ANTHOCYANIN EXPRESSION AND PHENYLALANINE AMMONIA-LYASE ACTIVITY IN *SORGHUM BICOLOR* AS INFLUENCED BY TEMPERATURE AND PLANT AGE

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ABSTRACT

The effects of temperature and plant age on anthocyanin production and Phenylalanine ammonia-lyase (PAL) activity in etiolated Sorghum bicolor seedlings were investigated in this study. The etiolated seedlings were exposed to various temperature combinations and sucrose in the presence and absence of light. After these treatments, they were analyzed for anthocyanin content or PAL activity.

Anthocyanin formation was not affected by temperature or sucrose in the absence of light. This indicated that light was required for the activation and/or synthesis of anthocyanin synthesizing enzymes. This was not the case for PAL which was stimulated in darkness when sucrose was added to the medium. It was speculated that the exogenous source of carbohydrates served as a substitute stimulus for light.

Anthocyanin formation was stimulated by temperature in the presence of and following exposure to light. Limited quantities of anthocyanin were produced at 30C 4 hours after seedling exposure to light. The effect was of a much greater magnitude 24 hours after exposure to light. The additional time period required for maximal anthocyanin production was attributed to the presence of a lag phase. It was further assumed that temperature affected the production and/or activity of anthocyanin synthesizing enzymes during this lag phase; a higher temperature being more effective than a lower temperature. Endogenous carbohydrate supply was also considered to be important. Anthocyanin production was high only when the seedlings had been exposed to low (10C) temperatures prior to light exposure. The low temperature was thought to decrease respiration rates of the seedlings and therefore make more substrates (e.g.

endogenous carbohydrates) available for anthocyanin formation during and following exposure to light.

PAL activity was greatly stimulated by a temperature of 30C 4 hours after the initiation of the light treatment but its activity decreased markedly 24 hours following exposure to light. It was assumed that light and high temperatures functioned as stimuli for the synthesis and/or activity of a PAL deactivating enzyme system. The effectiveness of this system to deactivate PAL was thought to be greatly increased during a 24 hour incubation period after exposure to light. This was the case only when the temperature was high (30C), therefore it was assumed that the activity and/or synthesis of the PAL deactivating enzyme system was hampered by low (10C) temperatures. Sucrose had a greater stimulatory effect on PAL activity at the lower temperatures. This might have been a result of the absence or low activity of a PAL deactivating enzyme system at low temperatures and/or the greater availability of endogenous substrates due to lower respiration rates.

As plant age increased, anthocyanin production and PAL activity decreased. It was postulated that this was a result of lower endogenous substrate levels due to longer respiration times. The older plants also may have produced greater quantities of anthocyanin and PAL inhibitors.

Maximal PAL activity was found to precede maximal anthocyanin production by a few hours. The trend was similar for minimal PAL activity and anthocyanin production. A lag phase was speculated as being responsible for this relationship. If this assumption is correct, our studies indicate that PAL activity might have some regulatory control over anthocyanin production.

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CHAPTER I

INTRODUCTION

Anthocyanins are plant pigments that are responsible for the majority of the red and blue colors of flowers and fruits. Frequently they are found in leaves and stems, and infrequently in roots of certain plants. They may be expressed as a normal physiological function of the plant, or abnormally as a result of plant stress (Wheldale, 1916).

A number of factors influence the formation of anthocyanins. Genetics is considered to be a primary factor which greatly determines the ability of a plant to form anthocyanin under various conditions. An example would be the Texas milo seedlings that are capable of producing twice as much anthocyanin as the Texas Dwarf White milo seedlings under a given set of conditions (Downs, 1964). Endogenous carbohydrate content plays a significant role in anthocyanin synthesis (Creasy, 1968). Conditions that influence the accumulation of carbohydrates usually affect the amount of anthocyanin produced. As an example, low temperatures might result in reduced respiration and increased accumulation of sugars and anthocyanin (Downs, 1964). Mechanical damage may operate to girdle a plant part and cause an accumulation of carbohydrates (Wheldale, 1916). Light exerts a most emphatic control over anthocyanin synthesis (Grill, 1967; Klein et al., 1957; Kolesnikov and Zore, 1957; Mohr and Hartmann, 1965; Kandeler, 1959; and Kandeler, 1960), and dependent on the plant material, phytochrome or a blue-absorbing photoreceptor seems to be involved (Smith, 1975).

The chemical structure of anthocyanin was determined in the early 1900s by Willstätter. Since that period, many other investigations have contributed toward what is now considered a rather complete picture of the structure of anthocyanin. The biosynthetic pathway of anthocyanin has also been fairly well established with the exception of determining its regulatory features. Also lacking is information pertaining to the importance of the A-ring synthesis (head-to-tail condensation of acetate units) as compared to the B-ring synthesis (Shikimic acid pathway) of anthocyanin formation.

Many studies have contributed toward a better understanding of the B-ring pathway of which Phenylalanine ammonia-lyase (PAL) was found to be a major, and possibly regulatory enzyme. Most of the studies have related to light and its effects on PAL (Zucker, 1965; Engelsma, 1967; Zucker, 1969; Engelsma, 1969; Engelsma, 1968; Engelsma, 1970; Bellini and Poucke, 1970; and Bellini and Hillman, 1971). Few have investigated the effects of temperature on PAL activity (Engelsma, 1968; and Engelsma, 1970). To our knowledge, no work has been conducted to determine the relationship of PAL activity and anthocyanin production based on temperature. Therefore, to further facilitate the understanding of this relationship, it was the intent of our study to evaluate the synthesis and/or activity of these compounds as influenced by temperature and plant age. It was assumed that high temperatures would act to increase respiration rates and result in lower levels of endogenous carbohydrates required for anthocyanin synthesis. The opposite effect would be anticipated at lower temperatures. Plant age determines total respiration time, therefore an older seedling would be expected to

possess less endogenous substrates due to longer periods of respiration. The opposite was expected to be true for the younger seedlings.

CHAPTER II

REVIEW OF LITERATURE

Anthocyanins

Anthocyanins are considered the most conspicuous of the natural flavonoid compounds. They constitute the principle red, violet and blue pigments of plant parts. These pigments are polyphenolic flavylium salts derived chiefly from six aglycones (anthocyanidins), namely, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, by glycosidation of the hydroxyl group at position 3.

The Role of Anthocyanins in Plants

Anthocyanins are visually conspicuous in many plants and found in different tissues of the same plant. They are found in stems, leaves, flowers and fruits of certain plant species, yet there is no general agreement as to its physiological role in the plant.

A possible function of anthocyanin is to attract bees, insects, and other animals to flower parts for pollination, but does not explain why some plants are brightly colored when there is not a need for cross pollination. Also, many plants have brightly colored fruits that are too large for birds to consume and are inaccessible to animals.

Stanko (1956) has reported that anthocyanins may have a regulatory role in the opening of stomata of plant leaves. Examination of Kazakhstan plants showed that plants that contained anthocyanin retained in their protein-lipid complex some 33-51% water, while plants devoid of anthocyanin retained 20-44% water.

Further work was conducted by Shakhov and Stanko (1962) to determine the physiological role of anthocyanins in plants in northern regions. Their findings indicated that plant leaves which contained anthocyanins had higher temperatures, higher water contents, higher transpiration, and a greater water-retaining capacity than those without. They explained that the physiological significance of anthocyanins in plants might be related to a photoenergetic role; by absorbing radiant energy not directly utilized by photosynthesis it furthers a more productive utilization thereof in circumstances of low temperatures.

Anthocyanins and anthocyanidins extracted from corn leaves inhibited the aerobic oxidation of indole-3-acetic acid by peroxidase (Voronkov, 1970). The inhibiting effect of these compounds was caused by the flavonoid group, and there was no destruction of auxin oxidase or formation of a stable complex. This might indicate that anthocyanins could serve a protective role towards indole-3-acetic acid in certain plants.

Methods for Separating and Identifying Anthocyanins

The structures of flavonoid compounds were not determined until the 1890's when Perkin of the United States and Von Kostanecki of Germany did research in this area. Approximately 20 years later Willstätter and Everest were able to obtain anthocyanin in the crystalline form. They isolated cyanin from cornflower and proposed the terms anthocyanin and anthocyanidin for the glycosides and aglycones respectively. Since the work of these two men, research in this area has progressed rapidly. Methods commonly utilized for separation and identification studies are zone electrophoresis (Markakis, 1960), thin

layer chromatography (Lees and Francis, 1971), column chromatography (Fuleki and Francis, 1968), and spectrophotometry (Fuleki and Francis, 1968; Lees and Francis, 1971; Fuleki and Francis, 1968a; and Fuleki and Francis, 1968b).

Factors that Affect Anthocyanins

Effect of pH

Anthocyanins are stable only in strong acid solutions (Jurd, 1963; Jurd and Geissman, 1963; Timberlake and Bridle, 1967). At higher pH, anthocyanins lose a proton and then rapidly hydrate to yield colorless chromenols. The pH range at which this reaction generally occurs is pH 2-5. Upon acidification the chromenols immediately regenerate the anthocyanin cation. In contrast to the relative stability of chromenols from anthocyanins, chromenols derived from anthocyanidins are very unstable and readily decompose irreversibly. At pH 2-5 it has been observed that the cyanidin pigment decomposes with liberation of B ring as 3,4-dihydroxy benzoic acid (Jurd, 1972). Based on the reactions that occur at higher pH values, most workers use extracting solutions that are buffered at pH 1 for anthocyanin extractions (Fuleki and Francis, 1968a; Fuleki and Francis, 1968b; Fuleki and Francis, 1968c; and Lees and Francis, 1971).

Anthocyanin-metal complexes

Anthocyanins are known to form stable colored complexes with metals at higher pH values (Ansen et al., 1969; Bayer, 1966, Chandler, 1970). Ansen and Siegelman (1957) observed that aluminum caused a marked shift in absorption maximum toward the longer wavelengths with

a resultant color change from red to blue for anthocyanin. They explained that the shift was probably attributable to the formation of 5-membered rings on chelation with aluminum ion. Jurd and Ansen (1966) also observed that cyanidin 3-glucoside forms a colored complex with aluminum ions at an optimal pH 5.5. The cyanidin-aluminum complex remained stable even under continued exposure to diffuse light.

Further studies by Yasunori et al. (1966) reinforced the postulation that stable anthocyanin complexes can be formed with metals. Their work showed that anthocyanin pigments found in eggplant were unstable in alkaline media to heat and light but stable in the presence of aluminum, iron, calcium and sodium ions.

Effect of carbohydrates

Anthocyanins are products of the shikimic acid pathway. Therefore, a relationship between endogenous carbohydrate content and anthocyanin formation might be anticipated. Several sugars were used in a test conducted by Szweykowska et al. (1959) to determine the effects on anthocyanin synthesis in red cabbage and radish. They found that mannose, raffinose, maltose, glucose, lactose and sucrose were able to promote anthocyanin synthesis. Margna (1970) worked with buckwheat and found that isolated hypocotyls or cotyledons were stimulated in the production of anthocyanins by sugars. But the presence of sucrose, glucose, fructose, or mannitol in water used for seed germination and in the culture medium for seedling cultivation reduced the content of anthocyanin.

Sucrose was found to be the most effective in promoting anthocyanin formation in corn endosperm tissue cultures, glucose was found to be

the least effective and fructose intermediate (Straus, 1959). However, glucose supported the greatest plant growth and sucrose the least. Increases in pigment content was observed as the concentration of sucrose in the medium was increased from 0.05M to 0.20M. Stimulation of anthocyanin synthesis by an exogenous supply of sucrose was also observed by Creasy (1968) who worked with strawberry leaf disks.

Effect of growth hormones

Indole-acetic-acid and 2,4-D reduced anthocyanin yield in excised dark grown sorghum seedlings (Vince, 1968). Auxin increased the duration of the lag phase before anthocyanin synthesis began as well as the rate of synthesis. Anthocyanin synthesis did not begin until auxin enhanced elongation of the internodal section ceased or slowed down. Vince speculated that it was unlikely that the effect of IAA on anthocyanin synthesis resulted from the increased utilization in growth substrates needed for anthocyanin formation.

Gibberellic acid was found to inhibit anthocyanin and flavonoid synthesis markedly in both growing and non-growing cultures of two species of Spirodela (Furuya and Thimann, 1964). Initially, very low concentrations of GA_3 (10^{-6} to 10^{-7}M) promoted anthocyanin formation in the first 7-10 days, but after 3 weeks a concentration as low as $3x10^{-6}$M caused 50% inhibition. It was also observed that at these levels, gibberellin had no effect on growth, and that inhibition of anthocyanin synthesis was greater under lower light intensities. The results of Vince's (1968) studies seem to parallel the works of the afore-mentioned men. She also observed the GA_3 decreased anthocyanin content. The effect was found to be greater in sections incubated with

coleoptiles attached, and therefore it was speculated that GA_3 acts by increasing the concentration of endogenous auxins.

Ethylene was found to both promote and inhibit anthocyanin synthesis in Sorghum vulgare (Cracker et al., 1971). The rate of anthocyanin formation was found to be dependent upon the time of ethylene treatment in relation to light exposure and the stage of anthocyanin synthesis process. Plants that received ethylene treatment during the early lag phase of anthocyanin synthesis had higher anthocyanin content after 24 hours. Plants that received ethylene treatment after the lag phase had lower anthocyanin content after 24 hours. Synthesis of anthocyanin pigments in etiolated cabbage seedlings was found to be influenced by ethylene at concentrations higher than 10 ppb (Kang and Burg, 1972). Stimulation of anthocyanin synthesis by brief red illumination was completely prevented by applied ethylene and indoleacetic acid which stimulates ethylene production. It was suggested that in part, photo-induced anthocyanin synthesis is due to lowered ethylene content in light-treated tissue.

Effects of light

Light is known to exert a most emphatic control over anthocyanin synthesis (Grill, 1967; Klein et al., 1957; Kolesnikov and Zore, 1957; Mohr and Hartmann, 1965; Kandeler, 1959; Kandeler, 1960). Except in a few instances, anthocyanin will usually not be formed in the absence of light. In those few instances, the amount is increased manyfold when the plant material is illuminated.

Milo seedlings did not produce anthocyanins when grown in the dark (Downs, 1964). However, if the seedlings were exposed to light, they

became a faint pink in about 6 hours. If the seedlings were placed in darkness for 20 to 24 hours after the light period, the internode section became an intense red. The amount of anthocyanin formed was found to be dependent upon the light intensity and the duration of exposure. It was also observed that at moderately high light intensities, milo seedlings accumulated anthocyanin at a constant rate for at least the first 16 hours of irradiation. At lower intensities, a lag period of 4 to 6 hours occurred before the linear phase of anthocyanin synthesis began.

Anthocyanin synthesis in milo seedlings was found to be controlled by two photoreactions (Downs and Siegelman, 1963). The first required more energy than the second and showed maximum response at 470 mμ. According to Downs (1964) the second reaction controls the effects of the first one and is a typical phytochrome response. The light intensity required is low, exposure time short, and a maximum inhibitory effect is obtained between 710 and 750 mμ. The effects of the far-red irradiation are reversed by a subsequent irradiation between 630 and 670 μm. Others such as Vince and Grill (1966), speculate that anthocyanin synthesis in turnip is a result of more than one reaction, presumably mediated by two forms of phytochrome and that the high energy reaction in blue light does not appear to depend on phytochrome.

Riboflavin

Riboflavin completely reversed the inhibition of anthocyanin formation by methionine, thiouracil, and azaguanine (Thimann and Radner, 1958). When the plants were pre-illuminated, riboflavin increased anthocyanin formation in a subsequent dark period. This action was

enhanced by sucrose and diminished by the absence of CO_2. It was deduced that riboflavin acts, not as a photoreceptor, but as a dark catalyst to produce anthocyanins from sucrose or other precursors. The results of studies by Matsumoto et al. (1973) on Populus cells in suspension culture seem to be in conflict with Thimann's and Radner's postulation. They found that riboflavin was very effective for anthocyanin formation only under light exposure and ineffective in the dark culture. Therefore, it was suggested that riboflavin or its photochemical derivative might function as a photoreceptor.

Purines and pyrimidines

Purines and pyrimidines are thought to cooperate in the synthesis of anthocyanin (Thimann and Radner, 1962). Upon exposing Spirodela with analogs of both purines and purimidines, anthocyanin formation was inhibited. The most effective inhibitor was found to be 8-azaguanine which decreased anthocyanin formation by 50% at $3x10^{-7}M$.

Protein inhibitors

Protein synthesis was found to be necessary for maximum production of anthocyanin in Sorghum vulgare (Stafford, 1966). Light independent synthesis of apigeninidin and luteolinidin was inhibited by chloramphenicol and L-ethionine. The light induced synthesis of cyanidin was inhibited by actinomycin, azaguanine, chloramphenicol and ethionine. Lange and Mohr, 1965) also observed the inhibitory effect of actinomycin D on anthocyanin formation. Anthocyanin synthesis was completely blocked when actinomycin D was applied to Sinapsis alba seedlings before or at the onset of continuous dark red light, but when added 6 hours

after light exposure, synthesis was only partially inhibited. This indicated that actinomycin D blocked the formation of new kinds of messenger RNA. The continued synthesis of messenger RNA already being produced at the time of drug addition was permitted to a certain extent. This added support to the hypothesis that phytochrome 730 acts through the activation of potentially active genes.

Degradation of anthocyanins

Various enzymes have the capacity to degrade anthocyanins (Proctor and Creasy, 1969; Peng and Markakis, 1963; Peng and Markakis, 1963; Huang, 1956; Boylen and Hagen, 1969). The effectiveness of certain enzymes to degrade anthocyanins is dependent on pH. Gromeck and Markakis (1964) found that anthocyanins were degraded optimally at pH 4.5-5.5 by horse radish peroxidase in acetate buffer. The rate increased with H_2O_2 concentrations up to 10^{-3} to 10^{-4}M, above which it decreased. Another anthocyanin degrading enzyme called "anthocyanase" has been studied by Huang (1956). He studied the kinetics of the decolorization of anthocyanins by this enzyme. Further work was conducted by Wagenknecht et al. (1960) to determine the possible relationship between "anthocyanase" activity to scald in sour cherries.

Studies pertaining to the bleaching of anthocyanins have been of special interest to people involved with the commercial processing of fruits (Wagenknecht, 1960; Yang and Steel, 1958; Goodman and Markakis, 1965). Goodman and Markakis (1965) observed that sulfur dioxide was able to inhibit the degradation of anthocyanins by a polyphenol oxidase without bleaching the pigments. In tart cherry juice 30 ppm sulfur

dioxide was required for almost complete inhibition, and a reduction in pH produced inhibition with lower concentrations of sulfur dioxide.

Thermal decomposition of anthocyanins

The concepts involving the thermal decomposition of anthocyanins are not clear, but it is known that higher temperatures may lead to decomposition of these pigments possibly by the hydrolysis of the protective 3-glycosidic linkage (Singleton, 1972; Markakis et al., 1957). Meschter (1953) in studying the effects of carbohydrates and other factors on strawberry products found that high temperatures markedly decreased anthocyanin stability. It was calculated that the half-life of the pigment in strawberry preserves decreased from 1300 hours at 20C to 240 hours at 38C. A temperature of 20C was also found to be optimum for anthocyanin synthesis in milo seedlings in a study conducted by Downs and Siegelman (1963). Higher temperatures (30-35C) greatly inhibited the production of anthocyanins.

Summary

The formation of anthocyanins is dependent on several factors of which carbohydrate content, light, and temperature are of primary importance. Exogenous supplies of various sugars such as glucose, fructose, and sucrose have been shown to stimulate anthocyanin production in various plants (Szweykowska et al., 1959; Margna, 1970; and Straus, 1959). This stimulatory effect is probably attributable to the utilization of carbohydrates for the formation of the anthocyanin molecule. Light is necessary for the formation of anthocyanin in

sorghum seedlings and greatly stimulates the pigment production in many other plants. The effects of light seems to be related to a phytochrome response. High temperatures generally result in lower anthocyanin production, whereas, a temperature of 20C was optimum in milo seedlings (Downs and Siegelman, 1963). The lower pigment production at higher temperatures is probably a result of anthocyanin molecule decomposition. Phenylalanine ammonia-lyase (PAL) is an important enzyme required for the production of anthocyanin synthesizing substrates. Its activity is also influenced by the aforementioned factors, therefore, a literature review on PAL is appropriate to further evaluate the relationship between anthocyanin and PAL as affected by various conditions.

Phenylalanine Ammonia-lyase

Phenylalanine ammonia-lyase (PAL) was first isolated and studied by Koukol and Conn (1961). It is an enzyme associated with secondary metabolism in plants. PAL catalyzes the deamination of L-phenylalanine to trans-cinnamic acid and ammonia. In turn, the carbon skeleton of cinnamic acid serves as a major substrate for many pathways of phenolic compound biosynthesis in plants.

A reason for the increased interest in the study of PAL is that its activity can be stimulated by various factors within relatively short intervals of time. Also, the relative stable nature and the ease of assaying this enzyme possibly contributes to its popularity with researchers. Assay techniques may include spectrophotometry (Koukol and Conn, 1961; Zucker, 1965), radioisotope studies with (^{14}C) -

phenylalanine (Subba et al., 1967), or gas liquid chromatography (Vaughn and Anderson, 1971).

Isozymes of PAL

Phenylalanine ammonia-lyase has been isolated from several different plants including maize (M.W. - 306,000) (Marsh et al., 1968), mustard (M.W. - 300,000) (Schopfer, 1971), and potato (M.W. - 330,000) (Havir and Hanson, 1968).

Two forms of PAL with different properties have been found in a number of tissues. The isozymes in sweet potato show different sensitivities to phenolic inhibitors (Minimikawa and Uritani, 1965). Quercus pendunculata contains two forms of PAL which can be separated by DEAE - cellulose and which also possess different sensitivities to phenolic compounds (Boudet et al., 1971). PAL activity in different ammonium sulfate fractions may be found in light - and dark-grown mung bean seedlings (Ahmed and Swain, 1970). In contrast, PAL from light - and dark-grown plants of buckwheat, mustard, and soybean tissue cultures were found to be the same electrophoretically (Amrhein and Zenk, 1971; Schopfer, 1971).

