Tryptophol (TOL) and indolealdehyde (IAld).

2. Determination of the Collection Efficiency of the Preparative GLC System Using ^{14}C Labelled Standard Compounds

In order to determine the collection efficiency of the preparative GLC system, specific amounts of ^{14}C -labelled authentic growth substances were injected on the preparative column. These compounds were collected by a glass collection trap, fitted with a disc of glass fibre filter paper, which attached to the outlet of the collection port. Once the collection had been made, the contents of the trap were eluted with 10% methanol in ether and transferred to a scintillation vial, where the solvent was evaporated away with a stream of N_2 . At the same time, an amount of the ^{14}C -labelled growth regulator equivalent to that injected on the preparative column was also placed in a scintillation vial and the carrier solvent evaporated to dryness. 10 ml of TOX (Triton, Omnifluor, Xylene) liquid scintillation fluid was added to each vial to solubilize the residue and the radioactivity present was then determined by measuring the number of counts in each vial over a 10 minute period, using a Packard Tricard liquid scintillation spectrometer. The radioactivity in the vial containing material collected from the preparative column was expressed as a percentage of the activity of the respective control. This provided an estimate of the collection efficiency of the preparative GLC system (See Table 1).

3. Preparative Gas Chromatography of Acid and Neutral Ether Plant Extracts

Plant extracts ready for gas chromatography were removed from the storage desiccator and the dried contents were dissolved in ethyl acetate. The volume of solvent used in this procedure varied with the amount of material in the sample to be injected, but was usually between 200-300 μl . An aliquot of this dissolved sample, equivalent to between

50-100 g fresh weight of plant material (depending on the original sample size) was then injected on the preparative GLC column. The temperature programs described earlier were used to separate the components in the extract and glass collection traps were attached to the collection port of the GLC at those temperatures corresponding to the elution temperatures of the authentic growth regulators. After the collections had been made, the contents of each trap were eluted with 10% MeOH in ether and transferred to a clean 4 ml glass vial. The solvent was evaporated under a stream of N_2 and the vials were then stored in an evacuated dessicator over P_2O_5 until required for analytical GLC analysis.

(G) QUALITATIVE AND QUANTITATIVE ESTIMATION OF GROWTH-REGULATING SUBSTANCES BY ANALYTICAL GLC

Analytical GLC columns (2.1 m long, 4 mm bore) were packed with either 3% OV-101 or 3% Dexsil 400, both coated on acid washed chromasorb W, 80/100 mesh. Dexsil 400 columns were used for the analysis of acidic growth substances, while the OV-101 columns were used for the analysis of substances in the neutral ether fraction. The sample and reference columns were connected directly to the detector inlet in the gas chromatograph. Each trapped fraction, obtained after preparative GLC, was dissolved in a small volume of ethyl acetate (usually between 20-50 μ l) and the vial was then capped with a teflon-coated septum lid. This permitted removal of μ l volumes of each sample with a syringe without removing the cap of the vial. Further analysis of each collected sample was performed by isothermal chromatography at a temperature previously determined to be suitable for the best separation of the specific growth regulator being studied. The operating conditions and isothermal temperatures used in these analytical

GLC investigations are given below:

N₂ flow rate: 32 ml/min
 H₂ pressure: 20 psi
 Air pressure: 7 psi
 Attenuation: 100 x
 Chart speed: .5"/min

<u>Growth Regulator</u>	<u>Oven Temperature for Isothermal GLC</u>	<u>Column Packing Material</u>
IAA	205°C	3% Dexsil 400
4-Cl-IAA	235°C	"
IPA	215°C	"
ABA	235°C	"
GA ₇	270°C	"
GA ₃	290°C	"
TOL	180°C	3% OV-101
Xanthoxin	200°C	"
IAld	200°C	"

An aliquot (usually 1 μ l) of the trapped plant sample was injected on the analytical column at the appropriate temperature and such isothermal conditions normally gave a good separation of the various components in the mixture. A second, identical aliquot from the sample was then "spiked" by the addition of a very small volume (usually 1 μ l) of the authentic growth substance under investigation, which would be as the methyl ester derivative if it was an acidic substance. The concentration of the authentic growth substance solution used for "spiking" the second aliquot was approximately 1 μ g/ μ l, so that the 100 ng amount added to the aliquot was sufficient to give an appreciable enhancement of the area of the "suspected" peak observed in the GLC analysis of the first aliquot. This procedure of co-chromatographing the second and other aliquots from trapped samples with 100 ng amounts of one or more of the authentic growth substances permitted provisional identification of the growth regulator present in the plant sample.

Quantitative measurements of the levels of endogenous growth regulators present in the trapped samples was obtained by calculating the area of the relevant peaks in GLC scans of "unspiked" aliquots that were identified by co-chromatography. The area was determined by multiplying the peak height times the peak width, taken at half of the peak height. The area value was then converted to a ng quantity of an authentic substance by means of a standard curve, which had been prepared earlier. Using this aliquot value, it was then possible to calculate the amount of each growth regulator present in the total acidic or neutral ether extract. At this point, the amount of growth regulator per g fresh weight, per g dry weight or per organ could be determined. A sample calculation appears below:

1. The area of a "suspected" hormone peak in an analytical GLC scan was determined.
2. A ng quantity was read off the appropriate standard curve, for example: 10 ng.
3. If this 10 ng was present in a 1 μ l aliquot of the trapped fraction, and the total fraction had been dissolved in say, 50 μ l of solvent, then the total amount of growth regulator in the trapped fraction could be calculated as: $10 \text{ ng}/1 \mu\text{l} \times 50 \mu\text{l} = 500 \text{ ng}$
4. If the total equivalent fresh weight of the ether extract that was run on the preparative column was 50 g, then the concentration of the suspected hormone would be: $500 \text{ ng} \div 50 \text{ g} = 10 \text{ ng/g fresh weight}$.
5. This value could be converted to a per g dry weight or a per organ basis by multiplying by the appropriate conversion value. For example: the average fresh wt. per organ was .5 g for this sample $\therefore 10 \text{ ng/g} \times .5 \text{ g/organ} = 5 \text{ ng/organ}$.

(H) SOURCE OF CHEMICALS AND SEEDS1. Authentic Compounds

Substance	Supplier
3-Indoleacetic acid	Sigma
4-Cl-3-Indoleacetic acid	Dr. K.C. Engvild, Riso, Denmark
3-Indolepropionic acid	Regis Chemical Co.
cis,trans-Absciscic acid	Sigma
Gibberellic acid 3	Sigma
Gibberellic acid 7	U.S. Biochemicals
3-Indoleethanol (Tryptophol)	Sigma
3-Indolealdehyde	Sigma
cis,trans-Xanthoxin	Dr. F. Taylor, Wye College, University of London

2. Reagents for Derivatizations

Carbitol	Fisher Scientific
Diazald	Sigma

3. Solvents

Ethyl acetate	J.T. Baker
Diethyl ether	Canlab
Acetone	Canlab

4. Seeds of *Cucurbita pepo*, var. Golden Hubbard

Seeds were obtained from Ritchie Feed and Seed Ltd.,
York Street, Ottawa, Ontario.

EXPERIMENTAL RESULTS

(A) GROWTH DATA FOR SQUASH PLANTS GROWN IN DARKNESS OR UNDER 16 h PHOTOPERIODS OVER A 14 DAY PERIOD

1. Selection of var. Golden Hubbard as the Experimental Material

Considerable variability in the growth of the 8 squash varieties tested was observed 14 days after germination. As can be seen in figure 3, the ratio of the length of the hypocotyls of plants grown in the dark to those grown in the light was highest in two varieties, Green Hubbard and Golden Hubbard. However, the Green Hubbard seeds were found to show a much poorer germination rate during these tests than the Golden Hubbard seeds. It was decided that the variety to be used in these experiments would be the one that showed the most pronounced difference in growth under the two environmental conditions. If the difference in hypocotyl growth between etiolated and normal plants is due to differences in phytohormone levels, it was thought wise to choose a variety in which the difference between light and dark growth was maximal. Consequently, Golden Hubbard was the variety selected for all further experimentation. Although the hypocotyls of this variety did not grow as tall as many of the other varieties in the dark, the ratio of growth in the dark to that in the light was much greater than that of all the others.

