GROWTH AND PIGMENT ANALYSIS OF TISSUE CULTURES DERIVED FROM A GENETIC STRAIN OF SOYBEAN *GLYCINE MAX* (L.) MERRILL

A Dissertation

Presented To

The Faculty of the Department of Biology

College of Natural Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

John Kenneth Hemphill

December 1974

DEDICATION

This dissertation is dedicated to my mother and father, Anna Maria and Kenneth R. Kemphill, to whom I am indebted for their understanding, guidance, and support throughout my education. Their encouragement has been and always will be an inspiration for success.

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ABSTRACT

Growth studies and pigment analysis were made on tissue cultures derived from a genetic strain of soybean Glycine max (L.) Merrill. Optimal concentrations of growth hormones (NAA, 1 mg/l and kinetin, 1 mg/l), ascorbic acid (75 mg/l), δ-aminolevulinic acid (4 mg/l) and sucrose (30 gm/l) as supplements to a modification of Miller's salts (127) were determined on the basis of the following parameters: fresh and dry weight and chlorophyll formation. Other factors such as coconut milk, yeast extract, casein hydrolysate, a synthetic amino acid mixture and various carbohydrate sources were investigated in response to these parameters. It was evident that this callus strain required an exogenous supply of cytokinin, and glucose favored chlorophyll formation whereas in most cases fructose and sucrose supported both growth and chlorophyll formation. Also the data indicated that the physiological requirements for chlorophyll formation was inversely correlated with the requirements for rapid cell growth. Under the chemical environmental conditions employed, the callus variants demonstrated a degree of cytodifferentiation and genetic heterogeneity as observed by phase contrast microscopy.

The pigmentation of these cultured phenotypes which were maintained under chlorophyllous and dark-grown conditions varied in response to the physical as well as chemical

environmental conditions which were employed. Under continuous illumination the heterozygous variant in most cases demonstrated a greater potential for chlorophyll formation than the normal green and yellow phenotype. However, the dark-grown phenotypes demonstrated the characteristic ratio for chlorophyll synthesis as found in the parent material. The lethal mutant was more susceptible to photo-bleaching than the wild-type and light green phenotypes. This was evident by the increase in chlorophyll content under low light intensity. The greening ability of these variants demonstrated a two-phase accumulation period: one occurred at approximately day 3 while the second phase at day 12 corresponded with the deceleration phase of growth and the greening phase. The decline in chlorophyll b formation before the second accumulation phase was discussed in regards to plastid biogenesis of dark-grown callus cultures.

The implication of these findings are discussed in respect to callus growth and chloroplast biochemistry of these variants and other cultured genera.

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LIST OF ABBREVIATIONS

ALA	δ-aminolevulinic acid
2,4-D	2,4-dichorophenoxyacetic acid
IAA	Indole-3-acetic acid
LG	Light green
NAA	α-naphthaleneacetic acid
NG	Normal green
SDS	Sodium dodecyl sulfate
SA-Chl a	Specific activity - Chl a
SA-CHL b	Specific activity - Chl b
S.E.M.	Standard error of the mean
Y	Yellow

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INTRODUCTION

The elucidation of the genetic control and biochemical differentiation of the photosynthetic apparatus has been approached in several directions. One of the most frequent methods employed is the use of mutants which are defective in some aspect of chloroplast structure, function and/or pigmentation. Several chlorophyll mutants that possess a genetic lesion or some other blockage in the synthesis of chlorophyll (18,28,69,105,138,147,158,159,188) and carotenoids (3,78,204) have been studied. However only a few studies have characterized the specific biochemical blockage in the mutant under investigation (29,71,92).

Recent advances in tissue culture methods allow examining the many factors which influence cytological and biochemical differentiation of cells on chloroplast mutants, presumably coded by nuclear and extra-nuclear genes. Since none of these mutants have been analyzed under these conditions Venketeswaran and Mahlberg (196) initiated the investigations on N. tabacum L. which possess a genetic marker for or absence of chlorophyll synthesis under tissue culture conditions. This material for callus initiation was derived from progeny that were homozygous recessive for ws_1 while the second gene-factor, Ws_2, segregated as a Mendelian character in a 3:1 green-albino ratio. The nature of the callus growth and chlorophyll formation in the green and albino strains indicated that the level of chloroplast differentiation depends on chemical and physical factors employed under tissue culture

conditions (125,198). Supplementing with a carbohydrate source and other growth hormones, these authors demonstrated the cultivation of an albino callus *in vitro* (199). This method provided an experimental system whereby a lethal genetic character could be investigated.