Factors Influencing the Levels of PAL

Inhibitors

All PAL preparations are strongly inhibited by cinnamic acid (Havir and Hanson, 1968; O'Neal and Keller, 1970). Enzyme preparations from barley (Koukol and Conn, 1961) and Rhodotorula (Ogata et al., 1967) are sensitive to sulfhydryl inhibitors in contrast to preparations from potato (Havir and Henson, 1968) and maize (Marsh et al.,

1968) which are not. Other inhibitors include cycloheximide (actidione 10^{-4}M) which completely inhibited light-induced PAL synthesis in a study conducted by Hachtel and Schwemmle (1973). Acetylcholine was found to interfere with the phytochrome-controlled increase of PAL after brief light treatment (Saunders and Mclure, 1973). PAL from plants such as barley and potato (Camm and Towers, 1973) and grasses (Jangaard, 1973) were inhibited by p-coumarate. Other compounds that Jangaard found to be inhibitory are t-cinnamic acid, carbonyl reagents, hydroxylamine, nitromethane, and herbicides such as diuron, dalapon, amiben, and chloropropham.

PAL degrading or inactivating system

Zucker (1968) obtained data from his research on potato tubers indicating the possible presence of a PAL inactivating or degrading system. He found that cycloheximide (inhibits PAL synthesis) added to the disk after it was cut from the tuber did not affect the disappearance of the enzyme. However, if the cycloheximide was added to the disk after more than half the maximal enzyme content was formed, the enzyme disappeared from the tissue. Also, if cycloheximide was added at a concentration tenfold that required to completely inhibit enzyme synthesis after a maximal enzyme level was attained in the tuber disks, the subsequent loss of enzyme activity was prevented. Therefore, he postulated that 1) the early phases of induction involves the synthesis of enzyme protein in the absence of turnover, 2) that a system capable of degrading or inactivating the lyase subsequently forms in the tissue, and 3) that the formation of the degrading or inactivating system requires protein synthesis.

Ultraviolet light

Increases in PAL was observed after irradiating excised peas with short wave UV light (Hadwiger and Schwochau, 1971). The enhancement was explained as being attributable to new RNA and protein synthesis. This was substantiated to a certain extent when induction of PAL by UV light was readily inhibited by 6-methylpurine (potent inhibitor of all RNA synthesis) and cycloheximide when applied within 1 hour and 3 hours respectively after irradiation. Also proposed was that the control of this response occurs at the gene transcription level and depends on the conformational state of the double stranded DNA (possible that DNA becomes more transcribable either by dissociating a repressor component or by assuming a more desirable conformation for transcription).

It was speculated that UV light causes the conversion of trans-hydroxycinnamic acids to the cis-isomers in the hypocotyl of dark grown gherkin seedlings (Engelsma, 1974). Assuming that trans-hydroxycinnamic acids inhibit the development of PAL activity, it was further assumed that UV light indirectly was responsible for an increase in PAL activity in the irradiated seedlings.

Dichlobenil

Phenylalanine ammonia-lyase activity was increased by dichlobenil (2,6-dichlorobenzonitrile) in gherkin seedlings (Engelsma, 1973). Engelsma explained the response as a derepression of PAL and/or diminished inactivation of PAL. The basis of this study was that hydroxycinnamic acids, which are end products of the pathway in which

PAL is a key enzyme, repress PAL synthesis and/or induce a system which inactivates PAL. Therefore, if the hydroxycinnamic acids (which are normally present in cell compartments separated from compartments containing phenol oxidizing enzymes) were able to permeate through the membranes containing them, they would become available to oxidizing enzymes and result in the deposition of lignin-like material. Dichlobenil did have the ability to facilitate the above mechanism and therefore decreased the concentration of soluble hydroxycinnamic acids.

Divalent manganese ions

Divalent Mn ions were found to cause an increase in the level of PAL in gherkin hypocotyls (Engelsma, 1972). It is thought that Mn ions positively influence the removal of hydroxycinnamic acids which cause repression of PAL synthesis and/or inactivation. No other metal ion has been found to have this ability to form complexes with hydroxycinnamic acids.

Wounding

An increase in PAL activity in strawberry leaf disks was observed when the plant material was wounded in the presence of sucrose and light (Wong et al., 1974). The highest PAL activity was located nearest the wounded tissue. Without wounding, no increase in activity was observed when leaves were cultured in sucrose and light.

Growth hormones

The activity of PAL was higher in gibberellin-treated dwarf pea plants grown under white or red light than in untreated dwarf pea plants

(Cheng and Marsh, 1968). But gibberellic acid had no detectable effect on this enzyme when the plants were grown in darkness.

Abscisin II increased PAL activity two to four times that observed in the axes which had been incubated in buffer (Walton and Sondheimer, 1968). When the axes were incubated in buffer, the activity of PAL commenced after 5 hours and continued to rise until the 15th hour after which there was a rapid decline. But when the bean axes were incubated in 5 g/ml DL-abscisin II, there was a faster increase of enzyme activity after the 5th hour and a maximum was reached by 11 hours.

PAL activity increase in flavedo discs was attributed to the production of ethylene (Monselise and Kahan, 1969). It was found that the activity of PAL in the intact flavedo of mature grapefruit peel tissues was low. However, when the flavedo was cut into discs, the peel evinced upon incubation an increase in PAL activity. Increased PAL activity was also observed when the flavedo was treated with ethylene. Similarly, endogenous ethylene production of intact citrus fruit tissues is also low, and increases when the incubated flavedo is cut into discs. Therefore it was postulated that the increase of ethylene in the discs caused an increase in PAL activity. Some of the reasons were stated as follows. 1) PAL activity was induced by exogenous ethylene in intact fruits, but decreased when the fruit was removed from the ethylene atmosphere. 2) In the irradiated fruit there was a close correlation between ethylene production and PAL activity in the flavedo. 3) Carbon dioxide inhibits both ethylene production and PAL activities in the discs. 4) The appearance of PAL activity seemed to be due to de novo protein synthesis of which ethylene is considered to regulate.

There was an increase in extractable PAL activity due to ethylene in the discs of aged swede and parsnip disks (Rhodes and Wooltorton, 1970). The increase in PAL activity was found to be very sensitive to the concentration of ethylene; 1 ppm of ethylene was sufficient to give a half maximal response. It was stated that the ethylene induced increase in PAL activity was possibly due to de novo protein synthesis since it was inhibited by the presence of a range of inhibitors of RNA and protein synthesis. Also postulated was that ethylene produced by injured swede tissue could lead to the induction of PAL activity and the biosynthesis of phenolic compounds which play a role in forming some chemical or physical barrier to infection.

No correlation between ethylene production and PAL development in gherkin hypocotyl segments was observed by Engelsma and Van Bruggen (1970). They also found that ethylene did not increase PAL activity in intact hypocotyl tissue but did so in excised tissue. Therefore they concluded that if ethylene plays a role in PAL synthesis in excised gherkin hypocotyl tissue, it does so only in combination with the formation or disappearance of another factor. They claim that a heat-labile and nondialyzable inhibitor of PAL synthesis which is released after excision may be crucial for the ethylene effect on PAL synthesis in gherkin hypocotyl tissue.

Ethylene was found to enhance the activity of PAL in carrot root tissue (Chalutz, 1973). The addition of ethylene to the air stream increased PAL activity and total protein content of the carrot root discs until maximum activity was reached after 36 to 48 hours of incubation. The continuous presence of ethylene was required to maintain

a high level of activity. It was postulated that ethylene may combine with the induced enzyme or protect it from inactivation, or both. Chalutz concluded that his results support the hypothesis that the mode of action of ethylene may involve both de novo synthesis of the enzyme protein and protection or regulation of activity of the induced enzyme.

The addition of high 2,4-D concentrations to lag-and early exponential-phase cells of dark grown suspension cultures of Paul's Scarlet Rose retarded the development of PAL activity with a concomitant delay in polyphenol accumulation (Davies, 1972). But after the initiation of increased PAL activity, the early stage of development of the enzyme was less sensitive to high hormone levels than was polyphenol synthesis. Therefore, it was suggested that in addition to inhibiting the initiation of PAL synthesis, high auxin levels may also inhibit some other component(s) of the biosynthetic pathway. It was also found that high 2,4-D levels (10^{-3}M) did not inhibit the activity of PAL in vitro.

Temperature

There was an increase in the rate of synthesis of hydroxycinnamic acids in gherkin seedlings in response to light at higher temperatures, but the length of the period of enhanced accumulation was shorter (Engelsma, 1968). It was determined that a temperature range of 12.5 to 32C was optimal for phenol synthesis if the seedlings were continuously irradiated.

Gherkin seedlings exposed to temperatures below 10C in both dark-grown and pre-irradiated seedlings were found to respond with higher

levels of PAL activity (Engelsma, 1970). The activity was prevalent in the cold treatment or after transfer to higher temperatures; in the latter case, however, it was followed by a decline. The results were explained on the basis that at temperatures above 10C, a PAL inactivating system compensates for the synthesis of the enzyme and the end products (hydroxycinnamic acids) are involved in the induction and/or functioning or the inactivating system. But at lower temperatures there is a low rate of synthesis of these products. Therefore newly synthesized PAL is not inactivated and previously synthesized PAL is released from an enzyme-inactivator complex.

Light

The activity of PAL was greatly enhanced by the exposure of potato tuber tissue to white light during culture (Zucker, 1965). Ethionine and analogues of purines and pyrimidines inhibited enzyme activity. It was suggested that an actual synthesis of enzyme molecules occurs during the period of culture, and that the appearance of activity represents an induction of enzyme synthesis in the tissue.

Temporary changes in the level of PAL was detected in gherkin seedlings upon exposure to blue light (Engelsma, 1967). After a time lag of about 90 minutes the enzyme level increased but began to decline 180 minutes after irradiation. It was suggested that the light-induced increase in the level of PAL was due to de novo enzyme synthesis because when the plants were sprayed with cycloheximide prior to light treatment, enzyme increase was depressed. The increase in enzyme level was considered to be a function of light intensity, and one of the

initial processes involved is the transport of protomotive factor from the cotyledons to the hypocotyl. It was postulated that the decline in PAL level which followed the initial increase was probably due to enhanced enzyme inactivation.

The activity of PAL increased dramatically in xanthium leaf disks floated on sucrose in light (Zucker, 1969). An equally striking loss of enzyme activity was noticed when the disks were transferred from light to darkness. The data suggested that the enzyme was involved in synthesis and degradation. The use of cycloheximide as a protein inhibitor suggested that the induction of PAL synthesis was in the leaf. Light induction of PAL was possible in the absence of an exogenous supply of carbohydrate. This was explained on the basis that the tissue probably had a sufficient supply of endogenous carbohydrate to support an initial period of enzyme synthesis. But after a period of time, this supply was depleted and an exogenous supply was required. The photoperiodic regulation of PAL activity in the leaf disks was considered atypical. It was associated with the chlorophylls rather than phytochrome as the photoreceptors. The diurnal fluctuation in enzyme activity was attributed to two opposing responses of the xanthium system. One was the repeated induction of PAL synthesis that occurs during each light cycle and the other the rapid turnover of the enzyme. It was also found that xanthium leaf disks have the ability to synthesize PAL continuously during an extended period of illumination or to stop and synthesize repeatedly during alternating cycles of light and darkness. Therefore, unlike potatoes and gherkin seedlings, the repression of PAL if present, is reversible.

Exposure of gherkin seedling hypocotyls to alternating light-dark treatments resulted in corresponding PAL peaks and temporary increases in the rate of accumulation of hydroxycinnamic acids (Engelsma, 1969). The sensitivity to light was found to be inversely related to the amount of hydroxycinnamic acids that may have previously accumulated by preceding irradiation.

The repression of PAL activity in segments of gherkin seedlings was reported to be due to the end products of its reaction (Engelsma, 1968). A possible end product is p-courmaric acid which may have affected the ability of irradiation to lose its effect on the induction of PAL. Irradiation of the segments with blue light stimulated PAL synthesis, but so did the addition of glutathione to the incubation medium. It was assumed that these agents had different sites of action since irradiation antagonized the repressing effect of cinnamic acid, whereas glutathione did not.

Irradiation was still capable of inducing PAL synthesis at 10C in gherkin seedlings but the increase was not followed by an inactivation mechanism or the mechanism may have been incomplete (Engelsma, 1970). It was speculated that the synthesis of a PAL inactivating enzyme system may have stopped at a higher temperature than the PAL synthesizing system which seems to stop at around 8C. Irradiation of the seedlings at temperatures below 8C does not lead to an induction potential, therefore PAL synthesis does not occur at a higher incubation temperature, whereas, a relatively short cold period following the light treatment diminishes the effect of the latter. This may indicate that the low temperature treatment causes an interruption at

some point in the reaction sequence between the initiating photoreaction and PAL synthesis.

A rapid increase in PAL activity in etiolated radish seedlings exposed to far-red light was observed by Bellini and Poucke (1970). The activity rose sharply during 6 hours of irradiation and then decreased. They did not think that a lag phase was involved. The magnitude of the response to far-red in cotyledons was dependent on age. Forty-eight hours after sowing, irradiation during 6 hours caused sixfold activity increase. However, 24 hours later the increase was only fourfold.

Radish and mustard seedlings exposed to combinations of red light and darkness resulted in no difference in PAL activity from the dark control (Bellini and Hillman, 1971). Therefore, they concluded that the far red effect on PAL could not be explained solely by formation and maintenance of far red-absorbing phytochrome.

PAL synthesis was induced in 48 hour-old mustard seedlings upon exposure to red and far-red light (Schopfer and Mohr, 1972). This led to Schopfer's and Mohr's rebuttal to the postulation made by Bellini and Hillman. They claim that there is no reason why the far red effect on PAL synthesis in the mustard seedling "cannot be explained solely by formation and maintenance of Pfr." They feel that the discrepancy between their data and Bellini's and Hillman's could be attributed to the different sensitivities of the methods used. Also, they made a stipulation to discriminate between Pfr (ground state) and Pfr* (steady state concentration of the excited species) and to ascribe most of the effect of continuous far-red light to the action of Pfr*.

An induction and repression sequence was considered to be responsible for PAL activity in pea seedlings under the influence of continuous illumination (Smith and Attridge, 1970). All the light treatments used brought an early rise in extractable enzyme activity which reached a peak at 6 to 8 hours, followed by a further increase reaching a maximum at 12 to 14 hours. A decline followed the second peak in all cases with a return to the steady-state level maintained in dark-grown controls over the experimental period. It was suggested that two isozymes may be involved and sequentially induced. A more simple explanation was presented as the presence of separate mechanisms, e.g. enzyme activation and enzyme synthesis. The early rise in activity under continuous illumination was thought to be a phytochrome-mediated response. However, the later increases under continuous illumination was probably not phytochrome-mediated. A characteristic of their experiment was the presence of a 60 to 90 minute lag phase similar in magnitude to that observed by Mohr and his colleagues. This has been attributed to the time required for the de-repression of the gene for PAL. Also observed was PAL's activity dependence on the quality of light rather than intensity.

The control of PAL activity by photochrome in mustard seedlings was explained as the result of three processes, namely, Pfr-mediated enzyme synthesis, inactivation of PAL by an "inactivator", and eventual repression of enzyme synthesis (Weidner et al., 1969). During the period 1.5 to 12 hours after the onset of far red only enzyme synthesis occurs. The enzyme inactivation comes into play while enzyme synthesis continues at a constant rate. This antagonism of synthesis and

inactivation leads to a true steady state which is observed between about 24 and 27 hours after the onset of far red. After this period the rate of enzyme synthesis decreases and as a consequence, "inactivation dominates". They further explained that the results of the "secondary irradiations" with far red indicate that an "inactivator" of PAL does not have any direct influence on PAL synthesis.

Effects of carbohydrate levels

The addition of sucrose (0.15M) to strawberry leaf disks in darkness or light caused an increase in PAL activity, the increase being much greater in the presence of light (Creasy, 1968). The optimal concentration of sucrose for PAL activity in strawberry leaf disks ranged from 0.15M to 0.40M (Wong et al., 1974). A low but constant level of activity was detected in leaf disks maintained in 0.15M sucrose and in darkness. Light accelerated the rate of PAL increase but did not change the total level of enzyme activity which was determined by the sucrose concentration. Enzyme activity disappeared rapidly when leaf disks cultured in sucrose and light were transferred to darkness. It was suggested that an inactivating system was synthesized during the induction period, and the activity of the inactivating system increased as the induction period lengthened.

The Relationship of PAL to Flavonoid Synthesis

PAL activity increase was considered to be necessary to permit the high rates of flavonoid synthesis in strawberry leaf disks (Creasy, 1967). The correlation was not clearly defined because although light stimulated the increase in PAL activity, the increase was not altered

by short exposure to red or far-red light as was flavonoid synthesis. The accumulation of cinnamic acids was observed with the addition of phenylalanine, but there was no change in the amount of PAL produced. Based on this observation, he thought it unlikely that PAL is normally the rate limiting step in cinnamic acid biosynthesis and that the supply to phenylalanine would probably be rate limiting. However, a reduction of cinnamic acid synthesis did create a condition where cinnamic acids were rate limiting for flavonoid synthesis. PAL was inferred to be a key enzyme in the biosynthesis of anthocyanin by Matsumoto et al. (1973). They studied the effects of light, sucrose, and riboflavin on anthocyanin formation and PAL activity in Populus cells. A marked increase of PAL activity influenced by light and high sucrose concentrations occurred during the lag phase prior to anthocyanin formation. It was thought that this increase accounted for part of the increase in anthocyanin formation.

Although most of the literature indicate that PAL activity is related to flavonoid synthesis via the shikimic acid pathway, Swain and Williams (1970) have provided conflicting data. They observed the incorporation of ^{14}C-labelled L-phenylalanine and sucrose into caffeic acid. The results of their studies indicate that L-phenylalanine may not be an obligate precursor in the biosynthesis of phenylpropanoid compounds, including flavonoids.

Summary

PAL activity and/or synthesis is greatly influenced by light, temperature, and carbohydrate content. Blue light is effective in

stimulating PAL activity in gherkin seedlings due to de novo enzyme synthesis (Engelsma, 1967). Alternating light-dark treatments result in corresponding PAL peaks, and the sensitivity to light is dependent on the amount of hydroxycinnamic acids that accumulate by previous irradiation (Engelsma, 1969). Red and far-red light induce PAL synthesis in mustard seedlings (Schopfer and Mohr, 1972), therefore, phytochrome can control PAL synthesis and/or activity. Temperature has an indirect control over PAL activity by influencing the synthesis and/or activity of a PAL inactivating system (Engelsma, 1970). At high temperatures, a PAL inactivating system is synthesized and/or activated and compensates for PAL synthesis and/or activity. The opposite is true at low temperatures. Addition of an exogenous source of sucrose results in an increase in PAL activity in the presence or absence of light (Creasy, 1968). The increase is probably due to the conversion of sucrose to substrates required in the Shikimic Acid Pathway.

CHAPTER III

DETERMINATION OF ANTHOCYANIN CONTENT OF ETIOLATED SORGHUM SEEDLINGS AFTER EXPOSURE TO VARIOUS TEMPERATURES AND LIGHT TREATMENTS

Downs (1964) found that milo seedlings grown in the dark do not produce anthocyanin. Therefore light was necessary for its production. An illuminance of 1,200 ft-c to 2,400 ft-c was effective. However, at the lower intensities, a lag period of 4 to 6 hours occurred before the linear phase of anthocyanin began. Also, higher quantities of anthocyanin were produced in the internodes if a post incubation period in darkness for 20 to 24 hours followed the light period. Downs and Siegelman (1963) reported that the control of anthocyanin production in milo seedlings is dependent on two photoreactions. One photoreaction is thought to occur at 470 mμ and the other at wave-lengths greater than 600 mμ.

The temperature during the light period and the dark incubation period was found to influence anthocyanin production (Downs and Siegelman, 1963). A temperature of 20C was optimal for both these periods. No detailed studies were conducted to determine the interaction of light and temperature on anthocyanin production.

Based on the possibility that temperature during light and dark periods may be influential in anthocyanin production, the following studies were conducted. These studies entailed the exposure of etiolated sorghum seedlings to different temperatures before, during and after a light period.

Materials and Methods

Sorghum bicolor variety ST-6 was used in these studies. Seeds were soaked in running water for a minimum of 1 hour to enhance imbibition of water as well as to leach out possible germination inhibitors. They were transferred to a 0.5% sodium hypochlorite solution for 15 minutes to be surface-sterilized and then washed with distilled water. Thirteen seeds were planted in each 250 ml flask containing 50 ml Bacto-agar (0.65 gm/100 ml). The flasks were stoppered with cotton plugs to reduce possible contamination, wrapped in aluminum foil to exclude light, and placed in a walk-in temperature controlled room (25C $\pm$ 1C) for seed germination and etiolated plant growth.

Effects of Pre-light Temperature Treatments

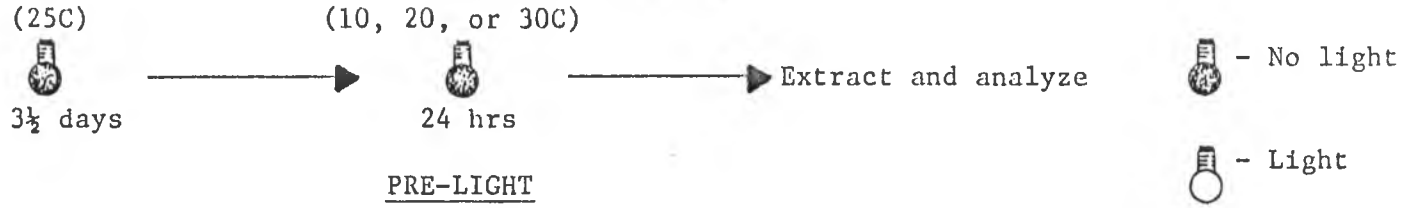
Flasks containing seedlings to be treated were removed from the 25C chamber and transferred to dark temperature control chambers set at either 10C, 20C, or 30C (figure 1). They were left in these chambers for 24 hours then transferred to a darkroom and analyzed for anthocyanin content.