2. Changes in the Morphology and Growth of the Plants

Changes in the growth and development of squash seedlings grown under the two environmental regimes are shown in figure 4a. 4-5 days after planting the seeds, the seedlings had grown to the stage at which the hypocotyls were just beginning to push through the surface of the

Figure 3. Comparison of the growth of the hypocotyls of 8 varieties of squash seedlings after 14 days in darkness or in light.
A = Table Ace, B = Green Hubbard, C = Acorn,
D = Spaghetti, E = Crookneck, F = Buttercup,
G = Patty Pan, H = Golden Hubbard.

DIFFERENTIAL GROWTH OF SQUASH
VARIETIES IN LIGHT AND DARKNESS
AFTER 14 DAYS

Figure 4a. Photograph of squash seedlings grown in light and darkness over a 2 week period after germination.

Figure 4b. Graph of the increase in hypocotyl length of squash seedlings grown in light and darkness over a 2 week period.

vermiculite mixture. At the 5th-day stage, the seedlings were opaque white and had very short hypocotyls; in most cases the seed coat still covered much of the cotyledons and the hypocotyl was bent into a "hook." By the sixth day, however, the hypocotyls of plants that had been moved into the light growth chamber had begun to synthesize chlorophyll, as indicated by the light green colour of the hypocotyl and cotyledons. In contrast, plants left in the dark had longer, white hypocotyls and yellow cotyledons. Over the next several days, the hypocotyls of the etiolated plants grew rapidly, extending greatly in length; the cotyledons, however, remained yellow in colour and a small hook was still present at the point of attachment to the hypocotyl. In contrast, the plants grown in the light had much shorter hypocotyls that were light green in colour. The cotyledons of these plants were dark green and had expanded greatly in size. Furthermore, there was no hypocotyl hook present, and the cotyledons had opened up. The changes in the average length of etiolated and light-grown hypocotyls through the 14-day period after planting are shown in figure 4b.

In terms of fresh weight gain over the 14-day period, the hypocotyls of etiolated plants increased much more rapidly than did their light-grown counterparts. After 14 days of growth, the fresh weight of the dark grown hypocotyls was 3.3 times greater than those grown in the light, (see figure 5a).

As seen in figure 5b, dry weight gain in the dark grown material also occurred at a greater rate than in the light; however, the difference in the average dry weight per organ was less pronounced after 14 days of growth than that observed for fresh weight.

Figure 5a. Graph of the increase in hypocotyl fresh weight of squash seedlings grown in light and darkness over a 2 week period.

Figure 5b. Graph of the increase in hypocotyl dry weight of squash seedlings grown in light and darkness over a 2 week period.

FRESH WT

DRY WT

(B) GROWTH OF SQUASH HYPOCOTYLS UNDER RED, BLUE, AND WHITE LIGHT
OR IN DARKNESS AFTER 10 DAYS

After 10 days of growth under monochromatic light, darkness or white light, distinct differences in the length, fresh weight and dry weight per hypocotyl were observed. As can be seen in figure 6, the light-grown hypocotyls had the lowest average length, fresh weight and dry weight of all treatments, while those grown in the dark were consistently the highest for the same growth parameters. The hypocotyls of plants grown under continuous blue light appeared to be similar in appearance to their dark-grown counterparts. A few differences were noticed, however; these plants were faint green in colour and the hypocotyls were slightly shorter. Red light treatment appeared to induce effects on hypocotyl growth somewhat similar to those observed in plants grown under full-spectrum light. The average length, fresh weight and dry weight of these hypocotyls was lower than both the etiolated and blue-light grown material, but was greater than the white-light grown organs. The cotyledons and hypocotyls of these plants were faint green in colour, and their morphology most closely resembled the hypocotyls of plants grown under white light.

(C) MORPHOLOGICAL CHANGES OBSERVED IN ETIOLATED PLANTS WHEN EXPOSED
TO CONTINUOUS WHITE LIGHT FOR 6 OR 24 h.

After 10 days of growth in the dark, the hypocotyls of squash plants were long and white, while the cotyledons were closed and light yellow in colour. Plants that had been exposed to continuous white light for either 6 or 24 h prior to the 10 day stage, were strikingly different in appearance. After only 6 h of light treatment, the hypocotyls had become pale green in colour, while the cotyledons were medium green and had begun to open. These effects were even more

Figure 6. Histograms showing the fresh weight, dry weight and length of squash hypocotyls grown for 10 days under red, blue or white light, or in darkness.

pronounced in those plants given 24 h of continuous white light. Slight expansion of the cotyledons (broadening and flattening) was noticed in this sample. Clearly, the light treatment rapidly stimulated the synthesis of photosynthetic pigments in the etiolated plants and promoted the observed morphological changes in the cotyledons.

(D) PREPARATIVE AND ANALYTICAL GAS CHROMATOGRAPHY OF PLANT EXTRACTS

After plant tissue samples had been harvested, solvent extracted, partitioned and the fractions derivatized as described in the Material and Methods section, preparative gas chromatography was performed. This procedure permitted the analysis of ether extracts equivalent to large amounts of plant tissue, in terms of equivalent fresh weight. Using glass traps to collect small, selected aliquots of the extract analysed on the preparative column, reasonably purified phytohormone samples could be obtained. Since the substances trapped in any one aliquot were of similar volatility and molecular weight, isothermal analytical GLC could be employed for the final identification and quantitative estimation of growth substances. This type of analysis was desirable because it permitted more rapid examination of the collected fractions than would have been possible using temperature programming procedures. A representative scan obtained from preparative GLC of a squash hypocotyl extract is shown in figure 7. The regions on this chromatogram corresponding to the elution temperatures of several authentic growth substances are indicated on the figure. The efficiency of the collection procedure for various authentic growth substances was determined by injection of ^{14}C -labelled compounds on the preparative column, followed by liquid scintillation counting of the trapped aliquots. The results of this calibration are shown in table 1. The efficiency of collection

Figure 7. Preparative GLC scan of a methylated ether extract of the hypocotyls of 14-day old light-grown squash seedlings.

Figure 8. Analytical GLC scans of an IAA fraction, collected during the preparative GLC analysis of a methylated, acidic ether extract of the hypocotyls of 14-day old light-grown seedlings.

Figure 9. Analytical GLC scans of a GA₇ fraction, collected during the preparative GLC analysis of a methylated, acidic ether extract of the hypocotyls of 14-day old light-grown squash seedlings.

Analytical GLC scan of the
GA₇ zone of the acid fraction
from 14-day light grown
squash hypocotyls

Suspected GA₇-Me

Suspected GA₇-Me plus
authentic standard

was lowest for IAA (ca. 68%), followed by GA₃ (ca. 83%) and ABA (ca. 95%). The variability observed in the collection efficiencies may have been due to differences in the relative stability of the three major groups of growth regulators or to differences in the amount of each compound bound to the column packing material and not eluted. Naturally-occurring growth regulators were identified in the plant samples by co-chromatographing aliquots of the sample with the appropriate authentic compound.

Figures 8 and 9 are representative of the GLC scans obtained when aliquots of the fractions, trapped off the preparative column, were re-chromatographed on analytical columns. The large increase in the area of specific peaks when the samples were co-chromatographed with authentic standards is evident in these figures. Although co-chromatography with authentic standards does not provide unequivocal identification of a growth regulator, the greater resolving power of GLC markedly improves the accuracy of co-chromatography compared to that which can be achieved by paper or thin-layer chromatography.

(E) CHANGES IN THE LEVELS OF GROWTH-REGULATING SUBSTANCES IN THE HYPOCOTYLS OF ETIOLATED AND LIGHT GROWN SQUASH

1. 3-Indoleacetic acid (IAA)

Changes in the levels of IAA in developing hypocotyls during the first 14 days of growth are shown graphically in figures 10a, b, c. When the levels of this growth regulator were expressed on a per g fresh weight or per g dry weight basis, a very similar pattern was observed. A

Figure 10a. Changes in the levels of IAA in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light or darkness.

Figure 10b. Changes in the levels of IAA in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light or darkness.