To date, a callus culture which is controlled by heterozygous trait with incomplete dominance for chlorophyll formation has not been investigated. Weber and Weiss (205) described a gene (y_{11}) tnat conditions the development of chlorophyll in soybean, *Glycine max* (L.) Merrill. After germination the progenies from the heterozygous parent (Y_{11} y_{11}) demonstrated a segregation of a phenotypic expression for chlorophyll synthesis in a Mendilian ratio of 1(NG):2 (LG):1(Y, lethal). This investigation reports on the establishment and maintenance of callus cultures derived from these soybean phenotypes which possess different phenotypic as well as genotypic complements for chlorophyll biosynthesis. The influence of various chemical factors on chlorophyll content and greening capabilities of those variants are presented.

LITERATURE REVIEW

Nutrition and Metabolite Biosynthesis of Plant Cells in Culture

Nutrition of Plant Cells in Culture. The variation in nutritional formulation employed in the cultivation of plant cells reflects the diversity of growth requirements of plant genera (132). It is routine in characterizing the nutrient medium by first determining the optimal basal medium for growth, and then establishing other criteria which reflect cell growth. These include the effect of growth hormones and other essential compounds, the best carbon source, the chromosomal behavior and other general criteria (47).

The assessment of the nutritional requirements involves the consideration of these major components: inorganic salts, organic compounds, and natural complexes (133). In most cases the inorganic requirement can be determined by culturing the plant system in question on one of the basic salt mixtures (86,93,130,211) or its modification (27,62,66, 137, 157). It appears that the ions K^+, Mg^{++}, Ca^{++}, NO_3^- and $H_2PO_4^-$ are essential for cell growth while Na^+, Cl^- and SO_4^{--} appear to be required at low concentrations (47).

Diversity in growth requirements becomes evident in the organic compositions of the medium. The carbohydrate requirement is satisfied generally with sucrose (2-4%).

Other carbon sources, glucose, fructose, raffinose, starch, cellobiose, galactose and maltose, have been shown to support growth in specific plant genera in culture (47). Several vitamins have been employed in growth medium with thiamine, inositol, nicotinic acid, and pyridoxin as the ones most commonly used (133). Thiamine is critical especially at low cytokinin levels (47). Subsequently, the addition of this vitamin has been shown to be not as essential since the induction of thiamine synthesis occurs in the presence of increased cytokinin levels (49). The requirement for the other vitamins have been shown to be tissue specific (47,132). Specific amino acids or their amides are also known to be tissue specific (132,133). These organic compounds are active in only the L—isomers and in certain combinations these acids are antagonistic to each other (132,133).

In 1957 Skoog and Miller postulated a model of growth regulation by an auxin and cytokinin (166). The basic elements are that quantitative interaction exist between an auxin and cytokinin and that this interaction as influenced by other factors regulates the extent and form of cellular growth. Exceptions to this model are divided into four additional groups: tissue which require only an auxin, tissue which do not require an auxin, tissue which require only a cytokinin and tissue which require only the addition of a natural complex (47,217). The most common auxins employed are IAA, NAA, and 2,4-D which are listed in order of increa-

sing potency (47) and are added over a wide concentration range (0.01-30 mg/l)(47,174). It is interesting that 2,4-D has the potential to eliminate the cytokinin requirement in some soybean (57,128,214) and tobacco callus tissue (214). The traditional cytokinins employed are of synthetic nature: 6-furfurylaminopurine (kinetin) and 6-benzylaminopurine (benezyladenine) with the latter being slightly more active and both being applied over a wide concentration range (0.01-10 mg/l) (47,174). The naturally occurring cytokinins, 6-Δ^2-isopentenyl aminopurine, the most active cytokinin, and zeatin are seldom employed due to their commercial value (118,132).

Complex mixtures such as coconut milk contain growth-promoting factors that when added in combination with vitamins and growth hormones stimulate cultivation of certain plants or tissue which otherwise could remain unresponsive on chemically defined media (132,169). Until the growth-regulating substances in these natural sources have been identified, it remains a necessity to employ such additives. The main disadvantage of complex mixtures is their variability from one batch to the next.