Effects of Pre-light and Light Temperature Treatments

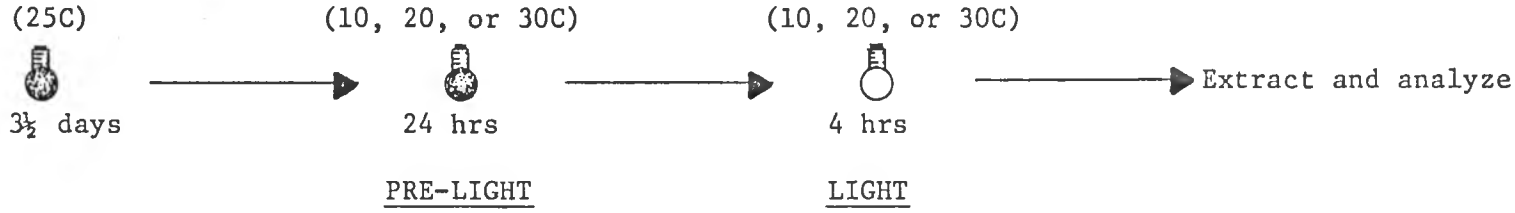
The same procedure as the pre-light temperature treatments was utilized with the exception of an additional treatment. After the pre-light temperature treatment, the seedlings were placed in a growth chamber having temperatures of either 10C, 20C, or 30C. The aluminum foil was removed from the flasks and the seedlings were exposed to light (2,400 ft-c to 3,000 ft-c) for 4 hours (figure 1). At the

Figure 1. Experimental procedure for anthocyanin and Phenylalanine ammonia-lyase studies.

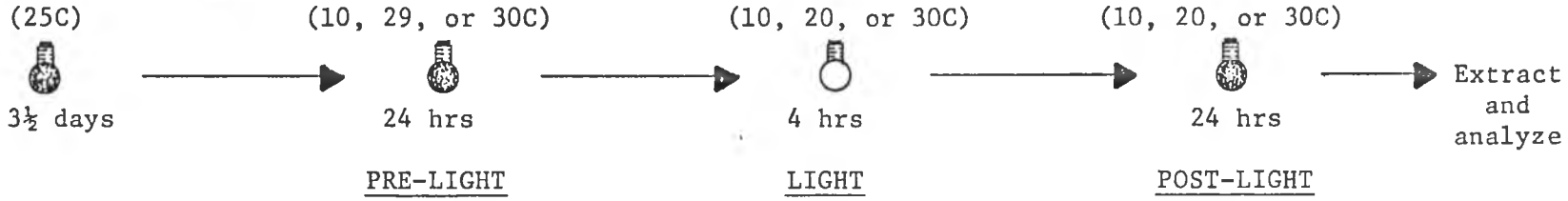
EXPERIMENT I - PRE-LIGHT TEMPERATURE TREATMENT



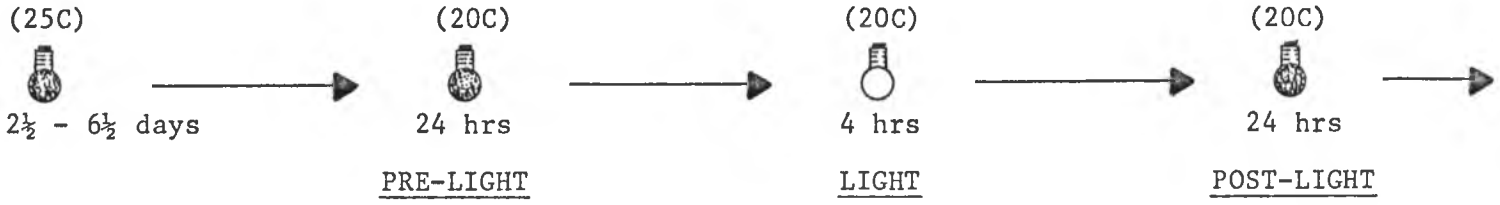
EXPERIMENT II - PRE-LIGHT, LIGHT TEMPERATURE TREATMENT



EXPERIMENT III - PRE-LIGHT, LIGHT, POST-LIGHT TEMPERATURE TREATMENT



EXPERIMENT IV - PLANT AGE



termination of this treatment the flasks were covered with aluminum foil, taken to a darkroom, and then analyzed for anthocyanin content.

Effects of Pre-light, Light and Post-light Temperature Treatments

The same procedure as pre-light and light temperature treatments was applied with the exception of a post-light temperature treatment. Following the light temperature treatment, the seedlings were wrapped with aluminum foil and placed in 10C, 20C, or 30C temperature controlled chambers for 24 hours (figure 1). A final transfer was made to a darkroom where they were analyzed for anthocyanin content.

Effects of Plant Age

The seedlings were left in the 25C chamber for 2½, 3½, 4½, 5½, or 6½ days before transfer into a 20C chamber for 24 hours. The seedlings were then transferred into a growth chamber set at 20C and exposed to light for 4 hours. The flasks were covered with aluminum foil and transferred to a 20C chamber for 24 hours. The treated seedlings were transferred to a darkroom and analyzed for anthocyanin content (figure 1).

Effects of Sucrose

Sucrose (0.01M) was added to the Bacto-agar medium prior to planting the seeds. The seedlings were then subjected to treatments involving the effects of pre-light temperature; effects of pre-light and light temperature; effects of pre-light, light and post-light temperature; and effects of plant age.

Anthocyanin Analysis

Treated seedlings were prepared for anthocyanin analysis in a darkroom. A 15 watt fluorescent lamp covered with two sheets of green cellophane provided the necessary illumination. Preliminary tests indicated that the light source had no effect on anthocyanin production. A maximum of six flasks containing seedlings were kept in the darkroom when preparing for analysis. The remaining flasks were placed in a refrigerator with a temperature setting of 6C. Pilot tests indicated that no apparent synthesis or degradation of anthocyanin occurred at this temperature.

Three of the 13 seedlings were randomly selected per replicate. It was determined that most of the anthocyanin was produced within 4 centimeters from the base of the coleoptile under the conditions of this study. Therefore, first internode sections 4 centimeters long from the base of the coleoptile were used for analysis. The internodal sections were immediately weighed and placed in a test tube (15.5 cm x 2 cm) containing 6 ml 95% EtOH:1.5N HCl (85:15 v/v) with pH 1 (Fuleki and Francis, 1968). The plant material was macerated with a modified glass stirring rod. The test tube was then covered with parafilm and placed in a refrigerator at 1C for 24 hours to assure complete extraction. The extract was filtered through a Whatman no. 1 filter paper and the anthocyanin concentration was estimated by the optical density at 534 nm with a Unicam Sp 1800 Ultraviolet Spectrophotometer. The appropriate wavelength was determined after scanning several of the extract solutions for peak absorbance.

Analysis of Data

The data obtained were subjected to analysis of variance as a completely randomized design. The statistical results are reported in the appendix.

Results

Effects of Pre-light Temperature Treatments

This experiment was conducted to determine whether anthocyanin formation could be stimulated by different temperatures in the absence of light. Sorghum seedlings were analyzed for anthocyanin content immediately after exposure to a 24 hr dark incubation period at either 10C, 20C, or 30C. The results indicate that temperature was not a factor in determining anthocyanin production in this experiment. This is in agreement with Downs (1964) who found that Milo seedlings were incapable of producing anthocyanin in the absence of light.

Effects of Sucrose in Pre-light Temperature Treatments

Creasy (1968) speculated that the amount or rate of flavonoid synthesis probably depends on those factors regulating the supply of initial substrate (e.g. sugar), as well as factors influencing the levels of intermediate precursors (e.g. phenylalanine). The following investigation was conducted to determine whether an initial substrate such as sugar is a limiting factor in the absence of light.

Sorghum seedlings were planted in medium containing 0.01M sucrose and exposed to the same conditions as the previous experiment. The results were not statistically significant indicating that sucrose might not have been a limiting factor.

Pre-light, Light Temperature Combinations

The results of the previous experiments indicate that temperature had no effect on anthocyanin production in the absence of light. Therefore, the following experiments were conducted to evaluate the importance of temperature on anthocyanin production in the presence of light.

Sorghum seedlings were incubated in darkness for 24 hrs at either 10C, 20C, or 30C (pre-light temperature) then exposed to light for 4 hrs at either 10C, 20C, or 30C (light temperature). The seedlings were then analyzed for anthocyanin content immediately after the light incubation period.

The following results will relate to the production of anthocyanin as influenced by temperature during the pre-light period and temperature during the light period.

Effects of pre-light temperature

Temperature during the dark incubation period had no effect on anthocyanin production even when followed by a light incubation period at various temperatures (table 1).

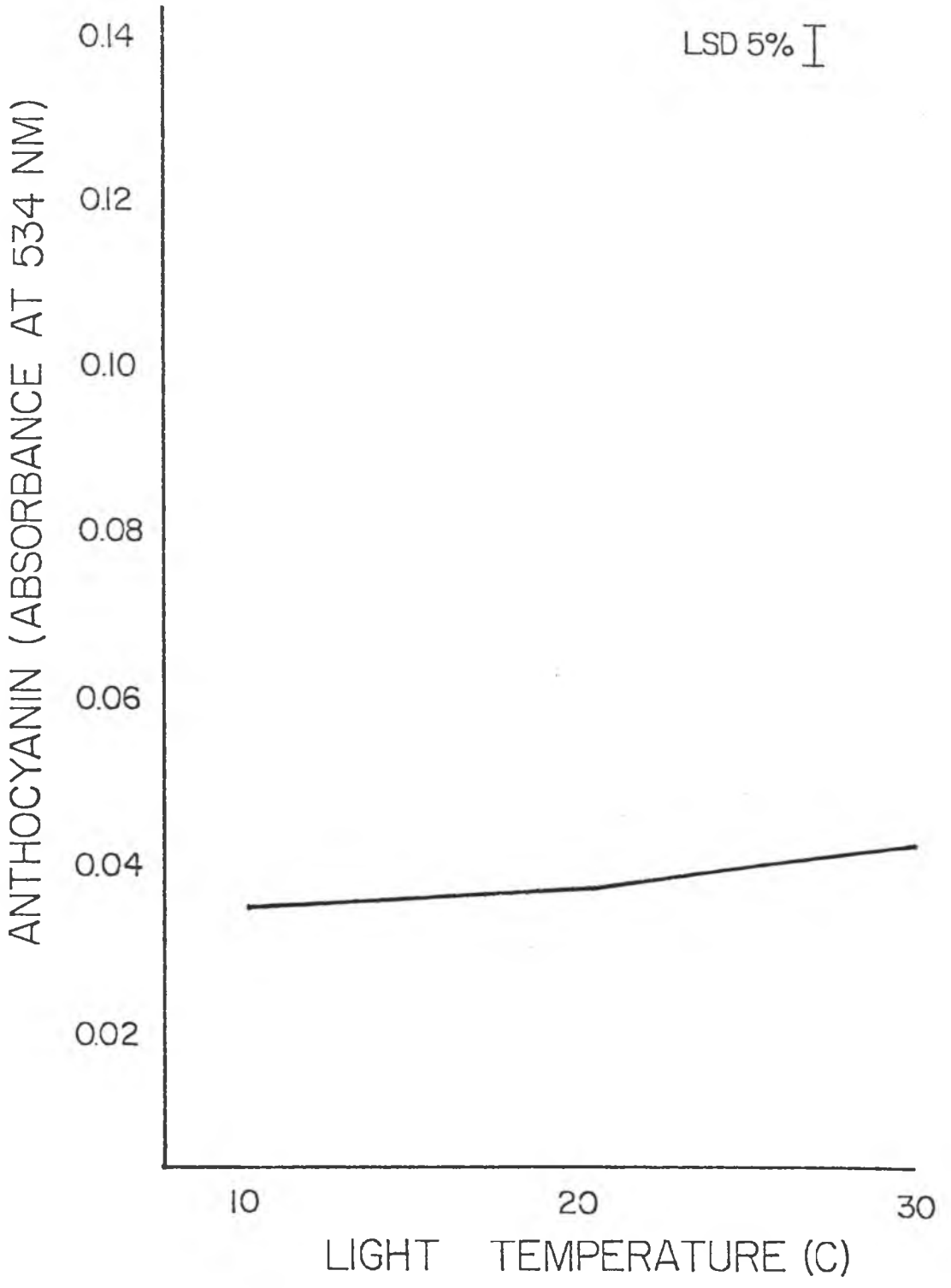
Effects of light temperature

Temperature during the light incubation period did influence the production of anthocyanin (figure 2). The trend showed an increase in pigment production with an increase in light temperature. The highest levels of anthocyanin were found in seedlings exposed to 30C while the lowest levels were found when the light temperature was 10C.

Table 1. Effect of sucrose on anthocyanin formation in sorghum seedlings during pre-light, light temperature treatments

Temperatures (C)		- Sucrose Anthocyanin production (O.D. at 534 nm)	+ Sucrose Anthocyanin production (O.D. 534 nm)	Stimulation in Anthocyanin production due to sucrose
Pre-light	Light			
10	10	0.037	0.035	-0.002
10	20	0.037	0.037	0.0
10	30	0.040	0.040	0.0
20	10	0.037	0.035	-0.002
20	20	0.040	0.038	-0.002
20	30	0.048	0.050	+0.002
30	10	0.037	0.037	0.0
30	20	0.038	0.037	-0.001
30	30	0.040	0.040	0.0

Figure 2. Effects of light temperature on anthocyanin production. Values for each temperature are means for combinations of pre-light, light temperatures.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
ANTHOCYANIN (ABSORBANCE AT 534 NM)
LSD 5%
10
20
30
LIGHT TEMPERATURE (C)

Effects of Sucrose in Pre-light, Light Temperature Combinations

A previous experiment demonstrated that sucrose was not effective in stimulating anthocyanin production in the absence of light. It was therefore the objective of this investigation to determine whether sucrose might affect anthocyanin production at various temperatures if the seedlings were exposed to light.

The experiments were designed in the same manner as those in a previous experiment with the exception that 0.01M sucrose was added to the growing medium. The results indicate that an additional source of carbohydrates failed to affect the production of anthocyanin in all combinations (table 1).

Pre-light, Light, and Post-light Temperature Combinations

Downs and Siegelman (1962) found that maximum production of anthocyanin took place during a 24 hr period following the exposure of Milo seedlings to light. Craker et al. (1971) found that light-stimulated anthocyanin synthesis in sorghum seedlings was preceded by a lag phase of approximately 8 hrs before a rapid formation of anthocyanin began. Based on the works of these people, it was decided to conduct experiments to determine the effects of temperature on anthocyanin production in sorghum seedlings during incubation periods before exposure to light, during light exposure, and after exposure to light. The incubation period after exposure to light could provide results to demonstrate the presence of a lag phase.

The treatments involved the sequential exposure of sorghum seedlings to either 10C, 20C, or 30C for 24 hrs in the absence of light (pre-light temperature), exposure to either 10C, 20C, or 30C for 4 hrs in the

presence of light (light temperature), and exposure to either 10C, 20C, or 30C for 24 hrs in the absence of light (post-light temperature). The seedlings were analyzed for anthocyanin content immediately after the post-light incubation period.

The following results will relate to the production of anthocyanin in seedlings exposed to three incubation periods as influenced by either pre-light temperatures, light temperatures, post-light temperatures, pre-light x light temperature interaction, light x post-light interaction, and pre-light x post-light interaction.

Effects of pre-light temperature

Temperature during the pre-light incubation period was influential in regulating the production of anthocyanin (figure 3). The trend as influenced by pre-light temperature seems to indicate that lower temperatures prior to light exposure are more stimulatory than higher temperatures. A pre-light temperature of 20C was the most effective, 10C was nearly as effective, and 30C was the least effective.

Effects of light temperature

Temperature during the light incubation period was highly influential in determining the production of anthocyanin. The trend, as illustrated in figure 4, shows that anthocyanin production increased as temperature increased. Pigment production was much higher in seedlings that were exposed to light at 30C than at 10C or 20C. Plants that were exposed to 20C light temperature produced a limited quantity of AC while those exposed to light at 10C were barely capable of producing the pigment.

Figure 3. Effects of pre-light temperature on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

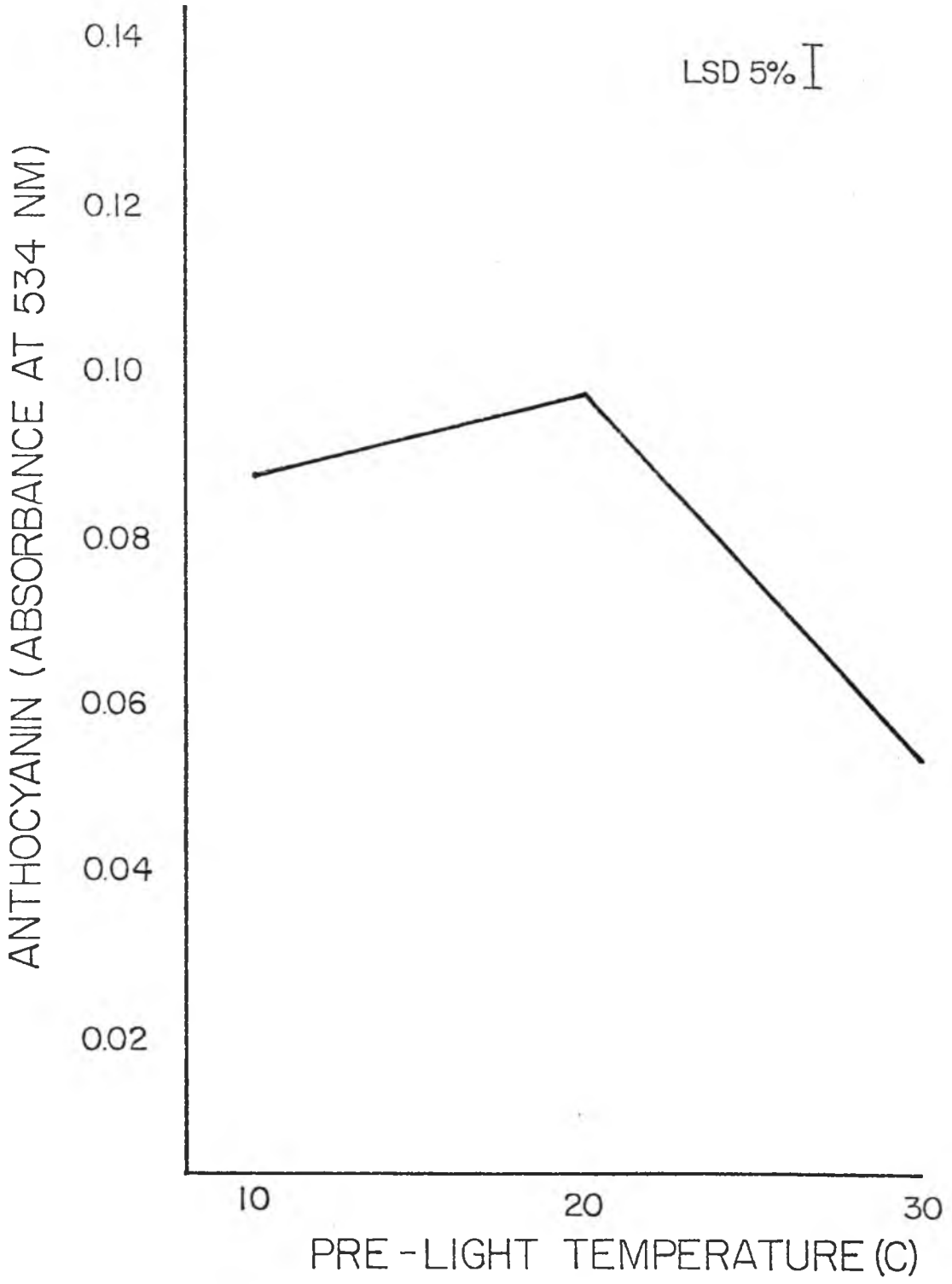
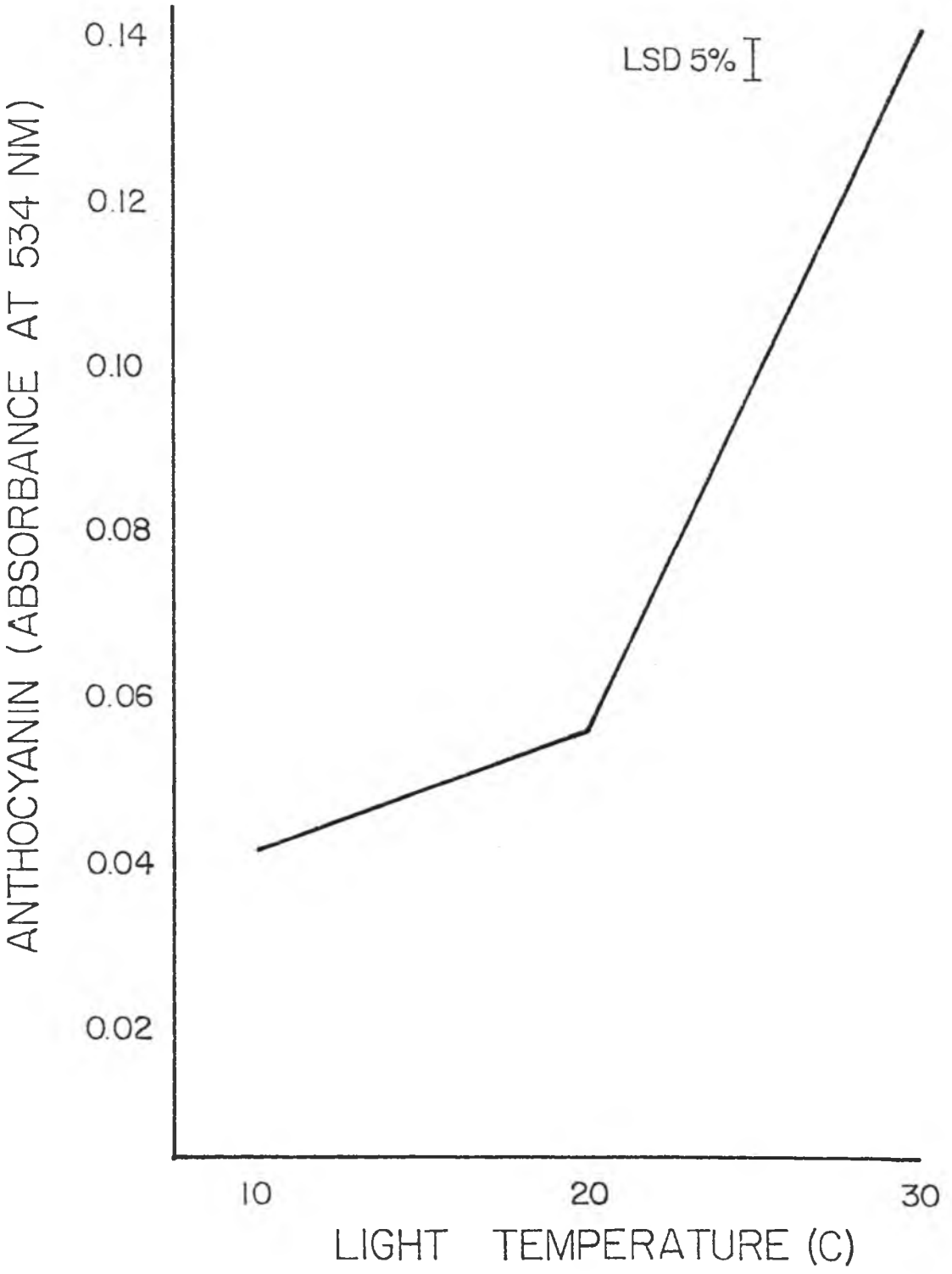

0.14
0.12
0.10
0.08
0.06
0.04
0.02
LSD 5%
ANTHOCYANIN (ABSORBANCE AT 534 NM)
10
20
30
PRE-LIGHT TEMPERATURE (C)

Figure 4. Effects of light temperature on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
LSD 5%
10
20
30
LIGHT TEMPERATURE (C)

Effects of post-light temperature

The results of this study are in agreement with those of Downs and Siegelman (1962). The trend of anthocyanin production as influenced by temperature during the post-light incubation period was non-linear (figure 5). Pigment production as a result of a 10C post-light temperature was minimal. A post-light temperature of 20C was the most effective, while 30C was intermediate.