IAA

NG PER G FRESH WT

IAA

NG PER G DRY WT

Figure 10c. Changes in the levels of IAA in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light or darkness.

rapid decline in IAA occurred in the dark-grown hypocotyls, while in the light grown material the levels remained relatively high until the 10th day after germination, at which time a decline in IAA began. When the data was expressed on a per hypocotyl basis, a somewhat different picture was presented. In this case, an initial rise in IAA occurred during the period from the 5th to the 10th day in light grown hypocotyls, followed by a decline towards the end of the experimental period. No such rise in auxin levels was apparent in the dark-grown organs. It is of interest to note that regardless of the manner in which the data was expressed, the levels of IAA in the light-grown tissue were higher than those found in the etiolated tissue in all but one case (and here the difference between the dark and light value is probably not significant).

2. 4-Chloro-3-Indoleacetic acid (4-Cl-IAA)

Although the occurrence of this compound has been conclusively demonstrated in the immature seeds of *Pisum sativum*, *Vicia faba* and, *Lathyrus latifolius* (Engvild et al, 1980), no 4-Cl-IAA was detected in any of the extracts of squash hypocotyl tissue, whether the plants were grown in light or darkness. It is concluded that if 4-Cl-IAA does occur in *Cucurbita pepo* seedlings, it is not present in sufficient amounts to be detected by GLC analysis.

3. 3-Indolepropionic acid

Changes in the levels of IPA in the light and dark-grown tissue were found to be similar to those recorded for IAA, but only when the data was expressed on a fresh weight or dry weight basis. As shown in figures 11a, b, c, the level of IPA declined throughout the experimental period in the etiolated tissue, while in light-grown tissue, the levels

Figure 11a. Changes in the levels of IPA in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light or darkness.

Figure 11b. Changes in the levels of IPA in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light or darkness.

IPA

NG PER G FRESH WT

IPA

NG PER G DRY WT

Figure 11c. Changes in the levels of IPA in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light or darkness.

IPA

NG PER HYPOCOTYL

dropped initially, then increased slightly and finally declined again toward the 14th-day stage. When expressed on a per organ basis, however, the data was far more complex. A large initial increase in IPA was noted during the early growth of the hypocotyls in the dark, followed by a decline during the 10th and 12th days of growth and a rise in the amount on the 14th day. The rise in IPA levels for the light-grown hypocotyls was more gradual and the peak occurred on the 12th day after germination, after which a rapid decline was observed. Thus, two rather dissimilar patterns of changes were observed with regard to the levels of IPA in the two types of hypocotyl tissues. However, the absolute levels of IPA, regardless of the way in which the data was expressed, were similar to those found for IAA.

4. Gibberellic acid 3 (GA₃)

Although collections were made on several preparative GLC analyses at the temperature range corresponding to that of authentic GA₃, no peaks co-chromatographing with GA₃ were detected during the subsequent analytical gas chromatography of the collected fractions. Therefore, it is concluded that GA₃ is not native to *Cucurbita pepo*, or that it may be present in such low levels in the hypocotyls of these plants that GLC detection was not possible. The latter possibility seems unlikely.

5. Gibberellic acid 7 (GA₇)

As can be seen in figures 12a, b, c, the levels of GA₇ in the light-grown material were considerably higher than in the etiolated tissue. This was the case, with one exception, irrespective of the way

Figure 12a. Changes in the levels of GA₇ in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light and darkness.

Figure 12b. Changes in the levels of GA₇ in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light and darkness.

GA₇

NG PER G FRESH WT

GA₇

NG PER G DRY WT

Figure 12c. Changes in the levels of GA₇ in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light and darkness.

in which the data was expressed. GA_7 levels increased rapidly during the first 3 days of exposure to light. The levels remained high until after the 12th day, when a rapid decline was observed. In contrast, GA_7 levels in etiolated tissues did not increase significantly during the experiment, but rather, remained stable at a low level throughout the 14-day period. Only when expressed on a per organ basis was a gradual increase in GA_7 observed; the levels, however, were consistently lower than those found in the light-grown tissue, with the exception of the 14th day sample. It should be noted that the levels of GA_7 in the hypocotyl tissues were, in general, higher than any of the other growth-promoting substances, and in some cases were more than ten times greater when expressed in terms of ng per g fresh weight.

A result common to all of the growth-promoting substances was that the levels of these compounds in the light-grown material was always higher than that present in the etiolated tissue, when the data was expressed on either a fresh weight or dry weight basis.

(F) CHANGES IN THE LEVELS OF GROWTH INHIBITING SUBSTANCES IN THE HYPOCOTYLS OF ETIOLATED AND LIGHT-GROWN SQUASH

1. *cis*,*trans*-Absciscic acid (ABA)

The changes in ABA concentration with time, when expressed in terms of the fresh or dry weight of the hypocotyls, were found to be similar for both types of tissue throughout the experiment. As shown in figures 13a,b,c, there was an initial rise in ABA levels in both light and dark-grown tissue at the 6th-day stage, followed by a decline over the next 2 days. After the 10th-day stage, ABA levels increased in the light-grown tissue. The ABA levels in the etiolated material also gradually increased towards the end of the experiment. In general,

Figure 13a. Changes in the levels of ABA in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light or darkness.

Figure 13b. Changes in the levels of ABA in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light or darkness.

Figure 13c. Changes in the levels of ABA in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light or darkness.

little difference was found in the amounts of ABA in comparable light or dark-grown samples of hypocotyls harvested after the 8th-day stage. When the data was expressed on a per organ basis, however, a different situation was observed. No significant increases in ABA levels were found for the etiolated or light-grown tissue until the end of the experimental period, at which point the level in the dark-grown organs exceeded that present in the light-grown tissue. Clearly, the changes that occurred in ABA levels through this experiment were complex and dissimilar, depending upon the way in which the data was expressed. The actual levels of this growth regulator in both types of tissue were very low, however, and never exceeded 8 ng per g fresh weight, which was considerably less than the concentrations of auxins and gibberellins found in the hypocotyls.

2. cis,trans-Xanthoxin.

The changes observed in the levels of xanthoxin are shown in figures 14a, b, c. When the data was expressed on a fresh weight or a dry weight basis, the levels in the dark and light-grown tissue were very similar throughout the experiment. The amount of xanthoxin present increased in both cases to a maximum, 10 days after germination, and then declined until the end of the experiment.

When the data was expressed on a per hypocotyl basis, as shown in figure 14c, a marked difference was seen in the xanthoxin levels in light and dark-grown tissue, as compared with the two graphs discussed earlier. The etiolated hypocotyls contained more xanthoxin than did the light-grown organs throughout the entire experiment, although the general pattern of increase and decline in the levels of this compound was similar for both light regimes. It is evident that any comparison of the changes occurring in the levels of xanthoxin in light and dark-

Figure 14a. Changes in the levels of xanthoxin in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light or darkness.

Figure 14b. Changes in the levels of xanthoxin in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light or darkness.

XANTHOXIN

NG PER G FRESH WT

XANTHOXIN

NG PER G DRY WT

Figure 14c. Changes in the levels of xanthoxin in squash hypocotyls
on a per organ basis during the first 2 weeks of growth
in light or darkness.

XANTHOXIN

NG PER HYPOCOTYL

grown tissues is highly dependant upon the way in which the data was expressed. Xanthoxin was present in relatively large amounts (up to as much as 130 ng per g fresh weight) in the hypocotyl tissue, making this growth regulator one of the most abundant of all those studied.

(G) CHANGES IN THE LEVELS OF SUBSTANCES INVOLVED IN THE BIOSYNTHESIS AND METABOLISM OF INDOLEACETIC ACID (IAA)

1. Tryptophol

Tryptophol (Indoleethanol) has been shown to be an important intermediate in one of the pathways of IAA-biosynthesis in the Curcubitaceae (Rayle and Purves, 1967). Since squash is a member of this family, it was thought that a study of the changes in this compound in relation to those for IAA would be of interest, even though tryptophol has not been shown to be active *per se* as a growth promoter in plants where it occurs naturally. Figures 15a, b, c, show the changes in tryptophol on a fresh weight, dry weight and per organ basis. In all cases, very little tryptophol was present in etiolated tissues, while the levels were quite high in light-grown material. An immediate rise in tryptophol was observed after the plants were moved to the light growth chamber, and high levels of this compound were found until the 10th day after germination. Beyond this point, the levels in light-grown hypocotyls dropped off slowly.