Other considerations such as the physical form of the medium, light and temperature of the in vitro environment have been critical in morophogenetic studies (132,133).

Metabolite Biosynthesis of Plant Cells in Culture. The accomplishment of biochemical differentiation in cultured cells without the recapitulating morphogenesis allows one

to investigate the biosynthetic potentialities of plant tissues as well as clarify the effects that certain biochemical events have on cells which undergo growth, differentiation, and morphogenesis (174). Accumulated evidence on the metabolic events in cultured cells have been demonstrated to be similar but not limited to those found in higher plants and micro-organisms (47,218). Therefore the product biosynthesis in the parent material frequently differs from its tissue culture system (218). The studies cited therein (218) indicated that the biochemical differentiation of plant cells in culture may not have the same genetic expression which occurred in the parent material. Possibly the metabolic events associated with differentiation and morphogenesis are not expressed under specific physical forms of the medium (218).

The products identified in callus cultures of various genera are numerous as reviewed by Carew and Staba (33) and Krikorian and Steward (112). Of particular interest are the pharmaceutical products; alkaloids, anti-biotics, steroids, and terpenoids. Other products formed by plant tissue cultures are tannins (12), lignins (48, 83,197) and pigments (7,33,47). Of particular interest to this investigation are the numerous studies involving chlorophyll and carotenoid biosynthesis in cultured genera (7,34,94,125,178, 179,193,196, 198). Venketeswaran and Mahlberg (125,196) have demonstrated that the chlorophyll a:b ratios of

chlorophyllous callus cultures are similar to normal leaves when grown under similar environmental conditions, however the total concentration of the cultured tissue was considerable less than whole leaves. Numerous factors such as light intensity (35), auxin (178) and kinetin concentrations (101), sucrose levels (52,101) and genetic inheritance (196) have been demonstrated to effect the chlorophyllous level in cultured genera. The carotenoid distribution in cultured tissue of 'Paul's Scarlet' Rose were qualitatively different than rose stem and leaves (213) and clonal callus cultures of Atropa belladonna var. Lutea (42). The total concentration of carotenoids in these cultured genera were considerable less than the parental material (42,213).

Biosynthesis of Photosynthetic Pigments In Higher Plants

Biosynthesis of Chlorophylls. Numerous studies of early workers have contributed extensively to our knowledge of porphyrin and chlorophyll biochemistry (10,27,55,70,119,152). Of particular interest to this investigation are the findings of Granick (72) and later Shemin and Russell (162). In investigating Chlorella mutants which had lost the ability to form chlorophyll and subsequently accumulated porphrins, Granick postulated a terminal pathway for chlorophyll a formation. At this time there were no assurance that these metabolites were actual intermediates for chlorophyll synthesis or side products of reactions. However the existence of these intermediates indicated a possible metabolic sequence and therefore served as a guideline for further studies.

By 1953 the classic study by Shemin and Russell (162) of protoporphyrin IX biosynthesis led to the use of labelled ALA in chlorophyll biosynthesis. ALA was originally believed to be formed within mitochondria from the succinate-glycine cycle (162) although according to Granick (73), it was not known if the synthesis of this metabolite occurred exclusively within mitochondria or if plastids can also produce ALA. Recently Wellburn and Wellburn (209) demonstrated the existence of ALA synthetase in plastids by following a light stimulated incorporation of labelled

glycine-2-^{14}C into chlorophyll a in an isolated intact etioplast preparation. However, Beale and Castefranco (15,17) found that glutamate, glutamine and α-ketoglutarate were found to be incorporated into ALA to a much greater extent than either glycine or succinic acid. This evidence which is not consistent with the concept that the succinate-glycine cycle synthesizes ALA indicates that this metabolite could possibly be produced by some other pathway.