Pre-light x light temperature interaction

Various combinations of pre-light and light temperatures were effective in influencing the production of anthocyanin (figure 6). The general trend for all combinations was an increase in anthocyanin formation with an increase in light temperature and a decrease in pigment formation with an increase in pre-light temperature. A 30C light temperature in combination with any pre-light temperature resulted in the production of higher levels of anthocyanin. Combinations that included pre-light temperatures of 10C were barely capable of producing the pigment. The most stimulatory combination included a 20C pre-light temperature and a 30C light temperature. The least stimulating combination included a 30C pre-light temperature and 10C light temperature.

Light x post-light temperature interaction

Anthocyanin production was affected by different light, post-light temperature combinations (figure 7). The trends were similar to those of the pre-light, light temperature combinations. The highest levels of anthocyanin were produced when a combination included a 30C light temperature. Lowest levels of the pigment were produced when a

Figure 5. Effects of post-light temperature on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

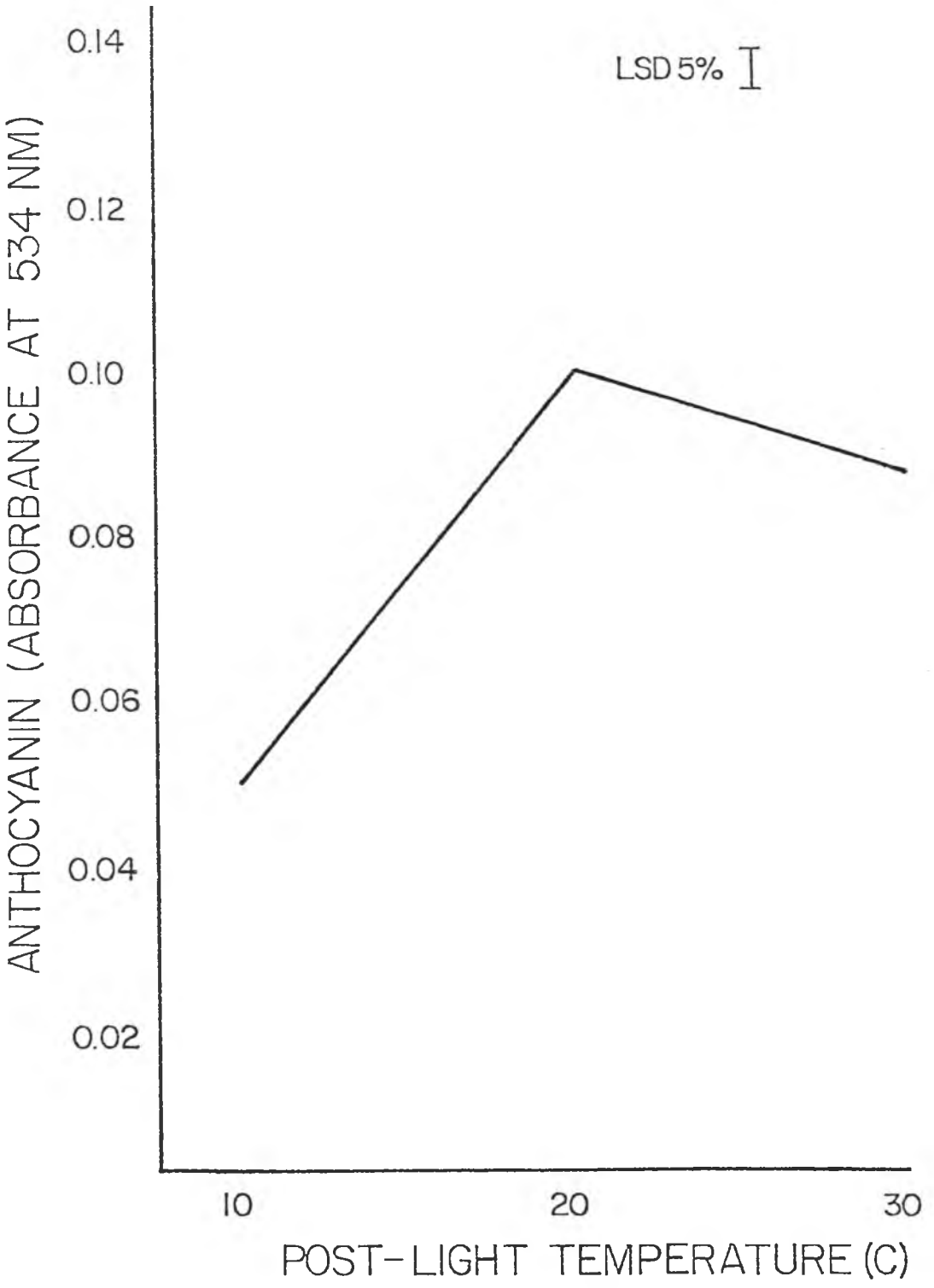

0.14
0.12
0.10
0.08
0.06
0.04
0.02
LSD 5%
ANTHOCYANIN (ABSORBANCE AT 534 NM)
10
20
30
POST-LIGHT TEMPERATURE (C)

Figure 6. Effects of pre-light, light temperature combinations on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

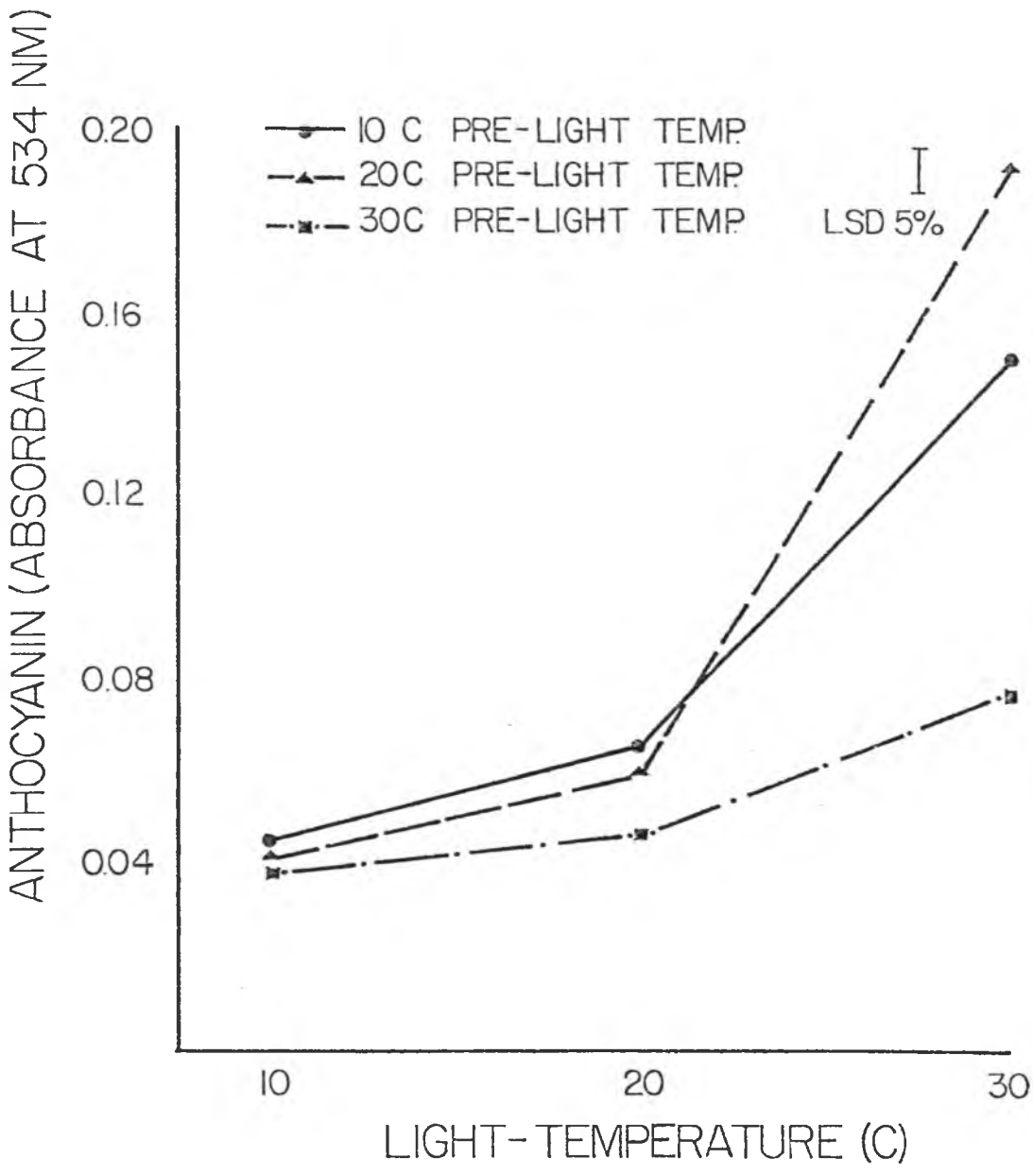
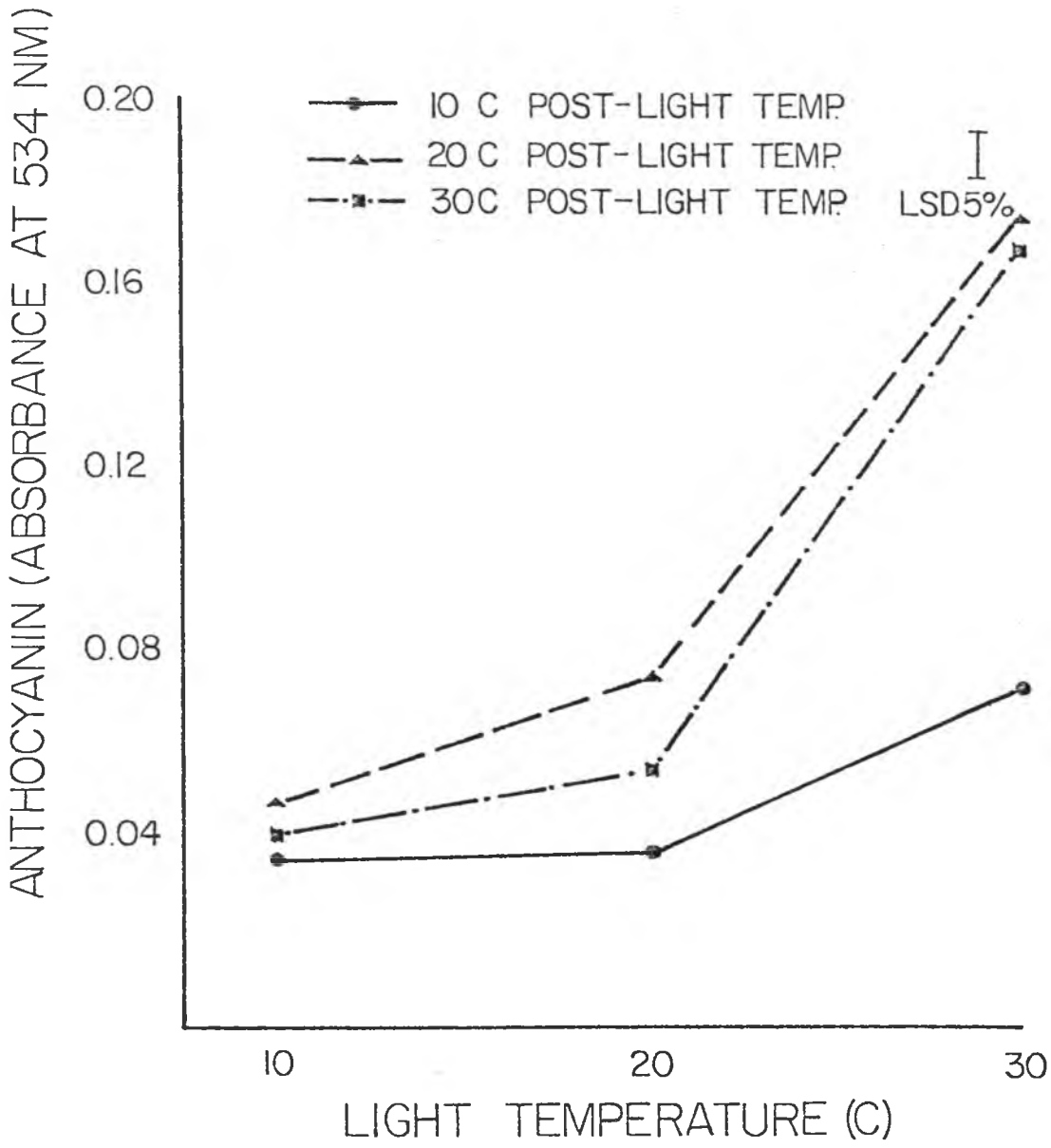

ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.20
0.16
0.12
0.08
0.04
10 C PRE-LIGHT TEMP.
20C PRE-LIGHT TEMP.
30C PRE-LIGHT TEMP.
LSD 5%
10
20
30
LIGHT-TEMPERATURE (C)

Figure 7. Effects of light, post-light temperature combinations on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.20
0.16
0.12
0.08
0.04
10 C POST-LIGHT TEMP.
20 C POST-LIGHT TEMP.
30C POST-LIGHT TEMP.
LSD5%
10
20
30
LIGHT TEMPERATURE (C)

combination included 10C light temperature. In general, anthocyanin production increased with an increase in light temperature for all light, post-light temperature combinations.

Pre-light x post-light temperature interactions

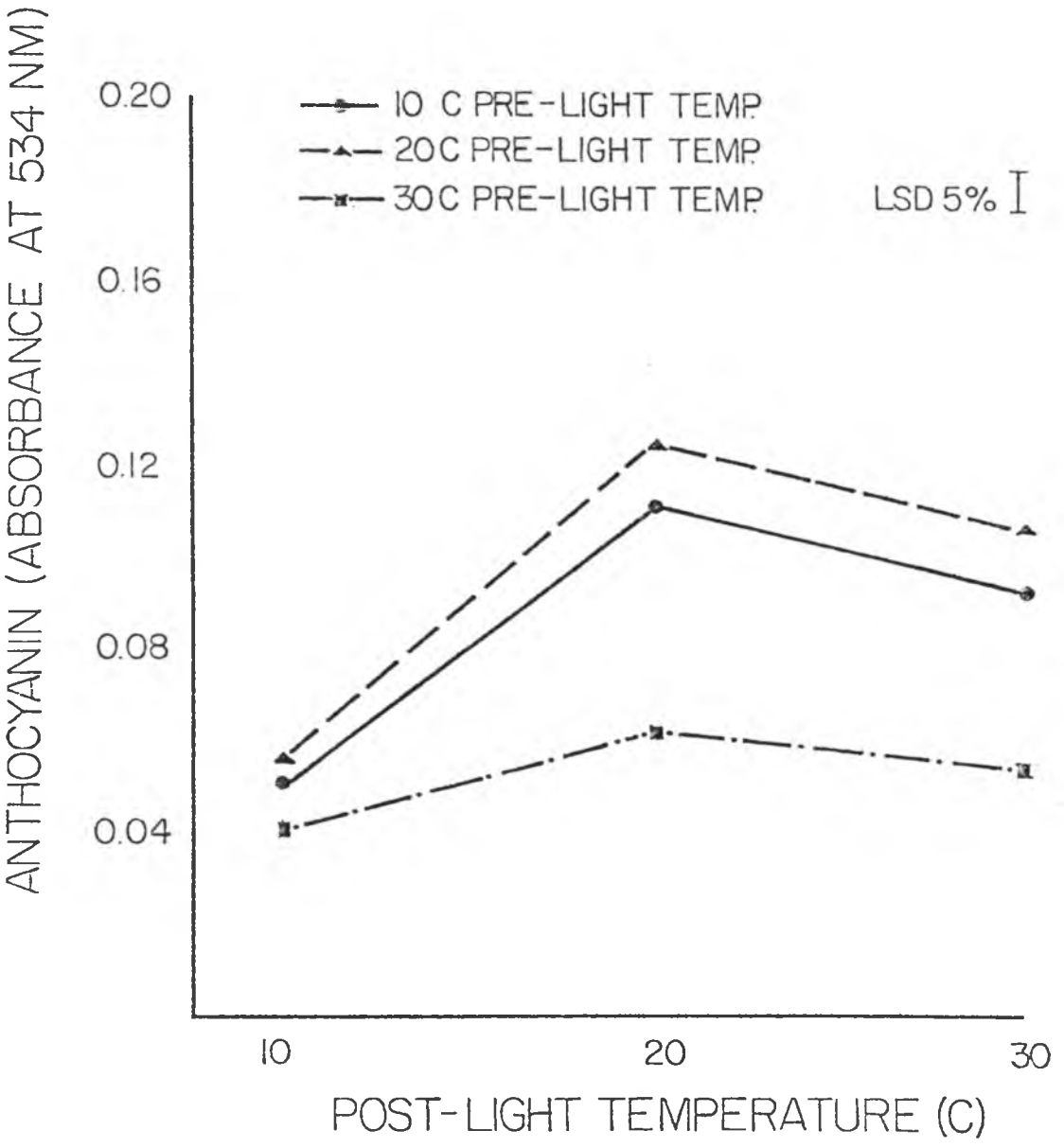
Various combinations of pre-light and post-light temperatures affected the production of anthocyanin (figure 8). The general trend was similar for all pre-light, post-light combinations. This was observed as a low production of anthocyanin in combinations with a 10C post-light temperature, higher production with a 30C post-light temperature, and maximum production with a 20C post-light temperature. A combination of 20C pre-light, 20C post-light temperature had the highest stimulatory effect while a combination of 30C pre-light, 10C post-light temperature had the least effect.

Effect of Sucrose in Pre-light, Light, and Post-light Temperature Combinations

Sucrose was not effective in stimulating anthocyanin production in the previous experiments. Neither of the experiments included the effects of a post-light incubation period which might be required for maximal anthocyanin production if a lag phase is present. Therefore, this investigation was conducted to determine the effects of sucrose in combination with different temperatures before, during, and after exposure to light.

The experiments were designed in the same manner as those in the previous section with the exception that 0.01M sucrose was added to the growing medium.

Figure 8. Effects of pre-light, post-light, temperature combinations on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.20
0.16
0.12
0.08
0.04
10 C PRE-LIGHT TEMP.
20C PRE-LIGHT TEMP.
30C PRE-LIGHT TEMP.
LSD 5%
10
20
30
POST-LIGHT TEMPERATURE (C)

The exogenous source of sucrose was effective in stimulating the production of anthocyanin in those treatments that included a pre-light temperature of 10C (figure 9). The effect of sucrose was negligible when the temperature during the pre-light period was 20C or 30C. Sucrose had no effect on anthocyanin formation during the light-temperature or post-light temperature incubation periods.

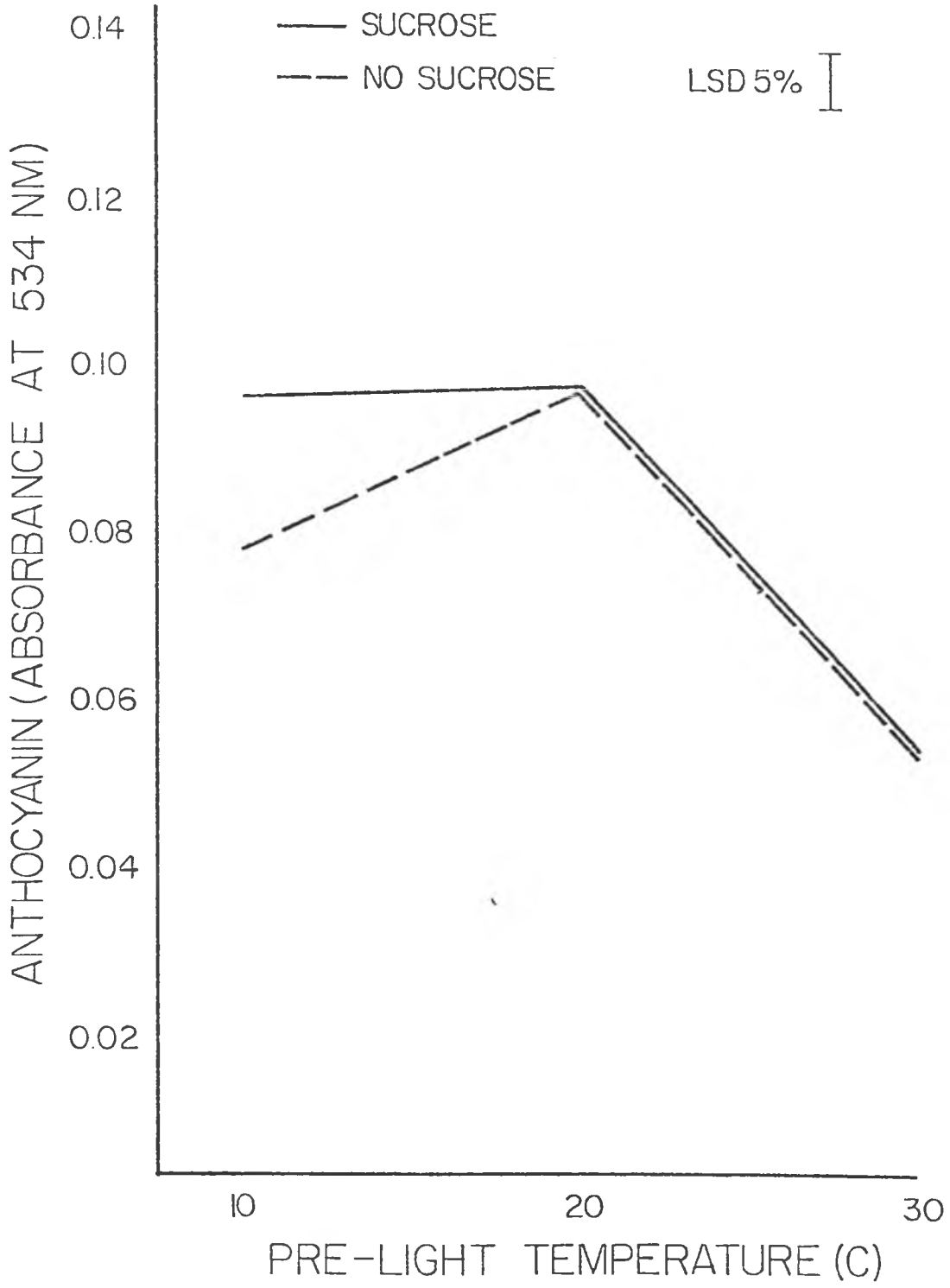
Effects of Plant Age

Anthocyanin production is probably dependent on an endogenous supply of carbohydrates, therefore factors which influence this supply could also affect the formation of anthocyanin. The endogenous levels of carbohydrates in the sorghum seedling would probably be dependent on: 1) respiration rate, 2) growth rate, 3) its ability to produce carbohydrates to replace whatever was utilized.