It should be noted that tryptophol was the most abundant auxin-related substance found in this experiment, with levels in light-grown tissue reaching a maximum of 77 ng per g fresh weight, 10 days after germination. The pattern of changes in the levels of IAA and tryptophol were very similar for the etiolated tissue, regardless of the way in which the data was expressed. With the light-grown material, although

Figure 15a. Changes in the levels of tryptophol in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light or darkness.

Figure 15b. Changes in the levels of tryptophol in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light or darkness.

TRYPTOPHOL

NG PER G FRESH WT

TRYPTOPHOL

NG PER G DRY WT

Figure 15c. Changes in the levels of tryptophol in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light or darkness.

TRYPTOPHOL

NG PER HYPOCOTYL

the values obtained for IAA and tryptophol were quite different, the pattern of changes in their levels were similar in that both substances were present in higher amounts than in etiolated tissue throughout most of the experimental period.

2. Indolealdehyde

Indolealdehyde is a stable metabolite of IAA which has been reported to be produced by enzymatically-mediated oxidation (Schneider and Wightman, 1974). As seen in figures 16a, b, c, the levels of indolealdehyde in light-grown tissues, when expressed on a fresh or dry weight basis, were consistently higher than those of etiolated material. In both cases, a maximal concentration was reached on the 10th day after germination, followed by a gradual decline over the next 4 days. Levels in etiolated tissue did not increase nearly as much, and maximal concentration was attained at the 8th day stage. When the data was expressed on a per hypocotyl basis, no major distinction between the levels in light-grown and etiolated organs were observed, and although the difference was very small, the etiolated hypocotyls contained more indolealdehyde than did the light-grown organs.

Comparison of the data for IAA and indolealdehyde indicated that the concentration range was similar for the 2 substances (2-12 ng/g fresh weight) but the pattern of change was quite different over the experimental period. This was especially true when the data was expressed on a per organ basis. However, one general similarity was that light-grown hypocotyls contained more of both substances than etiolated material throughout the entire experiment, when levels were expressed on a unit weight basis.

Figure 16a. Changes in the levels of IAld in squash hypocotyls on a per g fresh weight basis during the first 2 weeks of growth in light and darkness.

Figure 16b. Changes in the levels of IAld in squash hypocotyls on a per g dry weight basis during the first 2 weeks of growth in light and darkness.

INDOLE ALDEHYDE

NG PER G FRESH WT

INDOLE ALDEHYDE

NG PER G DRY WT

Figure 16c. Changes in the levels of IAl_d in squash hypocotyls on a per organ basis during the first 2 weeks of growth in light or darkness.

INDOLE ALDEHYDE

NG PER HYPOCOTYL

2 2
OF / DE

4

Table 2. The levels of plant hormones in the hypocotyls of
etiolated and light-grown squash seedlings.

- (a) per g fresh weight (b) per g dry weight
(c) per organ.

A

HORMONE		LEVELS IN ng/g FRESH WEIGHT					
		5-DAY	6-DAY	8-DAY	10-DAY	12-DAY	14-DAY
IAA	light		12.0	11.4	10.8	4.1	4.0
	dark	10.4	7.5	1.75	trace (<1)	trace (<1)	trace (<1)
4-Cl-IAA	light	No 4-Cl-IAA was detected in any of the plant samples					
	dark						
IPA	light		5.3	7.9	9.6	10.2	3.2
	dark	8.7	8.3	7.1	2.3	2.1	2.7
TOL	light		45.5	58.4	77.0	24.0	6.4
	dark	7.4	none detected	none detected	4.6	4.5	3.0
IAld	light		4.5	11.3	15.0	6.2	1.6
	dark	4.4	3.7	7.5	6.3	2.4	1.3
GA ₇	light		53.5	112.0	99.5	142.5	41.8
	dark	trace (<1)	trace (<1)	31.5	19.5	21.0	25.6
GA ₃	light	No GA ₃ was detected in any of the plant samples					
	dark						
ABA	light		7.1	4.3	1.8	3.4	5.1
	dark	trace (<1)	2.7	trace (<1)	1.2	1.9	5.2
Xanthoxin	light		40.9	108.4	128.3	44.3	10.9
	dark	59.7	55.0	84.9	119.8	63.2	9.2

(Average of 2 experiments, maximum range around average $\pm$ 17%)

B

HORMONE		LEVELS IN ng/g DRY WEIGHT					
		5-DAY	6-DAY	8-DAY	10-DAY	12-DAY	14-DAY
IAA	light		131	273	214	88	86
	dark	113	94	42	trace	trace	trace
TOL	light		496	1396	1525	516	138
	dark	81	trace	trace	126	129	94
IAld	light		49	270	297	133	35
	dark	48	46	179	173	69	41
IPA	light		58	189	190	219	69
	dark	95	104	79	63	60	85
GA ₇	light		579	2677	1970	3064	903
	dark	trace	trace	753	534	603	801
ABA	light		77	83	36	73	110
	dark	trace	34	trace	33	55	133
Xanthoxin	light		446	2581	2540	953	235
	dark	651	688	2029	3282	1814	288

C

HORMONE		LEVELS IN ng/hypocotyl					
		5-DAY	6-DAY	8-DAY	10-DAY	12-DAY	14-DAY
IAA	light		.674	2.37	2.58	1.18	1.29
	dark	.541	.755	.796	trace	trace	trace
TOL	light		2.56	12.2	16.0	6.90	2.10
	dark	.740	none detected	none detected	2.55	3.30	3.17
IAld	light		.250	2.35	3.59	1.78	.516
	dark	.250	.372	3.41	3.73	1.74	1.37
IPA	light		.298	1.65	2.29	2.93	1.03
	dark	.452	.835	3.23	1.36	1.52	2.85
GA ₇	light		3.01	23.3	23.8	40.9	13.5
	dark	trace	trace	14.3	11.5	15.3	27.1
ABA	light		.399	.896	.430	.978	1.64
	dark	trace	.272	trace	.710	1.38	5.49
Xanthoxin	light		2.30	22.6	26.7	12.7	3.51
	dark	6.00	5.53	38.6	66.4	45.9	9.72

(H) DIFFERENCES IN THE LEVELS OF GROWTH REGULATORS IN SQUASH
HYPOCOTYLS GROWN FOR 10 DAYS IN BLUE, RED, AND WHITE LIGHT,
OR IN DARKNESS

1. Growth-Promoting Substances

The effects of light quality on the levels of IAA and tryptophol in squash hypocotyls are shown in figures 17 and 18. It is immediately evident that on a fresh weight basis, white light was the most effective treatment in promoting the production of both tryptophol and IAA. The hypocotyls of plants grown in red light had considerably less IAA than those grown in white light, but the levels in this treatment were substantially more than for plants grown in blue light or darkness. When the data were expressed on a per organ basis, the levels of tryptophol and IAA in red and white-light grown hypocotyls were almost equal while the levels for the other treatments were much lower. It should be noted that, although the concentration of tryptophol was much higher (on a fresh weight basis) than that of IAA for both red and white light treatments, the relative abundance of the two compounds under the two light treatments was very similar.

In contrast, growth under red light was the least effective treatment for promoting IPA production. As seen in figure 19, levels of IPA on a fresh weight basis were highest for white light, followed closely by blue light and then dark treatment. Blue light treatment was nearly as effective as white light in stimulating IPA production in the hypocotyls. When expressed on a per organ basis, the level of IPA in the blue-light grown organs was considerably higher than that found after growth in white light.

Figure 17. The effect of light quality on the levels of IAA in 10-day old squash hypocotyl tissues.

Figure 18. The effect of light quality on the levels of tryptophol in 10-day old squash hypocotyl tissues.

Figure 19. The effect of light quality on the levels of IPA in
10-day old squash hypocotyl tissues.

Figure 20. The effect of light quality on the levels of GA_7 in 10-day old squash hypocotyl tissues.