Evidence for the *in vivo* synthesis of ALA in higher plants has been reported by many investigators (16,17,81, 152). Using an ALA dehydratase inhibitor, levulinic acid, chloroplasts have been shown to accumulate ALA in amounts equivalent to the inhibition of chlorophyll. The fact that ALA accumulates under these conditions provides an experimental system to study *in vivo* synthesis of ALA and elucidate the controlling mechanisms of chlorophyll synthesis. While characterizing this experimental system in *Chlorella*, Beale (16) demonstrated the existence of a labile enzyme involved in the formation of ALA which easily explains the difficulty in obtaining on *in vivo* ALA synthesizing preparation from green plants. Under greening conditions, cotyledons of barley and beans accumulated ALA when cultured in the presence of levulinic acid. This accumulation ceased when cotyledons treated with levulinic acid were returned to darkness, thus demonstrating a light requirement for not only ALA synthesis but also chlorophyll synthesis. This evidence substantiates

the model originally postulated by Nadler and Granick in 1970 in that the limiting factor for chlorophyll synthesis was a protein (or proteins) with a half-life of 1.5 hr. which was involved in the synthesis of ALA (134). Since cycloheximide as well as chloramphenicol inhibited ALA formation the synthesis of this metabolite may require both a cytoplasmic protein (enzyme) as well as a plastid protein (enzyme). The authors suggested that the rate limiting factor was ALA synthetase which was activated by light at the level of cytoplasmic translation. Phytochrome was suggested to be involved in regulating this enzyme activation (134) which was originally demonstrated by Price and Klein (148) and more recently by DeGreef and Caubergs and others (44 , see refs. therein). Once this enzyme system becomes functional, the conversion of ALA to protochlorophyllide occurred by non-limiting enzymes which are present in higher plant plastids (134). According to Granick, this model does not rule out the existence of a separate mechanism of feedback inhibition control of the synthesis of ALA by some intermediate of chlorophyll synthesis (134).

The chlorophyll intermediates from ALA to protochlorophyllide do not accumulate in chlorophyll mutants of higher plants except for protochlorophyllide (109). However, certain lethal nuclear mutants of barley can be induced to accumulate porphyrins by feeding with ALA in the dark (71). After characterizing the metabolic blockage of these mutants

and compiling data on Chlorella and other mutants of higher plants Gough purposed that chlorophyll-synthesizing enzymes of algae and seed plants consisted of at least two compartments in the chloroplast: one containing the enzymes converting ALA to protoporphyrin and the other converting protoporphyrin to protochlorophyllide. These compartments were considered to be independent from each other in that a lesion in one had no effect on the other. Also a defect in one component of the compartments was suggested to influence the activity of the others which explained why several intermediates accumulated in each nuclear mutant. This compartmentation of chlorophyll-synthesizing enzymes may be a requirement as dictated by the solubility properties of the intermediates and the nature of the enzymes. After the formation of coproporphyrin, succeeding intermediates are increasingly less water soluble. The enzymes in the first compartment are considered to be soluble (122,212) while Mg-protoporphyrin-S-adenosyl-Methionine methyltransferase, an enzyme from the second compartment, has been characterized as being membrane bound (149). Protochlorophyllide holochrome, a second component in this group, has been shown to possess photoenzymatic activity (134) and to be membrane bound within the prolamellar body (87).

Verification of this compartmentation was substantiated by Rebeiz, et al. using subfragments (105,000xg, 1 hr.) of isolated chloroplast from greening cucumber

cotyledons (151). When incubated with ALA and cofactors the soluble protein fraction demonstrated the ability to synthesize the free porphyrins (uroporphyrin, coproporphyrin and protoporphyrin), probably by their respective porphyrinogens (152). The green lamellar fraction possessed very limited biosynthetic activity in the presence of ALA and cofactors. However the addition of the soluble fraction to this preparation yielded substantial increase in free porphyrins and metal porphyrins. Based on this evidence Rebeiz concluded that the biosynthesis of free porphyrins (ALA to protoporphyrin) was synthesized by the stromal enzymes while the synthesis of metal porphyrins from ALA requires the membrane fraction (152).

After Mg chelation of protoporphyrin, the sequential biosynthetic steps leading to the formation of chlorophyll a are fairly well accepted as illustrated in Fig. 1. The following topics to be discussed are directly related to this investigation. The literature contains several comprehensive reviews on chlorophyll a formation (10,55,70,152).