The respiration and growth rate of plants can be regulated by temperature. Low temperatures usually result in low respiration and growth rates. High temperatures usually have the opposite effect. The ability of a plant to produce carbohydrates is dependent on the availability of substrates in the growing medium and light. Creasy (1968) found that photosynthesis was of extreme importance in controlling the synthesis of flavonoids in strawberry leaf disks probably because it affected the levels of endogenous carbohydrates.

This study was conducted to evaluate the possible relationship of plant age before exposure to light and anthocyanin production. It was assumed that plant age could influence the levels of endogenous carbohydrates due to differences in total respiration times. An older plant

Figure 9. Effects of sucrose on anthocyanin production. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


SUCROSE
NO SUCROSE
LSD 5%
0.14
0.12
0.10
0.08
0.06
0.04
0.02
ANTHOCYANIN (ABSORBANCE AT 534 NM)
10
20
30
PRE-LIGHT TEMPERATURE (C)

would have respired for a longer period of time than a younger plant and therefore result in less available endogenous carbohydrates for anthocyanin production. Due to the conditions of this study, the plants would be incapable of producing carbohydrates to replace what was lost through respiration.

Sorghum seedlings were incubated in the absence of light for periods varying from 3.5 to 7.5 days at 20C. They were then transferred to a growth chamber and exposed to light at 20C for 4 hrs (light temperature) and followed by another incubation period in the absence of light at 20C for 24 hrs (post-light temperature). Finally, the plants were analyzed for anthocyanin content.

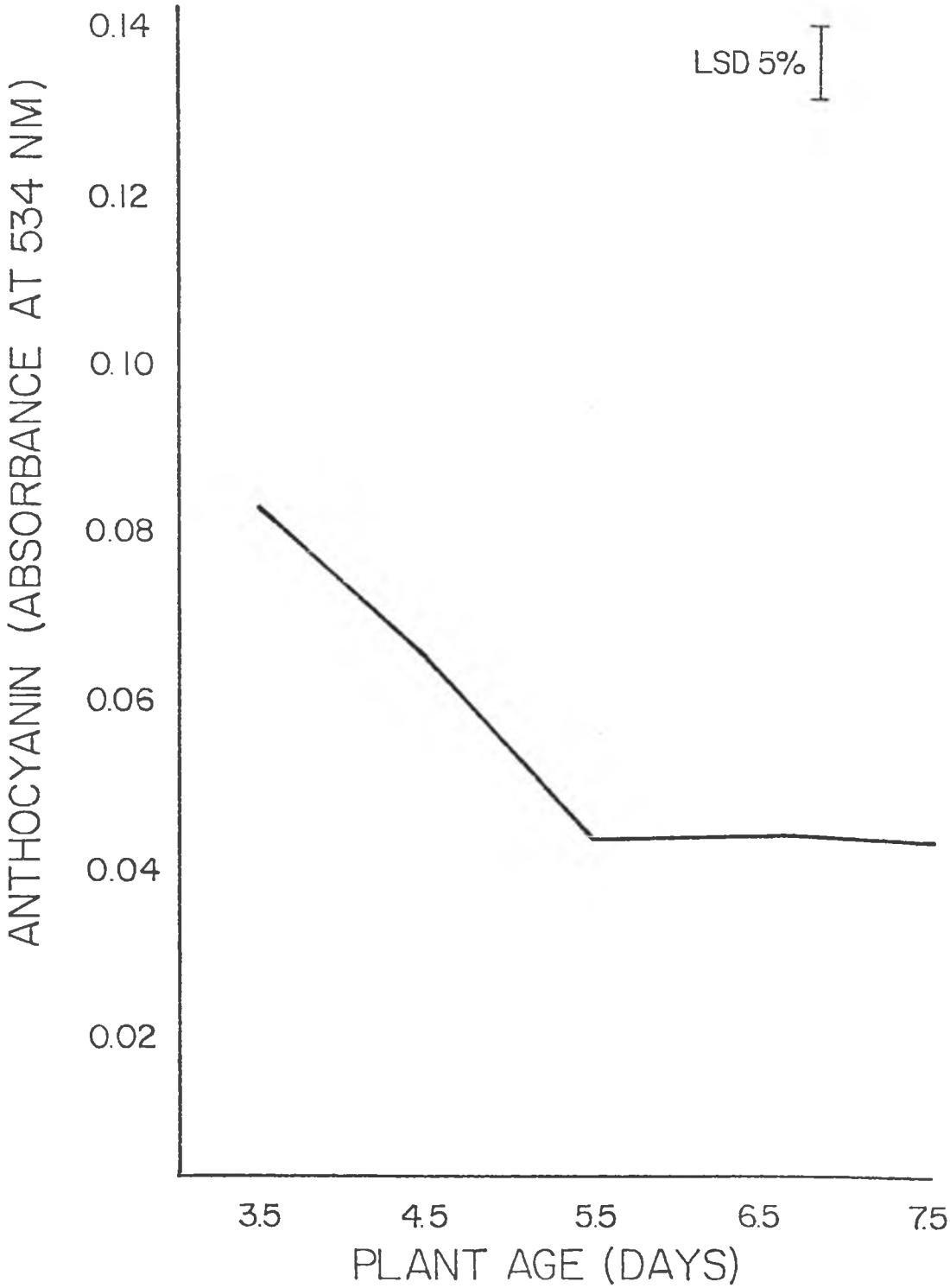
The seedling age prior to light exposure influenced the production of anthocyanin (figure 10). Highest levels of the pigment were found in seedlings that were 3.5 days old and the lowest levels in those that were 7.5 days old. There was a decrease in pigment formation as plant age increased from 3.5 to 5.5 days. There was no change in pigment formation from 5.5 to 7.5 days.

Effects of Sucrose in Plant Age Treatments

The results of the experiments in the previous section showed that plant age before exposure to light did have an effect on AC formation. This might indicate a relationship between endogenous substrate levels and plant age. To further investigate this possibility, experiments were conducted to determine the effects of an exogenous source of carbohydrate.

The experimental procedure was the same as that in the previous experiment with the exception that 0.01M sucrose was added to the

Figure 10. Effects of plant age prior to light exposure on anthocyanin production. Values for each time period are means for combinations of pre-light, light, and post-light temperatures of 20C.


ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
LSD 5%
3.5
4.5
5.5
6.5
7.5
PLANT AGE (DAYS)

growing medium. The results of this study indicates that sucrose had no effect on anthocyanin production for any of the treatments (table 2).

Discussion

Downs (1964) has shown that light is required for antohcyanin formation in milo seedlings, but another study indicated that temperature is also important (Downs and Siegelman, 1963). These works indicate that at least two environmental factors, light and temperature, are capable of regulating anthocyanin synthesis in milo seedlings. The results of this study further demonstrate the importance of these two factors.

Sorghum seedlings were incapable of producing anthocyanin in the absence of light even when exposed to different temperatures and sucrose. Light apparently was a more dominant controlling factor than temperature or carbohydrates under the conditions of this study.

Temperature did influence anthocyanin production during the light incubation period of the pre-light, light temperature combinations (figure 2). This is indicative of the importance of light for anthocyanin formation. According to Smith (1975), "light might act to selectively switch-on the transcription of the genes coding for the limiting enzymes in synthesis." If this is true, temperature during the light period might be influential in regulating the activity of these enzymes that are required for anthocyanin synthesis. Anthocyanin formation increased with an increase in temperature during the light period. This might be due to increased enzyme activity at higher temperatures which is in accordance with van't Hoffs' Law (Haas and Hill, 1928). Duke

Table 2. Effect of sucrose on anthocyanin formation in sorghum seedlings at different plant ages

Plant age (days)	- Sucrose Anthocyanin production (O.D. at 534 nm)	+ Sucrose Anthocyanin production (O.D. at 534 nm)	Stimulation in Anthocyanin production due to sucrose
3.5	0.092	0.076	-0.016
4.5	0.064	0.067	+0.003
5.5	0.043	0.045	+0.002
6.5	0.042	0.048	+0.005
7.5	0.040	0.047	+0.007

et al. (1976) encountered similar results with Zea seedlings. They observed a six to thirtyfold increase in anthocyanin formation at 25C in light as compared to 16C. It was implied that substrate availability might have accounted for the difference.

Sucrose had no effect on anthocyanin formation in the pre-light, light temperature combinations. This might have been a result of: 1) an inadequate period of time (4 hrs during the light incubation period) for the conversion of the exogenous sucrose to substrates required for anthocyanin formation; 2) an inability of the sorghum seedlings to effectively translocate the sucrose from the medium in sufficient quantities to stimulate anthocyanin formation; or 3) the incorporation of the additional substrate into some other products such as coumarins, phenols, flavonoids, or lignins (Smith, 1975).

Anthocyanin production was affected by temperature in those combinations that included three incubation periods (pre-light, light, and post-light temperature). Lower temperatures (10C-20C) during the pre-light incubation period were more effective in stimulating anthocyanin formation than 30C (figure 3). This might be a result of lower respiration rates and therefore decreased utilization of substrates. If this is true, more substrates would be available for anthocyanin synthesis during the light and post-light incubation periods. Higher temperatures during the pre-light incubation period would be expected to have had the opposite effect.

Temperature during the light incubation period did affect anthocyanin production (figure 4). The trend showed a strong increase in anthocyanin production at 30C in comparison to 10C and 20C. Figure 6

shows that high anthocyanin production was evident with a 30C light temperature only if the temperature in the pre-light incubation period was 10C or 20C. Anthocyanin production was very low in those treatments that included a 30C pre-light temperature followed by a 30C light temperature. It is possible that the lower pre-light temperature made available more substrates for anthocyanin synthesis during the light and post-light incubation periods. Furthermore, the higher temperature (30C) during the light incubation period might have been conducive toward higher activity of the anthocyanin synthesizing enzymes.

Figure 5 shows that temperature during the post-light incubation period of the pre-light, light, post-light temperature combination did influence anthocyanin production. According to Craker et al. (1971), light-stimulated synthesis of anthocyanin in sorghum seedlings showed an initial lag phase of 8 hrs after the dark grown seedlings were placed in the light. Based on this information, it seems that the seedlings should be exposed to a minimum of 8 hrs of ideal conditions for maximal anthocyanin production. These conditions could include light and temperature. If anthocyanin production is dependent on a lag phase of approximately 8 hrs, it might be assumed that most of the anthocyanin synthesis occurred during the post-light incubation period primarily because the light incubation period was only 4 hrs. Figure 2 seems to reinforce this idea, it shows that very little anthocyanin was produced during the light incubation period. Therefore, it is possible that low levels of anthocyanin were produced with a 10C post-light temperature due to lower levels of enzyme activity and/or synthesis. The higher post-light temperatures were probably adequate for anthocyanin producing enzyme activity and/or synthesis.

Figure 6 illustrates the interaction between pre-light and light temperatures of the pre-light, light, post-light temperature combinations. It seems that lower pre-light temperatures followed by higher light temperatures provide for maximal anthocyanin formation. The lower pre-light temperature might act to conserve substrates due to decreased respiration. This would make more substrates available once anthocyanin synthesis has been induced by light. The higher light temperatures could then facilitate increased enzyme activity and/or synthesis and therefore result in higher production of anthocyanin.

The importance of a high temperature during the light incubation treatments is further exemplified by figure 7. The optimum combination included a 30C light, 20C post-light temperature. A 30C light, 30C post-light temperature was nearly as effective. This illustrates the possible requirement of a high light temperature to initiate the production of anthocyanin. The high post-light temperature might serve to provide for the continued activity of the anthocyanin synthesizing enzymes. A 10C light, 10C post-light combination was ineffective probably because the temperature was too low for optimal enzyme activity.

Anthocyanin production was affected by various pre-light, post-light temperature combinations (figure 8). The general trend indicates that low pre-light, high post-light temperature combinations were effective in stimulating anthocyanin formation. The low pre-light temperature probably functioned to conserve the endogenous substrate supply due to decreased respiration rates and allowed for greater substrate availability during and after the induction of anthocyanin

synthesis. The high post-light temperature probably enabled the anthocyanin synthesizing enzymes to utilize the substrates that were available due to the substrate conservation effect of the pre-light temperature.

The data as expressed in figure 9 seem to be in conflict with the previously discussed sections. The greatest stimulation in anthocyanin production by sucrose was evident in those treatments that included a pre-light temperature of 10C. It was assumed that anthocyanin production would probably be stimulated by the addition of sucrose at the higher pre-light temperatures due to a lower supply of endogenous substrates. This was not the case, possibly because the exogenous supply of sucrose was insufficient to replace what might have been lost through respiration at the higher temperatures. Sucrose probably served to supplement the endogenous carbohydrate supply at the lower temperatures, and therefore resulted in higher anthocyanin production. The concentration of sucrose was not increased to test this hypothesis because pilot tests indicated that concentrations higher than 0.01M sucrose greatly inhibited plant growth. These results are in partial agreement with Stafford (1967) who found that sucrose did have an inhibitory effect on sorghum root growth. The reduction of root growth would probably result in an overall reduction in plant growth.

Plant age prior to light exposure definitely was important in relation to anthocyanin synthesis (figure 10). Since the sorghum seedlings were grown in an etiolated manner for a specified period of time before exposure to light, it was not possible for the seedlings to produce substrates (e.g. carbohydrates) to replace what was utilized by

respiration. Therefore, it is reasonable to assume that as the length of time prior to light exposure increased, the endogenous substrate level decreased and subsequently less anthocyanin was synthesized. This is in agreement with Duke et al. (1976) who worked with etiolated Zea seedlings. They found that anthocyanin production decreased as plant age before light exposure increased. They postulated that the decrease was due to a decline in potential for photoinduction of anthocyanin biosynthesis. It is also possible that an increase in plant age resulted in an increase in anthocyanin inhibitors. Finally, there might have been a deactivation or degradation of anthocyanin synthesizing enzymes (e.g. Phenylalanine ammonia-lyase).

Table 2 shows that sucrose had no effect on anthocyanin production at any plant age. As discussed earlier, the concentration of sucrose might have been too low to enhance anthocyanin formation, sucrose was not effectively translocated into the plant, or sucrose was utilized for the production of other compounds.

CHAPTER IV

DETERMINATION OF PHENYLALANINE AMMONIA-LYASE ACTIVITY OF ETIOLATED SORGHUM SEEDLINGS AFTER EXPOSURE TO VARIOUS TEMPERATURES AND LIGHT TREATMENTS

The effect of light on Phenylalanine ammonia-lyase (PAL) activity and/or synthesis has been studied by many people (Bellini and Poucke, 1970; Bellini and Hillman, 1971; Schopfer and Mohr, 1972; McClure, 1974; Smith and Attridge, 1970; and Weidner et al., 1969). The general conclusion seems to be that light does positively influence the synthesis of PAL. This increase can be observed even at temperatures as low as 8C (Engelsma, 1970).

Engelsma (1968) and Engelsma (1970) reported that PAL activity was higher at lower temperatures (10C) and decreased as temperature increased. It was postulated that the production of a PAL deactivating enzyme system is limited at the lower temperatures whereas PAL synthesis is not.

Creasy (1968) showed that there might be a relationship between PAL activity and anthocyanin synthesis. As PAL activity increased, anthocyanin content also increased. This relationship might have been anticipated based on the premise that PAL is an enzyme which is required for the production of flavonoids. However, according to their studies, Bellini and Poucke (1970) stated that light-induced PAL activity could not be the rate-limiting link in anthocyanin biosynthesis. They postulated that most or all of the enzyme activity is probably used for lignification of the vascular tissue.

Studies pertaining to possible relationships between anthocyanin production and PAL activity as influenced by different temperatures

seem to be lacking. Therefore an attempt was made in this study to follow PAL activity in sorghum plant tissue based on the same experimental designs previously used for anthocyanin studies in Chapter III. This would facilitate a comparison of anthocyanin formation and PAL activity.

Materials and Methods

The materials and methods for studying the effects of pre-light temperature treatments, effects of pre-light and light temperature treatments, effects of pre-light, light and post-light temperature treatments, effects of sucrose, and effects of plant age were the same as in the study of anthocyanin (Chapter III).

Phenylalanine Ammonia-lyase Assay

The following materials and methods were primarily the same as that developed by Saunders and McClure (1974). Flasks with the treated seedlings were transferred to a darkroom equipped with a 15 watt fluorescent lamp covered with two sheets of cellophane. Four seedlings were randomly selected from each flask. First internodal sections 4 centimeters long beginning from the base of the coleoptile were macerated with a mortar and pestle in cold 0.025M sodium borate:1.5N HCl (pH 8.8) plus 5 mM mercaptoethanol to make a total volume of 6 ml crude extract. The crude extract was filtered through Whatman no. 1 filter paper into small test tubes which were immediately covered with parafilm and placed in a refrigerator (1C) until ready to be analyzed.

The reaction mixture contained 1.2 ml sodium borate buffer solution (pH 8.8), 1 ml L-Phenylalanine and 0.8 ml of crude extract. The

reference contained 2.2 ml sodium borate buffer solution (pH 8.8) and 0.8 ml crude extract. The cuvettes containing the reaction mixture and reference were placed in a water bath with a temperature of 40C ± 1C. The reaction mixture and reference were incubated for 15 minutes at 40C before measurements at 290 mμ. Readings were taken at 10 minute or 15 minute intervals and returned to the 40C water bath between readings.

Analysis of Data

The same as in the study on anthocyanin (Chapter III).

Results

Effects of Pre-light Temperature Treatments

This study was conducted to determine the effects of temperature on PAL activity in the absence of light. The materials and methods were the same as those used in Chapter III with the exception that the seedlings were analyzed for PAL activity.

The results of the experiments indicate that PAL activity in sorghum seedlings was not affected by different temperatures during a dark incubation period (table 3).

Effects of Sucrose in Pre-light Temperature Treatments

In this study, 0.01M sucrose was added to the growing medium to determine the effects of an exogenous supply of carbohydrates on PAL activity. The materials and methods were the same as those used in Chapter III with the exception that the seedlings were analyzed for PAL activity.

Table 3. Effect of sucrose on PAL activity in sorghum seedlings during pre-light temperature treatments

Pre-light temperature (C)	- Sucrose PAL activity nmoles cinnamic acid/ 3 first internodes/min.	+ Sucrose PAL activity nmoles cinnamic acid/ 3 first internodes/min.	Stimulation of PAL activity due to sucrose
10	1.16	1.68	+0.52
20	1.37	1.62	+0.25
30	1.34	1.62	+0.28

LSD 5% = 0.036

Sucrose was effective in stimulating PAL activity at all pre-light temperatures (table 3). The greatest stimulation was observed in the 10C pre-light temperature treatment. The degree of stimulation during the 20C and 30C pre-light temperature treatments were equal.

Pre-light, Light Temperature Combinations

In order to determine the effect of temperature and light on PAL activity the following experiments were conducted. This entailed the same materials and methods as the study conducted in Chapter III with the exception that the sorghum seedlings were analyzed for PAL activity.

The following results will relate to the production of anthocyanin as influenced by temperature during the pre-light and light incubation periods.

Effects of pre-light temperature

PAL activity was influenced by temperatures during pre-light incubation periods (figure 11). The general trend showed an increase in PAL activity with an increase in pre-light temperature. A pre-light temperature of 30C was the most effective in enhancing enzyme activity while 20C was intermediate and 10C was the least stimulatory.

Effects of light temperature

Figure 12 shows that the trend of PAL activity as related to light temperature treatments was linear. PAL activity increased as temperature during the light period increased. A light temperature of 10C was the least effective in stimulating PAL activity, 20C was intermediate, and 30C was the most effective.

Figure 11. Effects of pre-light temperature on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light and light temperatures.