A comparison of the levels of GA₇ for each treatment is shown in figure 20. On a fresh weight basis, it is evident that white light was the most effective stimulus of GA₇ production. The 3 other treatments induced considerably lower levels of GA₇ which were not very different from each other. When the data was expressed on a per organ basis, however, the differences in GA₇ levels between the 4 treatments were far less pronounced, although the level for the white light treatment remained the highest. Unlike the situation for the indole compounds, no major stimulation of GA₇ production was observed under either red or blue-light treatment.

2. Growth-Inhibiting Substances

ABA levels in hypocotyl tissue grown under each of the 4 light regimes are shown in figure 21. In general, the levels of this compound were very low in all of the samples and particularly in etiolated and blue-light grown material. On a fresh weight basis, the concentrations of ABA in the red and light-grown tissue were almost identical; however, this was less apparent when the data was expressed on a per hypocotyl basis.

The levels of xanthoxin in the tissues grown under the same 4 conditions are shown in figure 22. In contrast to the results for ABA, xanthoxin was present in all of the samples at relatively high concentrations. On a fresh weight basis, xanthoxin levels in tissues grown under red light, white light and in the dark were all relatively high, while blue-light treatment resulted in the lowest amount. The situation was quite different, however, when the data was expressed on a per organ basis. In this case, levels in the dark and red-light treatments were similar, but were considerably higher than those present in the blue and white-light grown material.

Figure 21. The effect of light quality on the levels of ABA in 10-day old squash hypocotyl tissues.

Figure 22. The effect of light quality on the levels of xanthoxin in 10-day old squash hypocotyl tissues.

(I) THE EFFECTS OF DE-ETIOLATION ON ENDOGENOUS LEVELS OF GROWTH
REGULATORS IN SQUASH HYPOCOTYLS AND COTYLEDONS

1. Growth-Promoting Substances

As seen in Table 4, little or no IAA was found in either hypocotyl or cotyledon tissue grown under any of the light treatments. Since the level of IAA in 10-day old etiolated hypocotyls was found to be low in all earlier experiments, it was not surprising that IAA was not present in this tissue in sufficient amounts to determine its concentration accurately. After 6 or 24 h of continuous white light treatment, the IAA level in the tissue was still not high enough to permit accurate quantitation by GLC. However, as can be seen in figure 10, when hypocotyls were grown in the light for 10 days under successive 16 h photoperiods, a measureable level of IAA was found in the tissue.

The results recorded for tryptophol are shown in figure 23. The level of tryptophol increased slightly in the cotyledons when exposed to light for a 6 or 24 h period; however, the amount remained fairly constant in the hypocotyl tissue. It should be noted that the etiolated hypocotyl tissue used as a control in this experiment contained considerably more tryptophol than that present in similar material used for the light quality experiment, and that no marked increase in tryptophol was observed in the hypocotyls of plants after 6 or 24 h of continuous white light.

The changes in levels of IPA after the exposure of etiolated plants to light were the most striking result of this experiment. As seen in figure 24, large increases in the amounts of IPA in both the hypocotyl and cotyledonary tissues were observed after only 6 h of light exposure. The amount of IPA present in the hypocotyls increased greatly after 6 h of light treatment, and remained at a similar level after 24 h

Figure 23. The effect of de-etiolation of 10-day old squash seedlings on the levels of tryptophol in the hypocotyl and cotyledonary tissues.

TRYPTOPHOL

NG PER G FRESH WT

100

90

80

70

60

50

40

30

20

10

■ HYPOCOTYL

□ COTYLEDON

CONTROL

6 HR

24 HR

DURATION OF LIGHT EXPOSURE

Figure 24. The effect of de-etiolation of 10-day old squash seedlings on the levels of IPA in the hypocotyl and cotyledonary tissues.

Figure 25. The effect of de-etiolation of 10-day old squash seedlings on the levels of GA₇ in the hypocotyl and cotyledonary tissues.

Figure 26. The effect of de-etiolation of 10-day old squash seedlings on the levels of ABA in the hypocotyl and cotyledonary tissues.

Figure 27. The effect of de-etiolation of 10-day old squash seedlings on the levels of xanthoxin in the hypocotyl and cotyledonary tissues.

ABA

NG PER G FRESH WT

DURATION OF LIGHT EXPOSURE

XANTHOXIN

NG PER G FRESH WT

DURATION OF LIGHT EXPOSURE

of exposure. In the cotyledons, however, a sharp rise in IPA levels was found after only 6 h of exposure which declined somewhat in material that had received 24 h of light treatment.

Figure 25 shows the changes in levels of GA_7 observed in this experiment. Although some increase in the amount of GA_7 present in the cotyledons was noticed after de-etiolation, no significant increase in this growth regulator was seen in the hypocotyl tissue.

2. Growth-Inhibiting Substances

A short term enhancement of ABA levels was observed in both the hypocotyls and cotyledonary tissues after 6 h exposure to white light. However, the levels of ABA in both these organs declined, as compared with etiolated controls, when the light exposure was continued for 24 h, as shown in figure 26. Thus, it appears that short term exposure of 10-day old etiolated plants to white light results in an enhancement of ABA levels, but that this increase is only transitory. Xanthoxin levels were remarkably similar in both hypocotyl and cotyledonary tissues sampled from all the treatment conditions, (figure 27). Thus, no significant increase or decrease in the level of this substance was observed as a result of the de-etiolation treatment.

Table 3. The levels of plant hormones in squash hypocotyls grown for 10 days under blue, red and white light or in darkness.

Table 4. The levels of plant hormones in 10-day old squash hypocotyls and cotyledons after de-etiolation in white light.

HORMONE	BLUE		RED		WHITE		DARKNESS	
	ng/g F. Wt	ng/organ	ng/g F. Wt	ng/organ	ng/g F. Wt	ng/organ	ng/g F. Wt	ng/organ
IAA	trace	trace	2.8	1.6	5.2	1.2	trace	trace
TOL	10.7	5.4	20.5	11.4	42.7	10.2	trace	trace
IPA	7.5	3.8	trace	trace	8.3	1.9	1.4	0.8
GA ₇	10.0	5.0	7.5	4.2	38.3	9.2	12.4	7.3
ABA	trace	trace	1.2	0.7	1.1	0.3	trace	trace
Xanthoxin	34.5	17.3	74.5	41.3	72.1	17.2	63.2	37.4

(Average of 2 experiments, maximum range around average $\pm 12\%$)

HORMONE	HYPOCOTYLS ng/g F. Wt.			COTYLEDONS ng/g F. Wt.		
	Control	6 h	24 h	Control	6 h	24 h
IAA	trace	none detected	none detected	trace	trace	trace
TOL	12.6	13.1	8.8	41.0	54.0	57.0
IPA	trace	12.3	10.4	17.9	49.0	23.9
GA ₇	15.4	12.6	17.5	24.3	33.3	28.2
ABA	10.2	15.6	8.1	20.0	31.4	13.3
Xanthoxin	84.4	78.5	89.8	104.7	93.2	117.5

(Average of 2 experiments, maximum range around average $\pm 15\%$)

DISCUSSION

The rapid growth of stems in the dark and their slower growth in the light are phenomena that have been known to plant scientists for a considerable period of time. However, attempts to relate the process of etiolated growth and its photoinhibition to biochemical changes in the shoot tissue have not been frequently made.

Early work on the cellular morphology of etiolated tissues indicated that the rapid growth of stems in the dark was due primarily to increased cell elongation (Brotherton and Bartlett, 1918; Avery et al., 1937) compared with tissues grown in the light. The possibility that growth-promoting substances might be involved in etiolated growth was first investigated by Lockhart (1956). He found that exogenous application of gibberellic acid to light-grown stems resulted in length increases equal to that of etiolated plants. Thus, by implication, it appeared possible that endogenous hormones might play an important role in the rapid growth of dark-treated stems. Another possible explanation for the difference in growth could be that light stimulates the production of inhibitor substances which might reduce the rate of cell elongation in light-grown shoots. In the present study, an attempt was made to determine if such relationships exist.

The differences in the growth rate and morphology of etiolated and light-grown seedlings were apparent very soon after samples of 5-day old dark-grown seedlings were moved into the light. The decreased growth rate of the light-treated hypocotyls, compared with the etiolated controls, was considerable by the 8th-day stage, and this difference in growth rate continued throughout the remainder of the experiment (Figures 4a and 4b). The rapid growth of the hypocotyls in the dark was reflected in both the

fresh weight and dry weight increases in these organs. Thus, it seems likely that young, etiolated seedlings use much of their food reserves in sustaining the rapid growth of the hypocotyl, whereas light-grown plants of the same age with actively photosynthesizing cotyledons, put more of their energy reserves into promoting expansion of the cotyledons and the growth of the primary leaves. This would account for the observation that dry weight increases in the etiolated hypocotyls occurred at a faster rate than in the control material, even though no photosynthesis was taking place in the etiolated plants. The water content of the etiolated tissue was slightly higher than that of light-grown material (D = 96.8% vs L = 95.4% at the 14th-day stage), which might indicate that water uptake and retention in these plants was greater than for their light-grown counterparts.

For the determination of endogenous plant hormones, it was decided to analyse the ether-fractionated extracts of the hypocotyl tissues by gas-liquid chromatography, as this method provides a rapid, reproducible and highly sensitive method for the separation, identification and quantitation of plant growth regulators. GLC has been used successfully in previous studies carried out in this laboratory to determine changes in endogenous growth regulators (MacRae, 1978; Camirand, 1979; Wightman, 1977). However, a modification of the methods used previously was developed to improve the speed and effectiveness of the separation, identification and quantitative estimation of the different growth regulators by GLC. This modification involved the use of a preparative GLC step as a preliminary stage in the analysis. Preparative columns have a relatively wide bore (I.D. = 8mm) and can therefore be packed with large amounts of the stationary phase. This permits the injection of ether extracts from larger amounts of tissue, normally equal to 50-100 g fresh weight in the case of hypocotyl samples. Collection of specific fractions

from such an extract was facilitated by means of a splitter system attached to the end of the preparative column. When a linear temperature program was run, components of the crude ether extracts were separated on the basis of their molecular weight and volatility. Thus, the preparative GLC method provided a means by which an ether extract of a large tissue sample could be rapidly analysed, and at the same time, allow for the collection of specific fractions whose elution temperatures corresponded with those of the growth regulators expected in the ether extract under study. The need to work with less efficient and more time consuming preparative methods of analysis, such as TLC, was eliminated in this way.

After the preparative GLC step, trapped fractions were further analysed by isothermal GLC using analytical size columns. Again, this resulted in a considerable time saving in comparison with much longer temperature programming procedures. Tentative identification of one or more peaks in the GLC scan could be made after only 2 isothermal analyses of a trapped fraction (the first unspiked, and the second spiked with an authentic standard), which took about 30 minutes to complete. This compares favourably with the 1.5-2 h required for temperature programmed analysis. Since the re-solubilized fractions could be highly concentrated (eg. - 3-5 g F.Wt/ μ l), the ability to detect extremely low levels of growth regulators was enhanced. Furthermore, isothermal GLC on the analytical columns eliminated the problems related to column 'bleeding' that frequently occur at high temperatures when temperature programs are used. One final point in this regard is that the use of the two GLC steps for the growth regulator extract analysis permitted the employment of different stationary phases at each step, which further improved the separation of substances in the extracts.

A study of the levels of gibberellins in light and dark-grown squash hypocotyls indicated that GA₇ was present, but that GA₃ was absent

in both types of tissue (Table 2). It is possible that GA_3 is not a native gibberellin of *Cucurbita pepo*; however, GA_3 has been conclusively identified in extracts from other higher plant species, such as *Chenopodium album* (Hemphill *et al.*, 1972) and *Pharbitis nil* (Murofushi *et al.*, 1968). It therefore seems possible that GA_3 was not present in sufficient quantities to estimate its concentration in the hypocotyl tissue, or that it was rapidly converted to other forms during the extraction process. Whichever is the case, it seems unlikely that GA_3 has any role to play in the growth of squash hypocotyls in either light or dark conditions.

GA_7 was found to occur at substantially higher levels in the hypocotyls of light-grown squash than in the etiolated organs. The maximum difference occurred at the 12th-day stage, at which time GA_7 levels exceeded 140 ng/g fresh weight in the light-grown hypocotyls, compared with only 21 ng/g fresh weight in the dark-grown tissues. This was observed regardless of the way in which the data was expressed and clearly indicates that an enhancement of GA_7 levels does not occur in etiolated hypocotyls. These findings are in agreement with several earlier studies, in which the level of gibberellin-like activity was assayed in etiolated and light-grown pea (Kende and Lang, 1964), morning glory (Ogawa, 1964; Barendse and Lang, 1972), bean (Crozier and Audus, 1968), and lupin (Acton and Murray, 1974). In most of these studies however, identification of individual GA's was not attempted, making comparisons of the GA levels with those determined in the present study very difficult. However, a pattern does emerge which indicates that higher levels of gibberellins are probably not present in etiolated tissues, when compared with light-grown counterparts of the same age. This does not preclude the possibility that light may render the hypocotyl tissue less sensitive to gibberellin, as suggested by Barendse and Lang (1972) and by Acton and Murray (1974). In such a situation, endogenous gibberellin concentration would have to

be much higher in light-grown shoots to elicit a growth response equivalent to that observed in the dark.

It is unfortunate that GA₃ and GA₇ are currently the only commercially available gibberellins, as the lack of a supply of other authentic gibberellin compounds limited the number that could be examined in this study.

In the experiment using light regimes of different quality, no enhancement of GA₇ levels was observed when plants were grown for 10 days in red light. GA₇ was most abundant in the hypocotyls of plants grown in white light, and little difference was seen in the levels in blue, red and dark-grown tissue. This contrasts with the results from earlier work on barley (Reid *et al*, 1968), wheat (Loveys and Wareing, 1971) and pea (Neskovic and Konjevic, 1974). However, in all of the earlier studies, red light exposures were only given for short periods of time (not more than 10 minutes) to the excised leaves and upper shoot tissue of young etiolated plants. In the present investigation, plants were grown for a full 10 days in red light before the hypocotyls were harvested. Clearly, the differences in the duration of the light stimulus and the plant species studied, as well as the age and type of the tissue from which the gibberellins were extracted, could account for the apparent disagreement.

Reid and coworkers (1968) found that red-light induction of GA synthesis was very short-lived in the excised barley seedling system they employed. Also, the effect of a red light stimulus on an excised plant may not be typical of the effect observed when the same stimulus is given to an intact plant. Consequently, it appears unlikely that the long-term growth of squash seedlings in red light results in any enhancement of GA₇ and possibly other GA levels. Neskovic and Konjevic (1974) found no evidence of phytochrome-mediated GA synthesis in pea seedlings, so it is possible that if light is directly involved in GA biosynthesis, other

pigment systems may be involved.

A review of the literature failed to produce any studies of the effects of irradiation with blue light on endogenous gibberellin levels. The work presented here suggests that, like red light, long term exposure to blue light does not result in any enhancement in the level of GA₇ in the hypocotyl tissue. Since the hypocotyls of plants grown in blue light were longer than either of the other light treatments, this provides further support for the idea that enhanced endogenous gibberellin levels are not responsible for the rapid growth of etiolated hypocotyls or shoots.

When 9-day old etiolated plants were exposed to 6 or 24 h of continuous white light treatment, no major changes in the endogenous GA₇ levels were observed in either the hypocotyls or the cotyledons. It has been previously shown that GA is not involved in such photomorphogenic processes as the unbending of hypocotyl hooks (Kang and Ray, 1969). This would seem to indicate that the gross morphological changes observed after 'de-etiolation' were not directly related to rapid changes in the levels of this growth regulator.