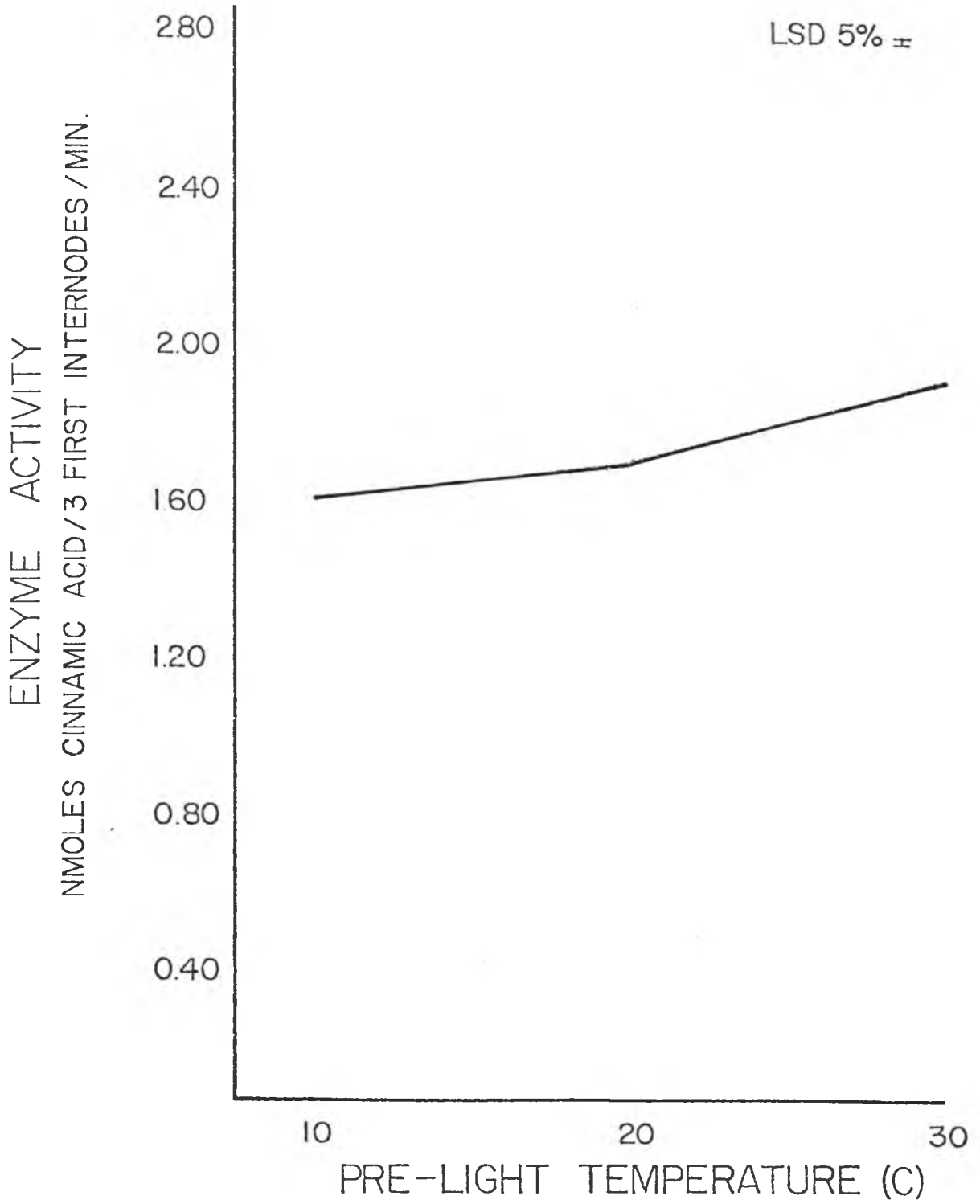
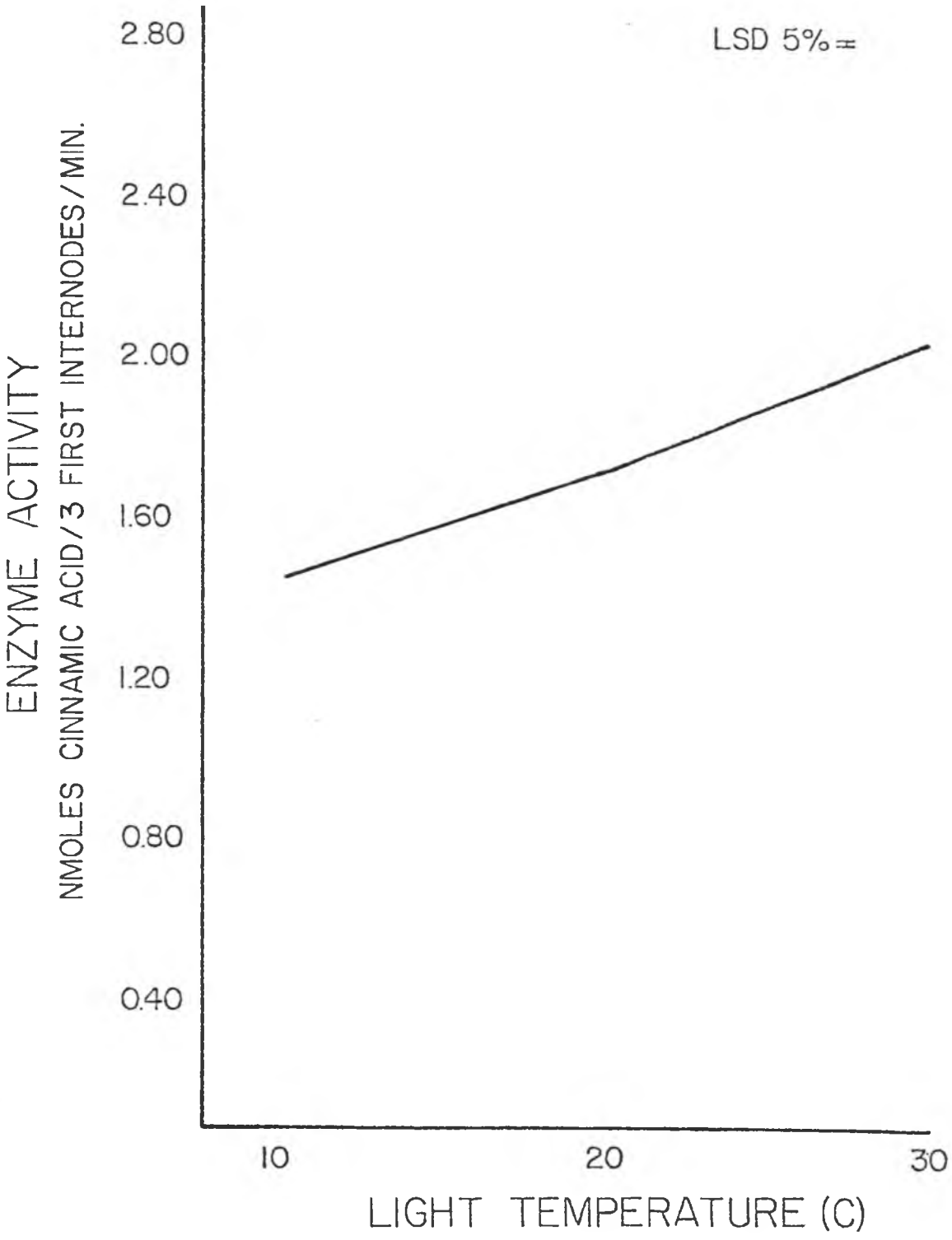

ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
LSD 5% H
10
20
30
PRE-LIGHT TEMPERATURE (C)

Figure 12. Effects of light temperature on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light and light temperatures.


ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
LSD 5% =
10
20
30
LIGHT TEMPERATURE (C)

Effects of Sucrose in Pre-light, Light Temperature Combinations

To determine the effects of an exogenous supply of sucrose on PAL activity during the pre-light, light-temperature treatments, 0.01M sucrose was added to the growing medium. The materials and methods were the same as those in Chapter III with the exception that the seedlings were analyzed for PAL activity.

Sucrose was effective in stimulating PAL activity for all temperature treatments (table 4). The greatest stimulation was observed in those treatments involving a temperature of 10C.

Pre-light, Light, and Post-light Temperature Combinations

The following study was performed to determine the effects of three incubation periods (pre-light, light, and post-light) on the activity of PAL. The materials and methods were the same as those in Chapter III with the exception that the seedlings were analyzed for PAL activity.

The following results will relate to PAL activity in sorghum seedlings exposed to three incubation periods as influenced by either pre-light temperatures, light temperatures, post-light temperatures, and light x post-light interaction.

Effects of pre-light temperature

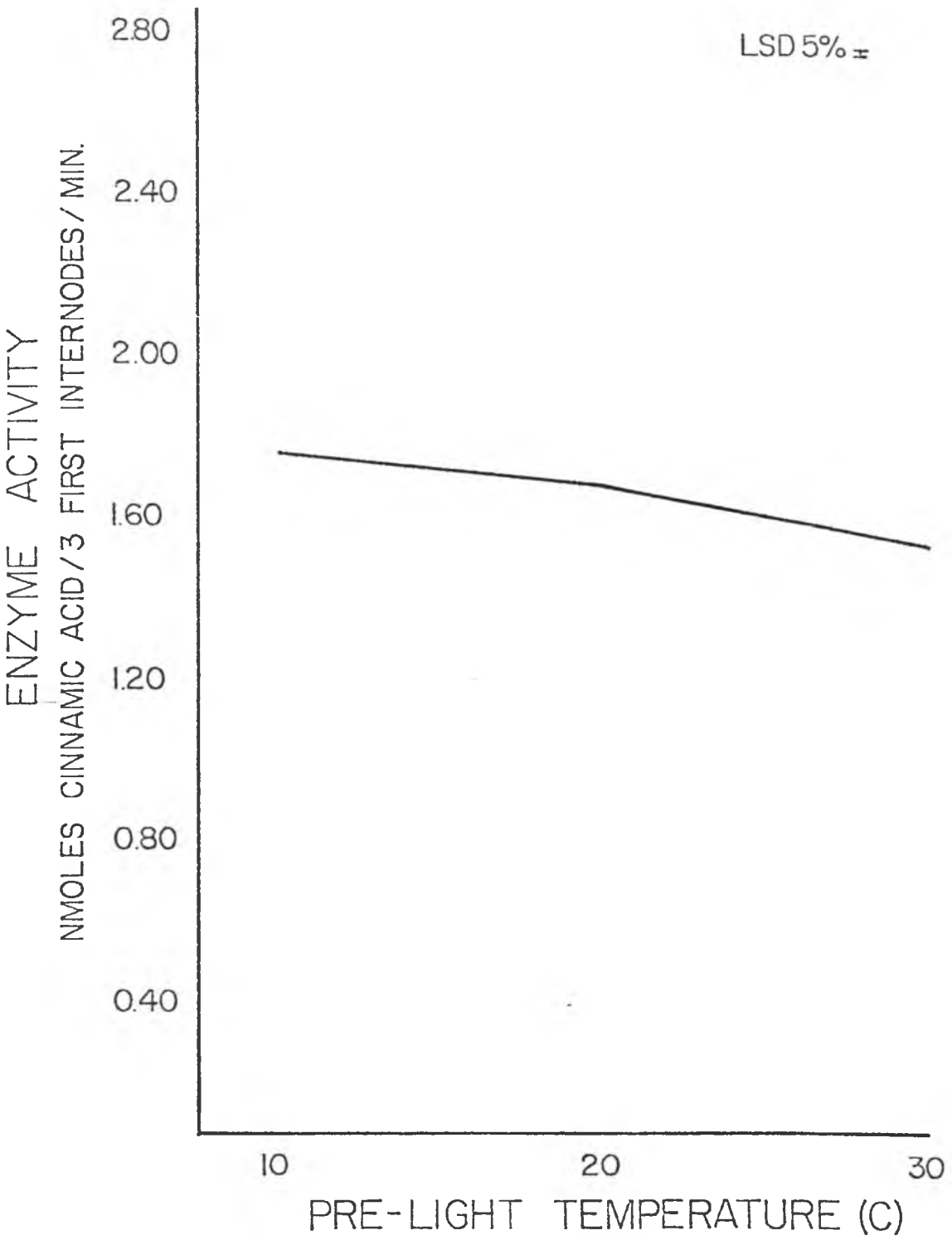
The general trend of PAL activity as influenced by different pre-light temperatures is shown in figure 13. A pre-light temperature of 10C was the most stimulatory while 20C was intermediate and 30C the least stimulatory.

Table 4. Effect of sucrose on PAL activity in sorghum seedlings during pre-light, light temperature treatments

Temperatures (C)		- Sucrose PAL activity nmoles cinnamic acid/ 3 first internodes/min.	+ Sucrose PAL activity nmoles cinnamic acid/ 3 first internodes/min.	Stimulation of PAL activity due to sucrose
Pre-light	Light			
10	20	1.41	2.06	+0.65
30	10	1.41	1.96	+0.55
10	10	1.13	1.51	+0.38
10	30	1.62	1.96	+0.34
20	10	1.31	1.65	+0.34
30	20	1.72	1.96	+0.24
20	20	1.47	1.65	+0.18
30	30	2.16	2.27	+0.11
20	30	2.09	2.09	0.0

LSD 5% = 0.029

Figure 13. Effects of pre-light temperature on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
LSD 5% =
10
20
30
PRE-LIGHT TEMPERATURE (C)

Effects of light temperature

Figure 14 illustrates the effects of temperature on PAL activity in sorghum seedlings as a result of exposure to a light incubation period. Temperatures of 20C and 10C were more effective in stimulating PAL activity than 30C. This seems to express a similar trend in PAL activity as the pre-light temperature data.

Effects of post-light temperatures

Temperature was highly influential in determining PAL activity during the post-light incubation periods (figure 15). Temperatures of 10C and 20C were equally effective in stimulating PAL activity. A post-light temperature of 30C seemed to be inhibitory, accounting for an equivalent of approximately one-half the enzyme activity in comparison to the other treatments.

Light x post-light temperature interactions

Light temperature incubation periods followed by 20C post-light temperatures seemed to be ideal for PAL activity (figure 16). A post-light temperature of 10C was nearly as effective and 30C seemed to be inhibitory.

The most stimulatory light, post-light combinations were 10C light, 20C post-light; 20C light, 20C post-light; and 30C light, 10C post-light. The least stimulatory were all light temperatures in combination with 30C post-light temperature.

Effects of Sucrose in Pre-light, Light and Post-light Temperature Combinations

The effect of an exogenous source of sucrose on PAL activity was investigated in this study. The materials and methods were the same as

Figure 14. Effects of light temperature on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

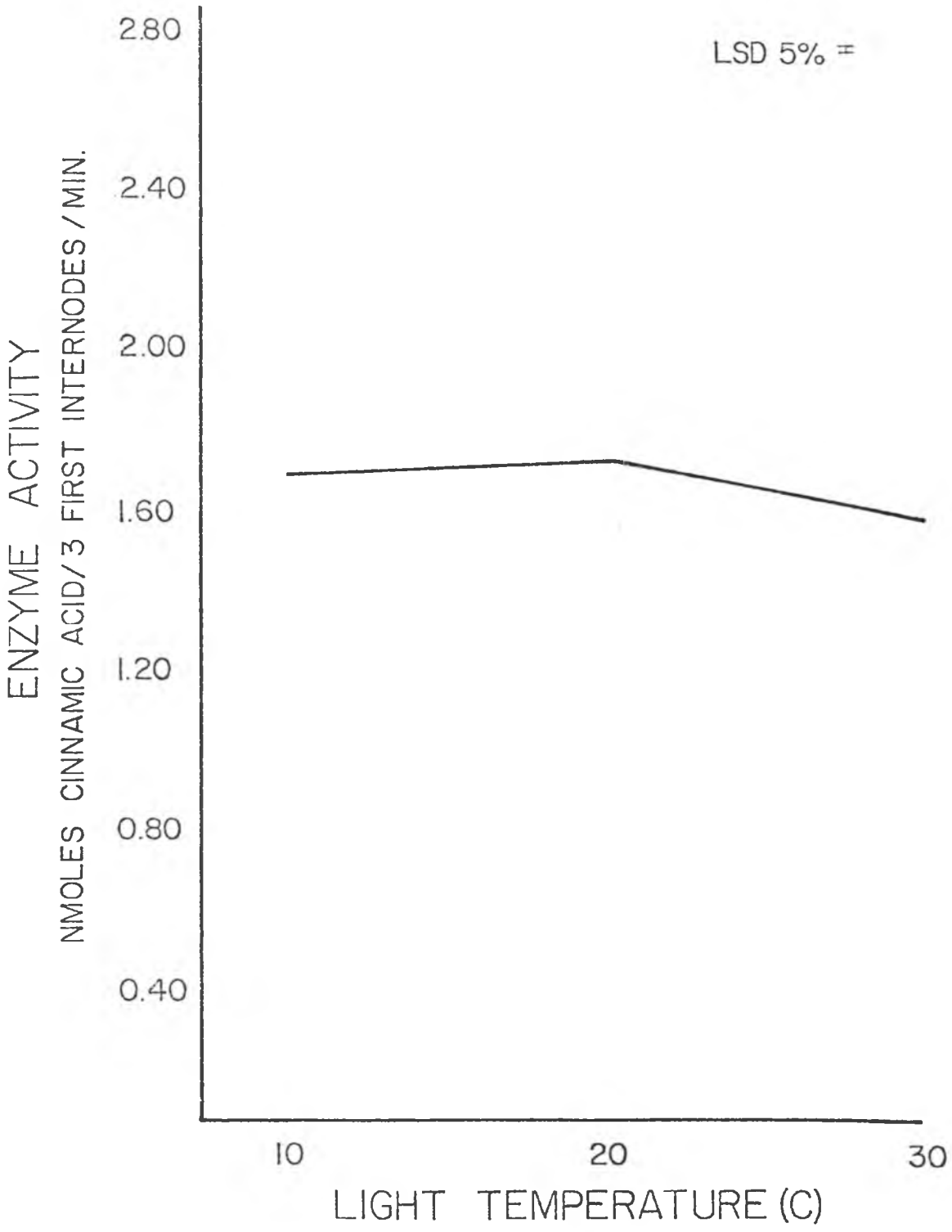

LSD 5% =
2.80
2.40
2.00
1.60
1.20
0.80
0.40
ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
10
20
30
LIGHT TEMPERATURE (C)

Figure 15. Effects of post-light temperature on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

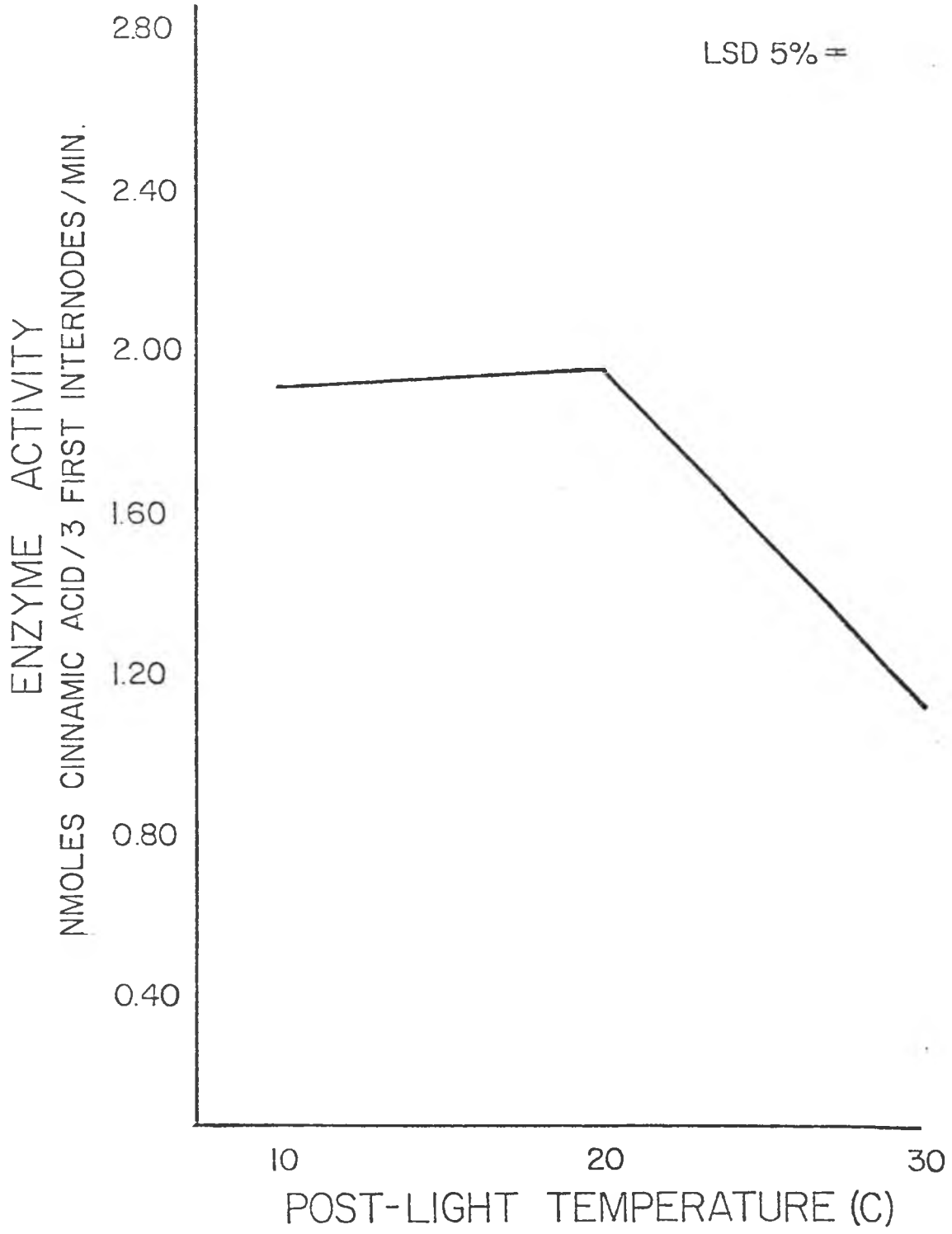
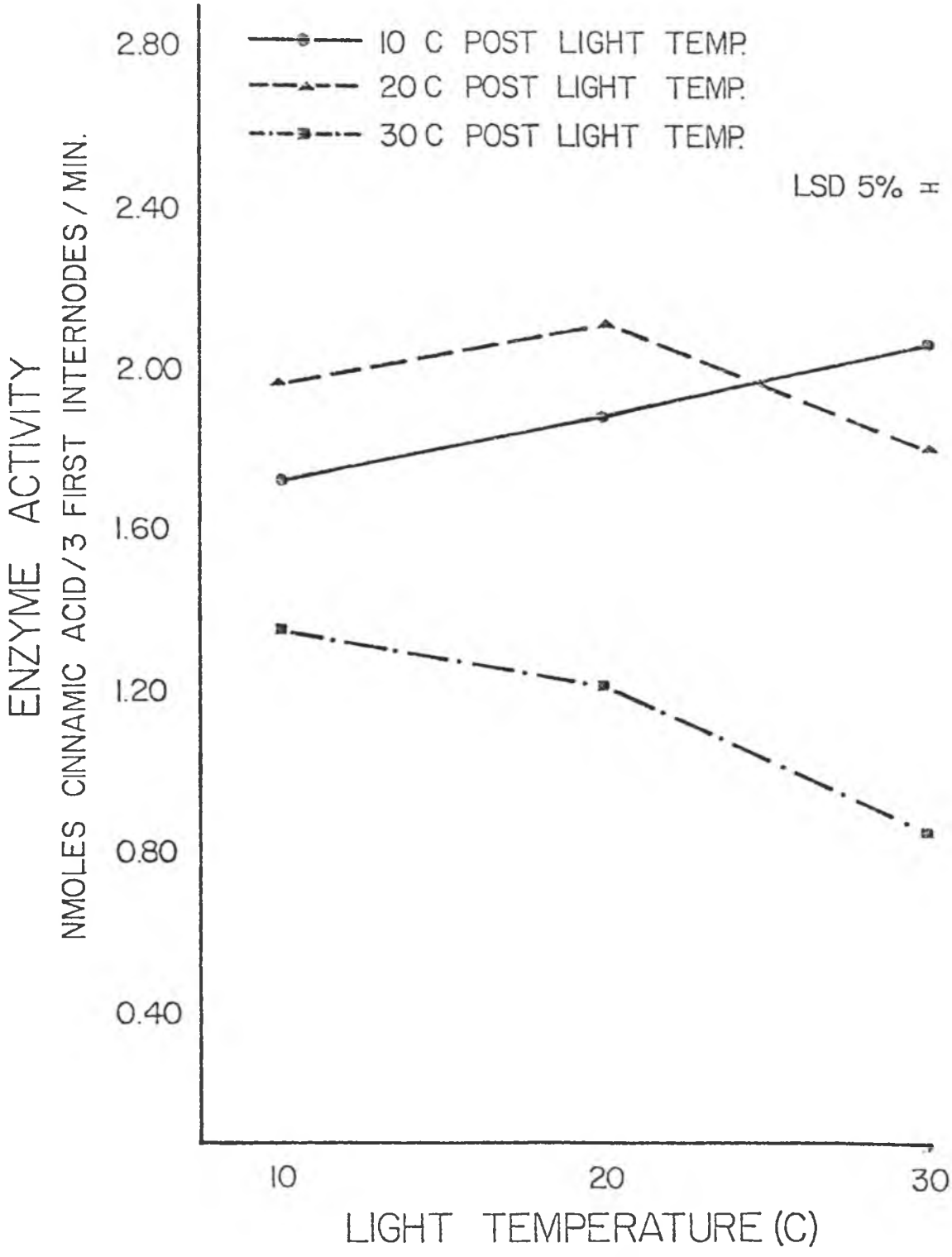

LSD 5% =
ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
10
20
30
POST-LIGHT TEMPERATURE (C)

Figure 16. Effects of light, post-light temperature combinations on Phenylalanine ammonia-lyase activity. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.