The changes in the levels of several indole auxins were also examined during the etiolated and normal growth of squash seedlings. IAA, its oxidative metabolite indolealdehyde (IAld) and tryptophol (TOL), an important biosynthetic precursor, were all present in higher concentrations in the light-grown tissue than in etiolated hypocotyls (Table 2). The level of tryptophol increased very rapidly when 5-day old seedlings were moved into the light. The concentration continued to rise in such seedlings until 10 days after germination, after which a steady decline was observed. In contrast, no such increase in TOL concentration was observed in the etiolated tissues. The presence of tryptophol in light-grown cucumber shoots has been conclusively demonstrated by combined GLC and MS (Rayle and Purves, 1967). Wightman and Cohen (1968) partially

purified an alcohol dehydrogenase from mung bean seedlings which catalysed the reduction of indoleacetaldehyde to tryptophol in the presence of NADH. NADH-specific indoleacetaldehyde reductase was found to be associated with the cytosol fraction of cucumber hypocotyl and cotyledon tissue, while a second enzyme involved in this stage of auxin biosynthesis, requiring NADPH, was associated with a microsomal fraction (Bower *et al.*, 1976). Recent studies by Brown and Purves (1980) have shown that indoleacetaldehyde reductase activity is greatly dependant upon the co-substrate supplied to the system (NADH or NADPH). The requirement of the enzyme for NADH or NADPH might explain the low levels of TOL in the etiolated tissues, as the reducing power normally produced in photosynthesis would not be available to these tissues.

The initial level of IAA in the 5-day old hypocotyls (10.4 ng/g F.Wt) was very similar to that of TOL (7.4 ng/g F.Wt.). However, the IAA concentration did not increase in the light-grown hypocotyls over the next 5-day period, but rather remained stable at around 11 ng/g F.Wt. Beyond the 10-day stage, IAA levels in light-grown shoots declined appreciably. The data from the present study suggest that only a relatively small fraction of the TOL produced in light-grown hypocotyls is converted to IAA. In comparison, in etiolated hypocotyls, a steady drop in IAA levels occurred until the 10th day of seedling growth, at which time virtually no IAA was present in the system. Tryptophol was also present at low levels in etiolated tissue throughout the experiment. The observation that the levels of IAA in light-treated hypocotyls remained stable during the first 5 days of the experiment, while TOL concentrations increased rapidly during the same period, suggests that the enzymatic conversion of TOL to IAA is not greatly influenced by light or the concentration of TOL in the tissue.

The rise in the amount of indolealdehyde (IAld) in the light-grown hypocotyls up to the 10th day, followed by a gradual decline toward the end of the experiment, correlates well with the observed changes in the levels of IAA. The steady increase in the amount of IAld up to the 10th day may partially account for the lack of any significant increase in IAA levels during the same period. However, the maximum concentration of IAld was in the same range as that of IAA, which suggests that unless IAld is further metabolised to indolecarboxylic acid (ICA), a large portion of the TOL produced early in the experiment was not converted to IAA and then rapidly oxidized to IAld. Although a considerable amount of work has been carried out in the past, attempting to characterize the enzyme(s) responsible for the oxidation of IAA to IAld, a light-requirement for its activity has not been demonstrated. However, it has been shown that peroxidase and IAA-oxidase can exist in numerous isoenzymic forms (Schneider and Wightman, 1974) and that very few IAA-oxidases exist which do not also possess peroxidative activity. The data presented in this study indicate that IAld levels did rise in the etiolated tissue, and that this increase was especially noticeable when the concentration was expressed on a per organ basis. Therefore, it seems reasonable to suggest that the lower levels of IAld in etiolated hypocotyls were due primarily to differences in the IAA-substrate concentration between light and dark-grown tissue, rather than to de-activation of the oxidase system in the dark.

Although the general pattern of change in the levels of IPA were similar to the other indole compounds when expressed on a fresh weight or dry weight basis, the decline in the amount of IPA in the etiolated tissue was not as rapid (figure 11a, b). Consequently, when the data was expressed on a per organ basis (figure 11c), the amount of IPA in the etiolated hypocotyls rose more quickly than the levels found in the plants

grown in the light. The highest IPA concentration (ng/g F.Wt.) occurred on the 12th day after germination in the light-grown tissue, and this finding contrasts with the situation found for IAA, in which higher levels were present in the early part of the experimental period. However, the maximum levels of both growth regulators were comparable (~ 10 ng/g F.Wt.). A point of interest with regard to the changing levels of IPA is that immediately after transferring the 5-day old seedlings to a light-growth chamber, the concentration dropped. This initial decline was followed by several days of progressive increase in concentration to be followed with a sharp decline on the 14th day. The biosynthesis and metabolism of IPA in plants has received very little attention in past investigations of endogenous auxin substances. However, the lack of correlation of the pattern of IPA changes with that of TOL (which increased sharply immediately after light treatment of the seedlings) suggests that TOL is probably not the biosynthetic precursor of IPA.

The effects of irradiation with blue, red and white light on the endogenous levels of IAA, TOL and IPA in hypocotyl tissue were also studied. The data, when expressed on a fresh weight basis, indicate that white light was the most effective stimulus in terms of its promotive effect on the levels of these regulators (Table 3). When expressed on a per hypocotyl basis, the levels of both IAA and TOL were similar for both the white and the red light treatments. Blue light was much less effective than red in enhancing the levels of IAA and TOL in the hypocotyls. Etiolated tissue contained negligible quantities of both IAA and TOL. These results are in disagreement with other investigations of the effects of light quality on auxin levels in bean (Fletcher and Zalik, 1964) and corn (Briggs, 1963), which indicated that the levels of auxin were higher in etiolated tissues than in plants given any form of irradiation, and especially red light. However, later

studies with bean and lupin seedlings contradicted the earlier work, showing that auxin levels of etiolated material were lower than that found in tissue exposed to red light (Kang and Ray, 1969; Acton and Murray, 1974). It should be mentioned that in all of these earlier investigations, measurement of auxin activity was made by bioassay, so the levels observed could not be attributed to any specific growth regulator.

In the present study, it appears that white light and red light influence the levels of IAA and TOL to a greater degree than do blue light and darkness. In marked contrast, IPA concentration was significantly enhanced by exposure to blue light and white light, and appeared to be reduced by exposure to red light when compared with etiolated material. When expressed on a fresh weight basis, the levels of IPA in 10-day old hypocotyls were almost equivalent in plants grown in white or blue light, while on a per hypocotyl basis, the level was actually higher in the blue light grown material. This observation strongly suggests that blue light plays an important role in the promotion of IPA synthesis and/or accumulation in the hypocotyls of squash seedlings. Clearly, the influence of the spectral quality of a light treatment on IPA levels in squash differs fundamentally from that on IAA and TOL.

The results from the de-etiolation experiment with 9-10 day old dark-grown seedlings indicated that short term exposure to white light led to an enhancement of IPA levels in the hypocotyls and cotyledons, whereas no such stimulation was observed for IAA or TOL. Therefore, it appears that the light-mediated enhancement of IPA occurs rapidly in such etiolated squash seedlings. A conclusive explanation of these observations cannot be offered on the basis of the present experiments. However, when considered along with the data discussed earlier, the de-etiolation experiment does present additional evidence for two,

essentially different mechanisms through which light exerts its effects on IAA and IPA levels in squash hypocotyls.

Since the levels of the auxin substances examined were generally lower in etiolated plants than those found in light-grown material, it is clear that a greater degree of auxin stimulation cannot be offered as an explanation for the observed elongation of etiolated hypocotyls. However, it is possible that in the light, auxin levels are present in supra-optimal concentrations and consequently result in the inhibition of hypocotyl growth. The suggestion that auxin may act in the light as a brake to growth has been suggested by Acton and Murray (1974) and Edwards and Scott (1977). It has been demonstrated that levels of exogenously applied IAA which are stimulatory to the growth of stems in the light are inhibitory when applied to etiolated shoots. Thus, the higher levels of indole auxins found in the light-grown hypocotyls of squash may play an important role in the inhibition of growth in the light. The de-sensitization of hypocotyl tissue to auxin substances by light may also be proposed as an explanation for the observed differences in the endogenous levels of the indole compounds and the lack of correlation with hypocotyl growth. If this were the case, a much larger amount of such auxins as IAA and IPA would be required in the light in order to elicit a growth response equivalent to that in the dark.