10 C POST LIGHT TEMP.
20 C POST LIGHT TEMP.
30 C POST LIGHT TEMP.
LSD 5% =
ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES / MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
10
20
30
LIGHT TEMPERATURE (C)

those in Chapter III with the exception that the sorghum seedlings were analyzed for PAL activity.

The following results will relate to the activity of PAL as influenced by sucrose in different incubation periods.

Pre-light x sucrose interaction

Sucrose was effective in stimulating PAL activity during the pre-light incubation periods. The greatest stimulation was observed with a pre-light temperature of 10C, a lesser stimulation at 20C and the least at 30C (figure 17).

Light x sucrose interaction

PAL activity was stimulated by sucrose during the light incubation periods. The trend was similar to the pre-light x sucrose interaction. Highest stimulation of enzyme activity was apparent at 10C, a lesser stimulation at 20C and the least at 30C (figure 18).

Post-light x sucrose interaction

The effects of sucrose varied during the post-light incubation periods (figure 19). High enzyme activity stimulation was attributed to sucrose at 10C whereas the effect was much lower at 30C. Enzyme activity stimulation at 20C was slightly less than at 10C.

Effects of Plant Age

PAL activity as influenced by plant age was investigated in this study. The materials and methods were the same as those used in Chapter III with the exception that the seedlings were analyzed for PAL activity.

Figure 17. Effects of sucrose on Phenylalanine ammonia-lyase activity during the pre-light incubation period. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

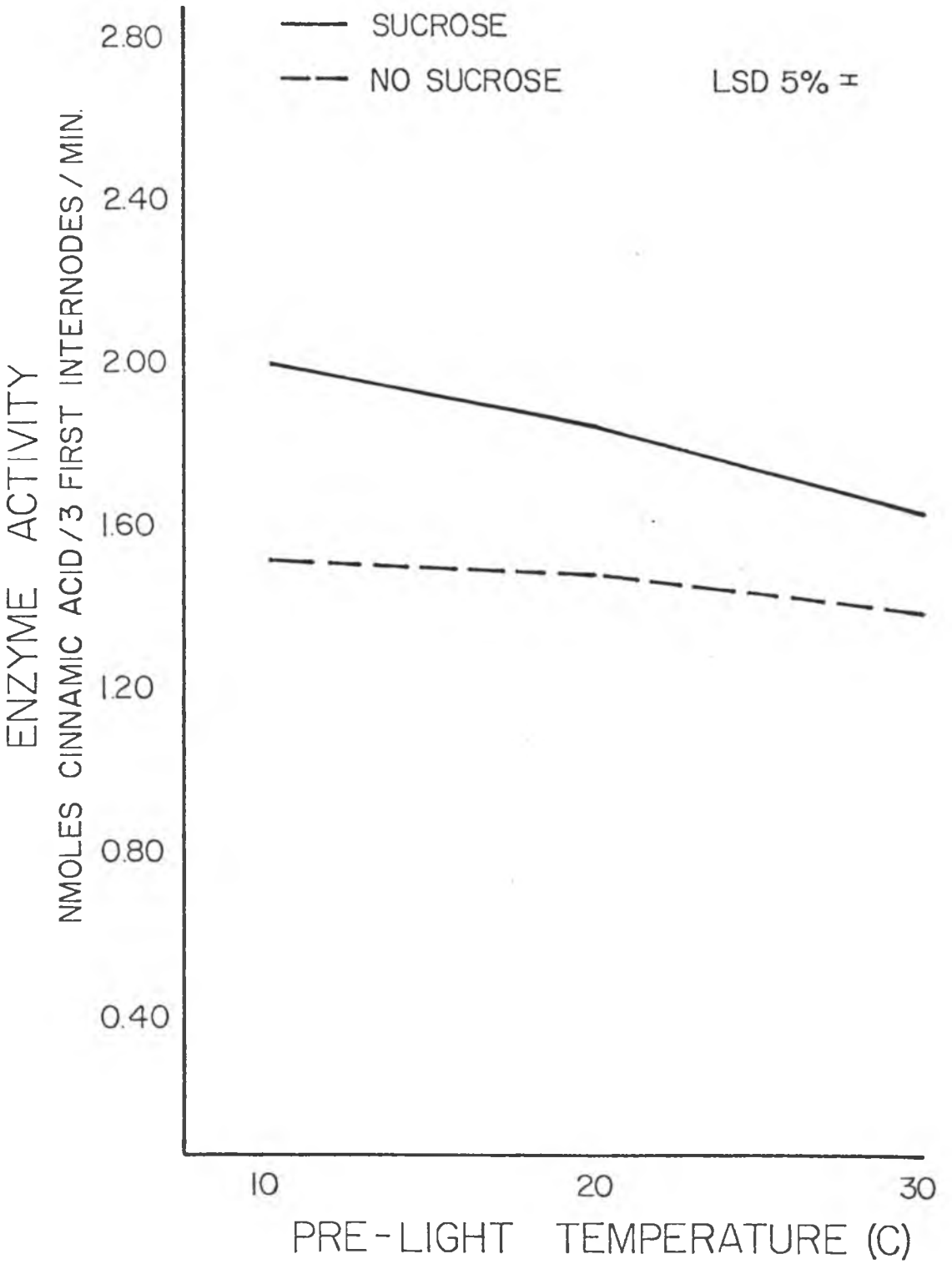

SUCROSE
NO SUCROSE
LSD 5% =
ENZYME ACTIVITY
NMOLES CINNAMIC ACID / 3 FIRST INTERNODES / MIN.
2.80
2.40
2.00
1.60
1.20
0.80
0.40
10
20
30
PRE-LIGHT TEMPERATURE (C)

Figure 18. Effects of sucrose on Phenylalanine ammonia-lyase activity during the light incubation period. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

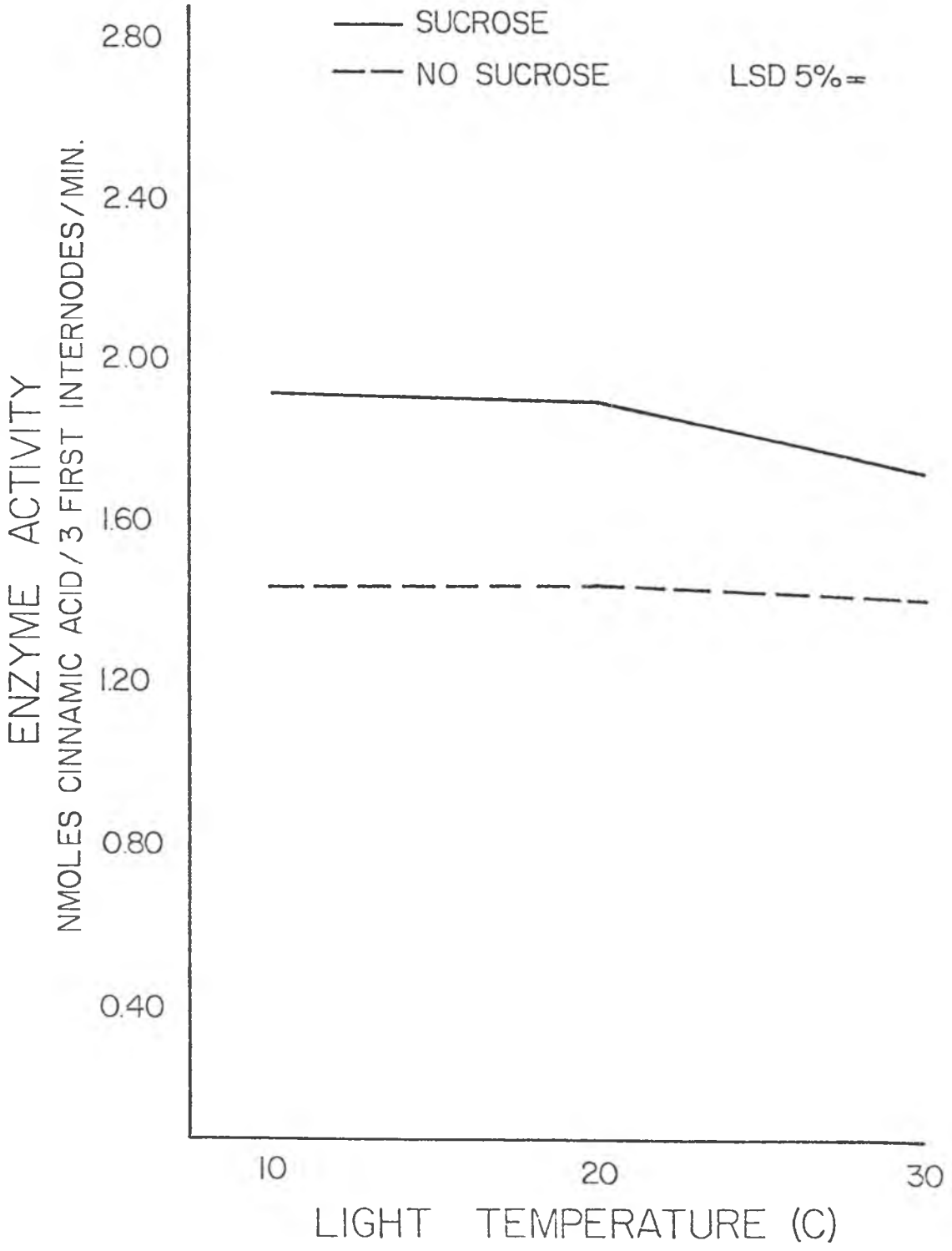

SUCROSE
NO SUCROSE
LSD 5%=
2.80
2.40
2.00
1.60
1.20
0.80
0.40
ENZYME ACTIVITY
NMOLES CINNAMIC ACID / 3 FIRST INTERNODES / MIN.
10
20
30
LIGHT TEMPERATURE (C)

Figure 19. Effects of sucrose on Phenylalanine ammonia-lyase activity during the post-light incubation period. Values for each temperature are means for combinations of pre-light, light, and post-light temperatures.

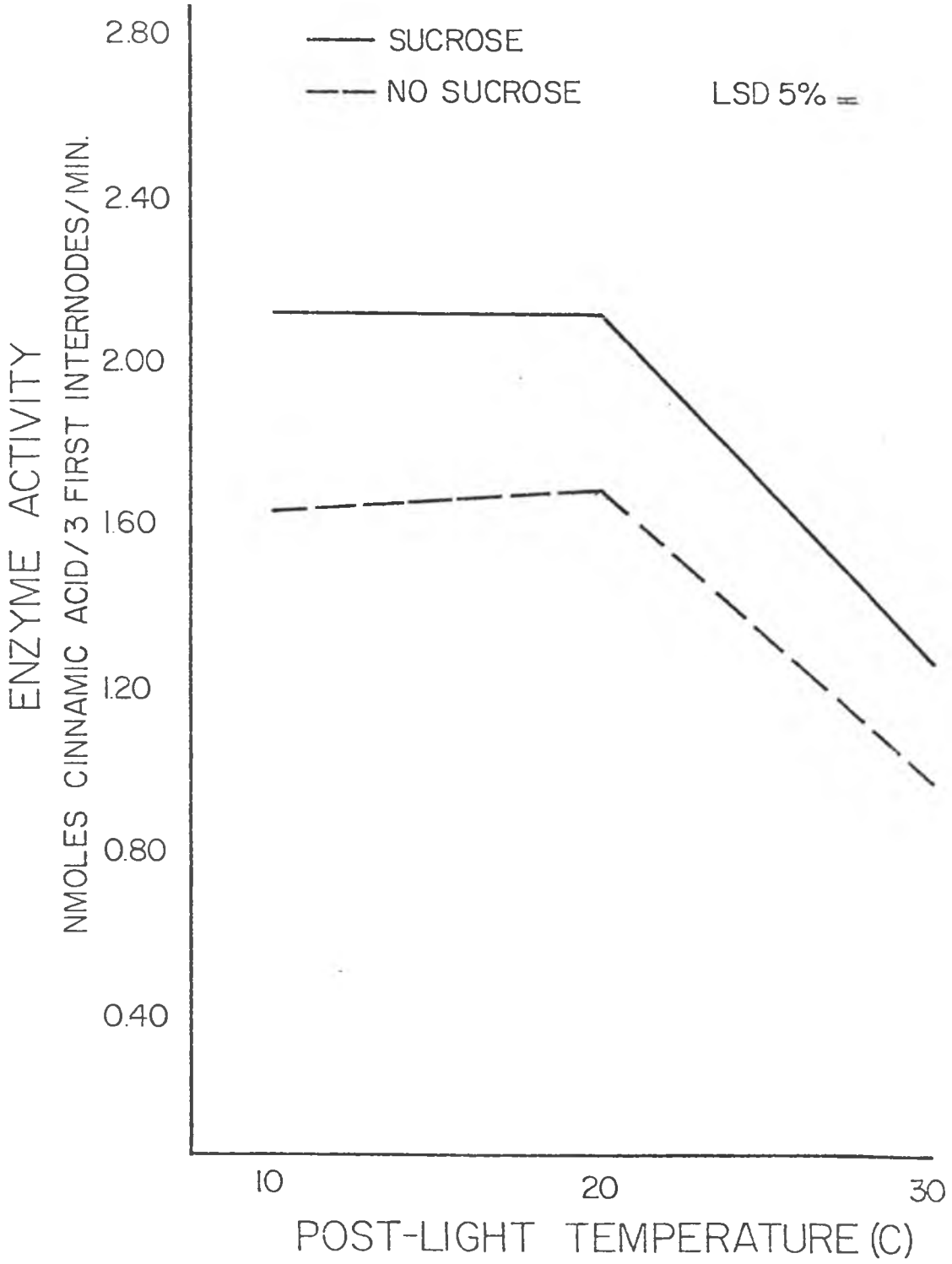

SUCROSE
NO SUCROSE
LSD 5% =
2.80
2.40
2.00
1.60
1.20
0.80
0.40
ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
10
20
30
POST-LIGHT TEMPERATURE (C)

Figure 20 shows that there was a decreasing trend in PAL activity as plant age increased before exposure to light. There was no difference in enzyme activity as plant age increased from 3.5 to 4.5 days. A marked reduction was observed as the plant age increased from 4.5 to 7.5 days. The highest enzyme activity was observed in the 3.5 day old seedlings and the lowest activity in the 7.5 day old seedlings.

Effects of Sucrose in Plant Age Treatments

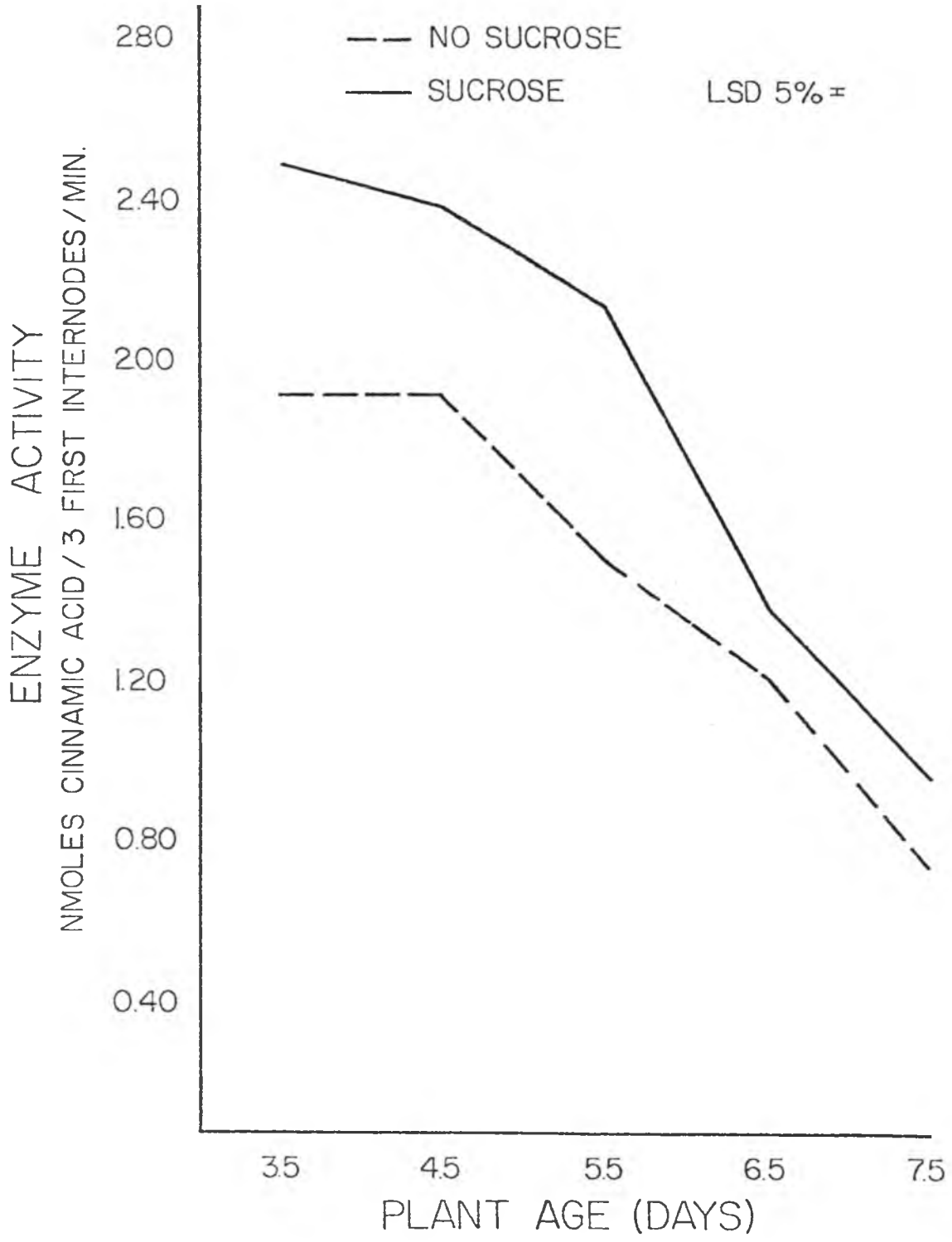
The effect of sucrose on PAL activity at different plant ages was studied. The materials and methods were the same as those used in Chapter III with the exception that the seedlings were analyzed for PAL activity.

The trend for PAL activity as affected by sucrose was similar to the minus sucrose treatments (figure 20). PAL activity was the highest in the 3.5 day old seedlings and lowest in the 7.5 day old seedlings. There was a moderate decrease in enzyme activity as the plant age increased from 3.5 to 5.5 days. The enzyme activity was much lower in the 6.5 and 7.5 day old seedlings.

Discussion

The results of various studies indicated that flavonoid synthesis could be affected by regulation of the levels of Phenylalanine ammonia-lyase (PAL) (Creasy, 1967; Creasy, 1971; Matsumoto, 1972; Saunders and McClure, 1973). These studies and most other studies have related to changes in PAL levels as a result of stimuli such as light. A few studies by Engelsma (1968) and Engelsma (1970) have demonstrated the

Figure 20. Effects of plant age and sucrose prior to light exposure on Phenylalanine ammonia-lyase activity. Values for each time period are means for combinations of pre-light, light, and post-light temperatures of 20C.


280
2.40
200
1.60
1.20
0.80
0.40
NO SUCROSE
SUCROSE
LSD 5% =
ENZYME ACTIVITY
NMOLES CINNAMIC ACID / 3 FIRST INTERNODES / MIN.
3.5
4.5
5.5
6.5
7.5
PLANT AGE (DAYS)

importance of temperautre on changes in PAL levels. The following data support the views that light and temperature are stimuli that can affect changes in PAL activity.

The activity of PAL, as influenced by temperature in the absence of light, seems to vary with the plant material. Engelsma (1969) worked with gherkin seedlings and found that PAL activity increased faster at 10C in the dark than at higher temperatures, but the effect was of a greater magnitude in the presence of light. Our study showed that PAL activity in etiolated sorghum seedlings was not affected by temperature in darkness (table 3). This indicates that temperature may not be a limiting factor on PAL activity under the conditions of this study.

Table 3 shows that sucrose was effective in stimulating PAL activity in the dark at all temperature treatments. This is in partial agreement with Creasy (1967) who worked with strawberry leaf discs. He found that PAL activity was greatly stimulated in the dark by 0.15M sucrose. It was suggested that sucrose functioned as a substitute stimulus for light. Creasy further postulated that light acted to provide for more carbohydrates via photosynthesis, and that it was probably the carbohydrate level that had stimulated PAL activity. It is not possible to speculate whether the increase in carbohydrate level increased PAL activity due to a de novo synthesis or by activation of existing enzymes, but it is possible to speculate that light is not required for its activity.

Pre-light temperature did have an effect on PAL activity for those treatments that included a combination of pre-light, light temperature incubation periods (figure 11). The trend was observed as an increase

in enzyme activity with an increase in temperature. From these data it is presumed that temperature prior to seedling exposure to light is effective in regulating PAL activity and/or synthesis. If this assumption is valid, higher temperatures might have acted to prime the availability and/or activity of PAL for the light incubation period.

The trend of PAL activity as influenced by temperature during the light incubation period of the pre-light, light temperature combinations was similar to, but of greater magnitude, than the pre-light incubation period (figure 12). This is in agreement with Cam and Towers, 1972; Zucker, 1965; Belli and Poucke, 1970; Schopfer and Mohr, 1971; and Creasy, 1968 who found that PAL activity was stimulated in various plant materials upon exposure to light. It has been agreed upon by most of these workers that at least some of the increase in PAL activity was due to de novo synthesis.

Table 4 illustrates the effect of sucrose on PAL activity in sorghum seedlings during pre-light, light temperature treatments. The greatest stimulation in enzyme activity was observed at the lower temperatures. This is a reversal of the results derived from those treatments that involved only pre-light incubation periods. The light treatment probably contributed toward these different trends. If this is true, then it seems as though some other factor was produced or activated by light at the high temperatures with the capacity to negate the stimulatory effects of sucrose.

A pre-light temperature of 10C was more effective than 20C or 30C in stimulating PAL activity in seedlings that were exposed to three incubation periods (figure 13). Based on Engelsma's theory, it is

possible that higher pre-light temperatures act to prime not only PAL activity for the incubation periods to follow, but also a PAL deactivating system. It can also be speculated that higher temperatures would probably result in faster utilization of carbohydrates by respiration and therefore decrease the substrate supply for PAL.

Temperature during the light exposure period effected PAL activity to a lesser degree than during the other incubation periods, but the trend remained the same (figure 14). PAL activity was higher when the seedlings were exposed to lower temperatures and lower when they were exposed to higher temperatures. Engelsma (1969) found that the transfer of gherkin seedlings grown in the dark at 10C to the light at 25C resulted in a faster drop in PAL activity than a transfer to 25C in the absence of light. The decline in PAL activity was partially inhibited when the seedlings were sprayed with cycloheximide 0.5 hr before transfer to the higher temperatures. This indicates that light might have acted to stimulate the activation and/or synthesis of a PAL deactivating system in the presence of higher temperatures.

Figure 15 illustrates a most emphatic effect of high temperatures on PAL activity during the post-light incubation period. There was a marked decline in PAL activity when the seedlings were exposed to a post-light temperature of 30C, whereas the enzyme activity was highest in those seedlings that were exposed to 10C and 20C. Engelsma (1969), who worked with gherkin seedlings, explained that at lower temperatures a PAL inactivating system was synthesized at a low rate and previously synthesized PAL was released from an enzyme-inactivator complex. Therefore, newly synthesized PAL was not inactivated. At higher

temperatures, it was postulated that the rate of the PAL inactivating enzyme synthesis compensated for PAL synthesis and resulted in lower PAL activity. If the PAL inactivating system is stimulated by light, it is conceivable that a lag phase for the synthesis of the inactivating enzyme might be involved. This assumption could account for the marked decline in PAL activity only during the post-light incubation periods at high temperatures. The decline probably was not observed during the other incubation periods because of insufficient time for its synthesis.

PAL activity was affected by light, post-light temperature combinations (figure 16). The more stimulatory combinations included a 20C post-light temperature while the least stimulatory included a 30C post-light temperature. As discussed earlier, the post-light incubation period seems to be the most influential on PAL activity, probably because of the presence of a PAL inactivating system that functions at higher temperatures. Based on this assumption, PAL synthesis and/or activity at 20C is probably functioning more efficiently than the PAL inactivating system. The opposite would be true for a post-light temperature of 30C. The most stimulatory combination included 10C light and 20C post-light temperatures. This further demonstrates the inability of the postulated PAL inactivating system to function effectively at the lower temperatures.

An exogenous supply of sucrose was effective in stimulating PAL activity during pre-light, light, and post-light incubation periods (figures 17, 18, and 19). The trend was similar for all treatments, showing a greater stimulation at the lower temperatures than at the higher temperatures. It is apparent that the possible activity of a PAL

inactivating enzyme system at the higher temperatures compensated for the stimulatory effects of sucrose. It is also possible that as indicated earlier, the higher temperatures may have resulted in higher respiration rates and faster utilization of substrates, therefore making less available for PAL.

Creasy (1968) speculated that the endogenous levels of carbohydrates in strawberry leaf disks was influential in determining PAL activity. He found that when the endogenous level of carbohydrates was high, sucrose was not able to stimulate PAL activity, but when the opposite condition existed, PAL activity was stimulated. The following data partially supports Creasy's work but from a different approach. It was decided that the endogenous levels of sucrose could be governed by different respiration times as determined by plant age. The older plants were presumed to contain less endogenous carbohydrates because of longer respiration times, the opposite would pertain to the younger plants. Figure 20 shows that PAL activity was essentially the same for the 3.5 day old and 4.5 day old seedlings. A rapid decline in enzyme activity resulted as the plant age increased from 4.5 to 7.5 days. These data might be indicative of a reduction in PAL activity with plant age due to less available carbohydrates during the light incubation period. Another factor that should not be overlooked is the possibility of the production of an inactivating enzyme system.

The previous postulation relating to PAL activity and carbohydrate levels as influenced by plant age was partially supported by the results as seen in figure 20. PAL activity was stimulated at all plant ages by an exogenous source of sucrose. The trend shows maximum stimulation

with the youngest plants and the least with the oldest plants. The results may be explained on the basis that the level of exogenous sucrose may have been lower than required to compensate for its utilization in the older plants.

CHAPTER V

CONCLUSION

The results of our studies indicate that light, temperature and plant age are important factors that can influence anthocyanin production and PAL activity. It was observed that anthocyanin could not be produced in the absence of light, which is in agreement with Downs (1964). The activity of PAL under the same conditions was also very low and in agreement with Engelsma's (1969) work.

Anthocyanin formation increased as temperature increased during the light incubation period. This trend was attributed to a possible increase in anthocyanin synthesizing enzyme activity which would be in accordance with van't Hoff's law. PAL activity supported this postulation, it showed an increase with an increase in temperature. Although temperature seems to have a regulatory role in these studies, the importance of light can not be overlooked. Smith (1975) discussed the importance of light on anthocyanin synthesis, and several people showed that light is a strong stimulus for PAL activity (Cam and Towers, 1972; Zucker, 1965; Bellini and Poucke, 1970; Schopfer and Mohr, 1971; and Creasy, 1968). It was not our intent to determine whether PAL was synthesized or merely activated from an inactive state. This could be investigated in future studies by spraying cychloheximide on the seedlings just prior to the light incubation period. If PAL activity remains the same during the light incubation period, it can be assumed that it was not being synthesized.

The inability of sucrose to influence anthocyanin production during the absence and presence of light was attributed to the following possibilities: 1) an inadequate period of time for the conversion of sucrose to substrates required for anthocyanin formation; 2) inefficient translocation of sucrose into the seedlings; or 3) the conversion of the additional substrate to some other product. Based on Craker et al's (1971) work, it is conceivable that a lag phase could account for minimal conversion of sucrose to anthocyanin. PAL activity was stimulated by sucrose in the dark and in the light, therefore it is apparent that sucrose was translocated into the seedlings. This does not negate the possibility that the quantity translocated was insufficient to enhance anthocyanin production because PAL would be expected to be more sensitive to levels of substrates. According to Smith (1975), even if the substrate were to reach PAL, there are numerous possibilities for its movement beyond this point (e.g. coumarins, phenols, flavonoids, and lignins). Radioactive studies can be utilized to determine how much sucrose was taken into the seedlings, but it would be nearly impossible to follow the movement of the labelled carbon within the plant because of its ubiquitous nature.

The trends were similar for anthocyanin production and PAL activity as affected by pre-light incubation periods for studies that included three incubation periods. The lower temperatures (10C and 20C) were more effective in stimulating the production of anthocyanin and PAL activity than 30C. It was assumed that carbohydrate levels and an inactivating enzyme system were the controlling factors. A high pre-light temperature was thought to increase respiration rates and

therefore reduce carbohydrate supply. A lower supply of carbohydrates would result in lower enzyme activity and anthocyanin formation. Also possible would be the synthesis of a PAL deactivating enzyme system at the higher temperatures which could deactivate PAL. This could be ascertained by spraying the seedlings with cycloheximide prior to exposing them to a 30C pre-light incubation period (Engelsma, 1969).

A temperature of 30C during the light incubation period resulted in maximal production of anthocyanin and minimal PAL activity. It is possible that 30C was ideal for maximum anthocyanin synthesizing enzyme activity exclusive of PAL. Initially, PAL may have made available substrates for other anthocyanin synthesizing enzymes, but due to the high temperature and light, a PAL deactivating enzyme system was probably synthesized. Therefore, PAL became deactivated toward the end of the light incubation period (Engelsma, 1969). Apparently, the amount of substrates that were initially produced by PAL was adequate to promote anthocyanin synthesis. Carbohydrate supply also might have influenced the trend of anthocyanin production. Only those treatments that included a low pre-light temperature followed by a high light temperature were effective in stimulating anthocyanin production. It was postulated that the low pre-light temperature served to conserve carbohydrates (due to low respiration rates) for anthocyanin production during the light and post-light incubation periods.

The effects of high temperatures on anthocyanin production and PAL activity during the post-light incubation period were greatly varied. Anthocyanin production was fairly high at 20C and 30C but PAL activity was very low at 30C. Anthocyanin production might have peaked during

the high post-light temperature period because of a lag phase (Craker, 1971) which was probably initiated during the light incubation period. PAL activity probably decreased because a PAL deactivating enzyme system was activated during the light incubation period. High PAL activity probably was not required during the post-light incubation period if it initially produced sufficient quantities of substrates. Other flavonoid synthesizing enzymes (Grisebach and Hahlbrock, 1974) may have required high temperatures during the post-light period for the continued synthesis of anthocyanin.

The greatest stimulation in both anthocyanin production and PAL activity by sucrose was evident at the lower temperatures. This may have been a result of higher carbohydrate levels and/or the absence of a PAL inactivating system. Lower respiration rates at the lower temperatures presumably resulted in more reserve carbohydrates. Lower temperatures were also considered undesirable for the synthesis of a PAL inactivating system (Engelsma, 1969). Stimulation of both PAL activity and anthocyanin production by sucrose indicates that the endogenous carbohydrate level might be a limiting factor in anthocyanin production.

Investigations pertaining to anthocyanin content and PAL activity as affected by plant age further strengthens the view that endogenous carbohydrate levels may influence anthocyanin production. As plant age increased, anthocyanin content and PAL activity decreased. This might be explained on the basis that the older plants were exposed to longer respiration times and therefore contained less endogenous carbohydrates for anthocyanin formation.

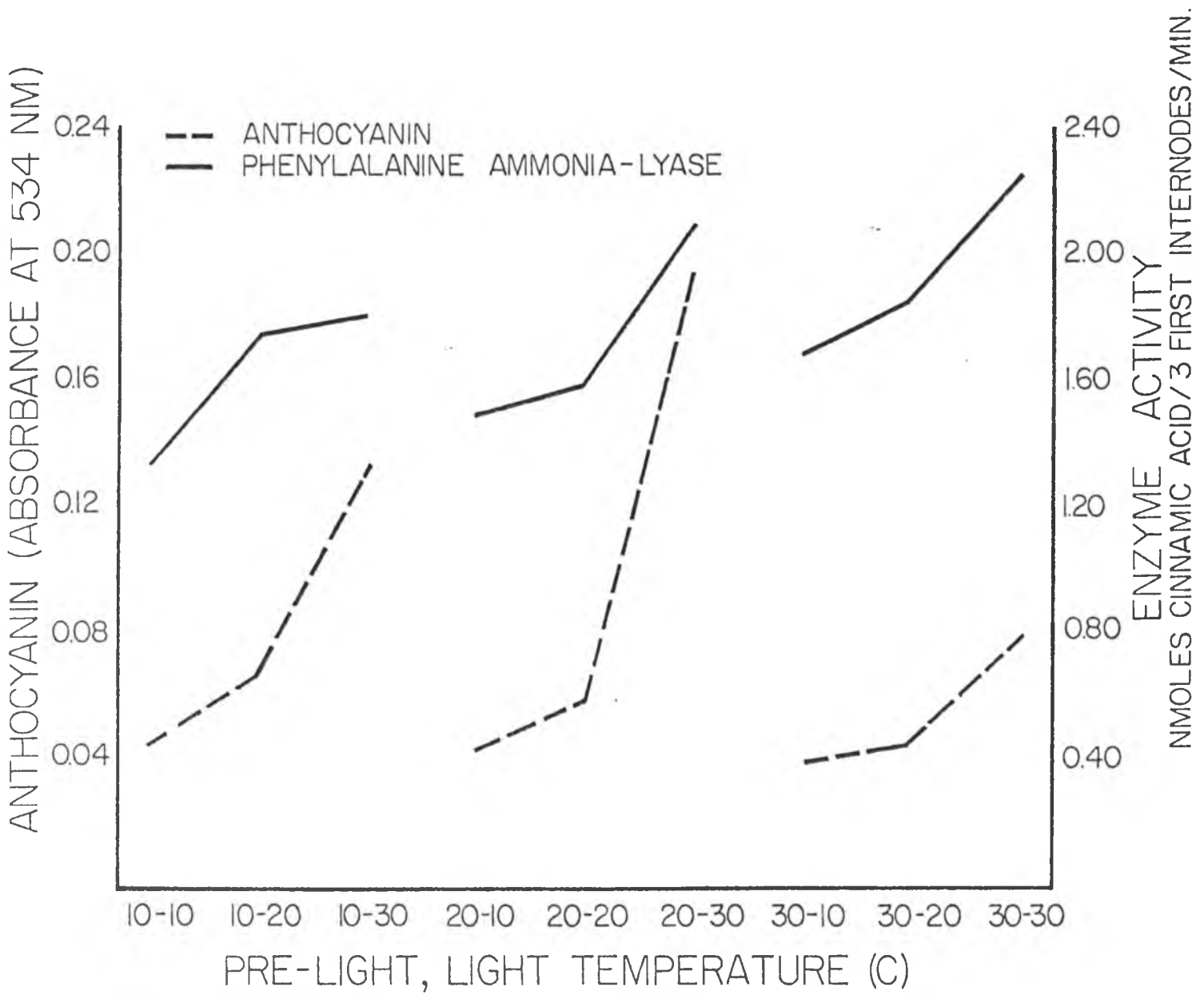
An exogenous supply of sucrose failed to effect anthocyanin production at all plant ages. Evidently the sucrose was translocated into the plants because PAL activity was stimulated, but as indicated earlier, the concentration might have been insufficient. The substrates that were produced by PAL from the exogenous supply of carbohydrates might have been utilized elsewhere.

It is not possible to provide any conclusive remarks in regards to the correlation between anthocyanin production and PAL activity by our studies, but a graph was constructed to evaluate the validity of assumptions made in the discussion sections (Appendix figure 20).

Higher quantities of anthocyanin were assumed to have been produced during the post-light incubation periods because of a lag phase. If this is true, and if PAL activity influences anthocyanin production, then maximal PAL activity should precede maximal anthocyanin production by a few hours. Minimal PAL activity should also precede minimal anthocyanin production. This relationship is illustrated in Appendix figure 21. The data for PAL activity immediately after the light incubation period are plotted on the same graph as the data for anthocyanin production 24 hours following the light incubation period. This time difference between PAL analysis and anthocyanin analysis should be more than ample for the completion of the lag phase. The data in each graph (Appendix figure 21) showed that PAL activity and anthocyanin formation were linearly correlated. The respective r values are: 0.71 for 10-10, 10-20, and 10-30; 0.98 for 20-10, 20-20, and 20-30; and 0.79 for 30-10, 30-20, and 30-30. Based on this relationship, and if the assumption that a lag phase exists is valid, then it is possible that PAL activity has some regulatory control over anthocyanin production.

APPENDIX

Appendix figure 21. Effects of temperature combinations on anthocyanin production and Phenylalanine ammonia-lyase activity. Values for each temperature combination for anthocyanin production are means of pre-light, light, and post-light temperature combinations. Values for each temperature combinations for Phenylalanine ammonia-lyase activity are means of pre-light, and light temperature combinations.


ANTHOCYANIN
PHENYLALANINE AMMONIA-LYASE
ANTHOCYANIN (ABSORBANCE AT 534 NM)
0.24
0.20
0.16
0.12
0.08
0.04
2.40
2.00
1.60
1.20
0.80
0.40
ENZYME ACTIVITY
NMOLES CINNAMIC ACID/3 FIRST INTERNODES/MIN.
10-10
10-20
10-30
20-10
20-20
20-30
30-10
30-20
30-30
PRE-LIGHT, LIGHT TEMPERATURE (C)

Appendix Table 5. Analysis of variance for effects of pre-light, light temperature treatments on anthocyanin production

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	53	0.001065	--	--
Treat	17	0.00085	--	--
Pre	2	0.00016	0.00008	1.33 ns
Light	2	0.00047	0.00024	4.00*
Sucrose	1	0.0000001	0.0000001	1 ns
Pre x Light	4	0.0002	0.00005	1 ns
Pre x Sucrose	2	0	0	1 ns
Light x Sucrose	2	0.00001	0.000005	1 ns
Pre x Light x Sucrose	4	0.00033	0.000083	1.38 ns
Error	36	0.000215	0.00006	

Appendix Table 6. Analysis of variance for effects of pre-light, light, and post-light temperature treatments on anthocyanin production

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	269	1.0410	--	--
Treat	53	0.9840	--	--
Pre-light	2	0.0940	0.0470	156.67**
Light	2	0.5103	0.2552	850.67**
Post	2	0.1250	0.0625	208.33*
Sucrose	1	0.0022	0.0022	7.33**
Pre x Light	4	0.1116	0.0279	93.00**
Pre x Post	4	0.0210	0.0053	17.67**
Pre x Sucrose	2	0.0060	0.0030	10.00**
Light x Post	4	0.0899	0.0225	75.00**
Light x Sucrose	2	0.0013	0.0007	2.33 ns
Post x Sucrose	2	0.0006	0.0003	1.00 ns
Pre x Light x Post	8	0.0150	0.0019	6.33**
Sucrose x Pre x Light	4	0.0044	0.0011	3.67**
Sucrose x Pre x Post	4	0.0011	0.0003	1.00 ns
Sucrose x Light x Post	4	0.0006	0.0002	1.00 ns
Sucrose x Pre x Light x Post	8	0.001	0.0001	1.00 ns
Error	216	0.0570	0.0003	--

Appendix Table 7. Analysis of variance for effects of plant age and sucrose on anthocyanin production

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	49	0.0175	--	--
Treat	9	0.0139	--	--
Age	4	0.0130	0.0033	33.00*
Sucrose	1	0.0	0.0	1.00 ns
Age x Sucrose	4	0.0009	0.0002	2.00 ns
Error	40	0.0036	0.0001	--

Appendix Table 8. Analysis of variance for effects of pre-light temperature treatments on PAL activity

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	17	0.010	--	--
Treat	5	0.0058	--	--
Pre-light	2	0.0001	0.00005	1.25 ns
Sucrose	1	0.005	0.005	12.50*
Pre x Sucrose	2	0.0007	0.00035	0.88 ns
Error	12	0.0043	0.0004	--

Appendix Table 9. Analysis of variance for effects of pre-light, light temperature treatments on PAL activity

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	53	0.0649	--	--
Treat	17	0.0533	--	--
Pre-Light	2	0.0078	0.0039	13.00**
Light	2	0.0249	0.0125	41.70**
Sucrose	1	0.0122	0.0122	40.7**
Pre x Light	4	0.0036	0.0009	3.00 ns
Pre x Sucrose	2	0.0017	0.0009	3.00 ns
Light x Sucrose	2	0.0016	0.0008	2.5 ns
Pre x Light x Sucrose	4	0.0015	0.0004	1.3 ns
Error	36	0.0116	0.0003	--

Appendix Table 10. Analysis of variance for effects of pre-light, light, post-light temperature treatments on PAL activity

Source variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	161	0.3834	--	--
Treatment	53	0.3515	--	--
Light	2	0.0058	0.0029	9.67**
Pre-Light	2	0.0122	0.0061	20.33**
Post-Light	2	0.2158	0.1079	359.67**
Sucrose	1	0.0541	0.0541	180.33**
Light x Pre	4	0.0017	0.0004	1.42 ns
Light x Post	4	0.0334	0.0084	28.00**
Light x Sucrose	2	0.0028	0.0014	4.67*
Pre x Post	4	0.0034	0.0009	3.00 ns
Pre x Sucrose	2	0.0034	0.0017	5.67**
Post x Sucrose	2	0.0026	0.0013	4.33*
Light x Pre x Post	8	0.0050	0.0006	2.00 ns
Light x Pre x Sucrose	4	0.0016	0.0004	1.33 ns
Light x Post x Sucrose	4	0.0040	0.0010	3.30 ns
Pre x Post x Sucrose	4	0.0032	0.0008	2.67 ns
Light x Pre x Post x Sucrose	8	0.0007	0.0001	0.33 ns
Error	108	0.0319	0.0003	--

Appendix Table 11. Analysis of variance for effects of plant age and sucrose on PAL activity

Source of variation	Degrees of freedom	Sum of squares	Mean square	Observed F
Total	29	0.0959	--	--
Treat	9	0.0882	--	--
Age	4	0.0731	0.0183	45.75**
Sucrose	1	0.0124	0.0124	31.00**
Age x Sucrose	4	0.0027	0.0007	1.75 ns
Error	20	0.0077	0.0004	--

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