Recently, changes in the levels of growth inhibitors in plants during etiolated and light growth have received attention in the literature. The possibility that the observed differences in the growth rate of light and dark-grown squash hypocotyls might be due to different levels of inhibitors was considered in the present study. The results indicate that the levels of the most physiologically active isomers of ABA and xanthoxin were slightly higher in the light-grown tissues, when the data was expressed on a fresh weight or a dry weight basis. When

calculated on a per hypocotyl basis. However, the levels of both substances were very similar during much of the experimental period and where differences were observed, the inhibitor levels were higher in the etiolated material. These observations indicate that light treatment does not markedly increase the levels of either of these growth regulators, which effectively precludes them from a major role in the photoinhibition of squash hypocotyl growth.

The results of the present study concerning ABA levels support previous findings with pea seedlings (Higgins and Bonner, 1974), in which little difference was observed in the levels of ABA in etiolated and light-grown shoots. However, no evidence for an increase in the level of xanthoxin in the light was obtained in the present work. This contrasts with earlier studies with pea (Anstis *et al.*, 1975) and sunflower (Thompson and Bruinsma, 1977). In the studies by Anstis *et al.* (1975), pea seedlings were exposed to 1 minute of red light treatment every 30 minutes, for a total of 24 minutes of intermittent light exposure each day. This differs greatly from the light exposures given to plants in the present experiments. Furthermore, these workers utilized a different extraction procedure than the one employed in the present study, and derivatized the plant extract thought to contain xanthoxin by acetylation, prior to analysis by GLC. It is possible that the differences in light treatment of the plants and in the extraction, purification and derivatization procedures could account for the apparent differences in the data between the present study and that of earlier workers. As mentioned previously, short term exposure to red light is thought to enhance the endogenous levels of GA's, but no such increase was found to occur with long-term light treatment. Perhaps a similar situation exists with respect to xanthoxin. Nonetheless, Thompson and Bruinsma (1977) found a higher level of neutral

inhibitory activity in sunflower hypocotyls grown under 16 h photoperiods than those grown in the dark. In this study, however, identification of xanthoxin was not made by GLC. Several zones of inhibitory activity were found to be present in a neutral ether extract examined in another investigation of plant inhibitor levels, as determined by bioassay estimation (Hasegawa and Miyamoto, 1978). Therefore, it is possible that the activity in sunflower hypocotyl extracts, attributed by Thompson and Bruinsma to the presence of xanthoxin, was actually due to several inhibitor substances. No evidence for the light enhancement of xanthoxin-like activity was observed in recent work by Goto (1978a) with etiolated and light-grown bean seedlings. Furthermore, most of the neutral and acidic inhibitory activity was present in the cotyledons of the plants, and diffusion of these substances into the hypocotyl was not observed after exposure to light. This would appear to support the suggestion made earlier, that it seems unlikely that increased concentrations of xanthoxin or ABA in squash hypocotyl tissue are responsible for the photoinhibition of growth.

Spectral quality did not appear to change appreciably the levels of ABA, or xanthoxin in the hypocotyls of 10-day old squash. ABA levels were very low for all of the treatments, but were highest under red and white light. Xanthoxin, on the other hand, was considerably more abundant in the hypocotyl tissue, reaching concentrations as high as 74 ng/g F.Wt. However, only plants grown under blue light had a substantially different level of xanthoxin (expressed on a F.Wt. basis) compared with other light treatments. On a per organ basis, xanthoxin concentration in red light and darkness was substantially greater than that present when seedlings were grown under blue or white light. The inconsistency of the data obtained in this experiment, depending on how the data is expressed, makes any statement regarding the effect of light

quality on inhibitor levels (and especially of xanthoxin) in squash difficult. However, it appears that, in most cases, marked changes in the levels of inhibitors in squash hypocotyls do not occur in response to changes in spectral quality of the light treatment.

Unfortunately, very little information about this question is available in the literature. Recent work by Loveys (1979) indicated that while the stems of tomato plants grown under red light were shorter and had higher ABA levels than those grown under far-red light, the changes in levels of ABA were much slower than the change in the growth rates under these two environmental conditions. This suggests that ABA does not function as a hormonal substance involved in the regulation of the light-induced growth response.

The de-etiolation of 10-day old seedlings with 6 or 24 h of white light exposure resulted in only very slight changes in the levels of xanthoxin in both the cotyledons and the hypocotyls. Zeevaart (1974) found that the xanthoxin concentration of spinach stem tissue remained ~~constant~~ when the day length and humidity to which plants were exposed was altered. This is in agreement with the observations made in the present study, and provides further evidence that light does not play a major role in the stimulation of xanthoxin production in plant tissue. ABA increased in both the cotyledons and the hypocotyls after 6 h of light, but declined when exposure was increased to 24 h. Thus, no consistent pattern of light stimulation of ABA was observed after de-etiolation of the seedlings. Zeevaart (1971) found that spinach seedlings contained higher levels of ABA at the end of the photoperiod than at the beginning. However, these results are not directly comparable with those of the present experiment, as the work with spinach did not involve a de-etiolation effect.

In summary, it appears that the endogenous inhibitors ABA and xanthoxin are not greatly affected by light when other environmental parameters are kept constant. It is possible, however, that other substances, such as phenolic acids, may play a role in the light-stimulated inhibition of growth. Many of these compounds are known to have inhibitory activity on IAA-mediated growth (Leopold and Kriedemann, 1975) and future studies in this direction may provide important information concerning the regulatory mechanisms for the photoinhibition of plant growth.

SUMMARY AND CONCLUSIONS

When seedlings of Golden Hubbard squash were grown in darkness or light, marked differences in their morphology and hypocotyl growth were observed. The hypocotyls of etiolated plants grew far more rapidly than the light-grown controls. The cotyledons of light-grown seedlings expanded in size during the early stages of growth, when they became the principal photosynthetic organs of the plants. The cotyledons of etiolated seedlings displayed no similar development.

The possibility that the enhancement of growth in etiolated hypocotyls might be due to either higher endogenous levels of auxin and gibberellin substances, or to lower levels of inhibitors than light-grown organs was examined in the present study. Preparative and analytical GLC were the methods by which growth regulators in plant tissue were estimated.

In nearly all cases, the levels of the auxins and gibberellins studied were higher in the light-grown material than in the etiolated tissue. A general pattern of decline in the concentration of IAA and IPA was observed in the dark-grown tissue throughout the 2 week experimental period. This contrasts with the situation for light-grown organs, in which the levels of these substances remained constant or increased through most of the period under study. Gibberellin A₇ also increased in the light to a much greater degree than in the dark. No gibberellin A₃ was found in either treatment condition. These results suggest that higher levels of auxins and gibberellins are probably not the cause of the rapid growth of hypocotyls in the dark.

Furthermore, no evidence was obtained which would support the suggestion that the growth of light-grown hypocotyls is slower than that in the dark because of significantly higher endogenous levels of inhibitor substances. The levels of both ABA and xanthoxin were similar for both

experimental conditions throughout the experiment and where differences were found, the amount of inhibitor was only slightly higher in the light than in the dark.

Light quality and de-etiolation were found to have little effect on the levels of gibberellins and inhibitors in ten-day old squash hypocotyls and cotyledons. However, it was demonstrated that while tryptophol and IAA levels were stimulated primarily by red light, IPA levels were most effectively enhanced by irradiation with blue light. IPA was also shown to be the only substance in which a rapid enhancement was observed when 9-day old dark-grown seedlings were "de-etiolated" for either 6 or 24 h with white light. Taken together, these observations strongly suggest that the mode of light-enhancement of IAA and IPA levels differ fundamentally from one another. The lack of correlation of the levels of tryptophol and indole aldehyde with those of IPA suggest that its biosynthetic pathway involves different intermediates than those involved in the biosynthesis of IAA.

In conclusion, it appears that the etiolated growth of squash hypocotyls cannot be explained by a hormonal control mechanism involving those auxins and gibberellins examined in the present study, or the inhibitors ABA and xanthoxin. It is possible that cytokinins or ethylene, which were not studied in this work, play an important role in the hormonal control of the growth of hypocotyls in the dark. Alternatively, etiolation may be the result of a complete loss of hormonal control of growth in the plant. In any case, further study of the relationship between light as an environmental stimulus, and the endogenous levels of growth regulating substances will be necessary to determine whether plant hormones are directly involved in etiolation and photoinhibition.

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