The formation of protochlorophyllide from added ALA takes place in isolated etioplasts and greening plastids which indicates that all the enzymes involved are located within this organelle (152). The accumulation and photoreduction of protochlorophyllide under etiolated conditions occurs in a structural complex associated with a protein, the holochrome protein (22). Following chromatographic

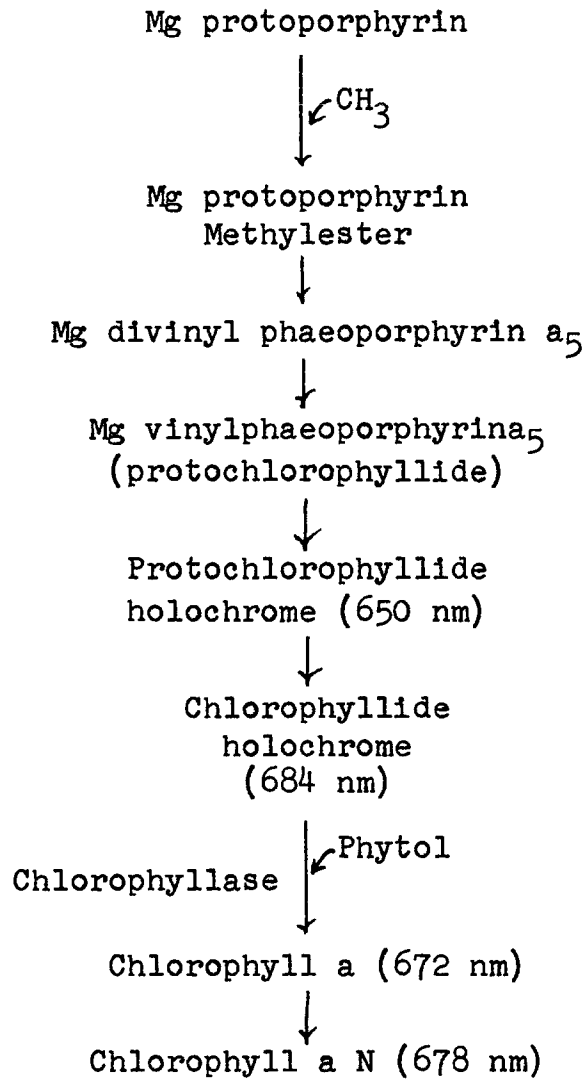


Figure 1. Biosynthetic pathway of chlorophyll a from Mg-protoporphyrin (70).

preparation by Sephadex gel filtration and saponin, the photoactive protochlorophyllide holochrome from bean and pea has a molecular weight of approximately 170,000 in comparison to 51,000 to 75,000 for the protein complex from barley (88). This larger pigment-protein complex was suggestive of an existence of a dimer or trimer unit but the unpublished data referred to by Henningsen, et al. (88) was inconsistant with this concept. The photoactive state of protochlorophyllide are believed to exist in three forms in etioplasts; one (650 nm, λ max) being easily converted to chlorophyllide upon illumination, second (636 nm, λ max) when combined with a photoenzyme can be converted to protochlorophyllide 650 and then to chlorophyllide and the third (628 nm, λ max) not being photoactive (65,98,180). Recently Kahn and Nielsen (99) suggested that the absorption maxima at 635-637 (Protochlorophyllide 636 nm, λ max) and 650 nm of photochlorophyllide exists as a single species in the pigment-holochrome molecule but the evidence does not rule out the possibility that two distinct species exist which are in dynamic equilibrium in vivo (99). The photoconversion of saponin protochlorophyllide holochrome from dark-grown barley seedlings involves an absorption maximum shift of 641 to 646 nm (most likely the 650 nm protochlorophyllide species) to 677 to 679 nm which is characteristic of the first chlorophyllide a molecule formed after illumination (88). No change in the molecular weight of the holochrome was observed. Also

the red shift 678 to 684 nm which occurs immediately after the formation of chlorophyllide a (678 nm) in dark grown seedlings (203) was not observed in the holochrome preparation (88). The direct shift from 678 to 672 nm occurred depending on temperature and detergent concentration and corresponded to a dissociation of the holochrome subunits with a molecular weight range of 63,000 to 29,000. This dissociation change was proposed to release the photoenzyme involved in the photoconversion process from the chlorophyll-protein complex thus allowing the photoenzyme to be used again. The spectroscopic and structural changes observed with the holochrome preparation was considered by the authors to be related to the reorganizational development which occurs in the prolamellar body during greening (88).

The photostimulus required for chlorophyll synthesis in angiosperms but lacking in gymnosperms can be substituted by substances found in the megagametophyte of a gymnosperm (26). Bogdanovic (26) transplanted wheat embryos onto pine megagametophytes which were then subjected to dark conditions. Pigment analyses of primary leaves of these transplanted embryos showed that the conversion of protochlorophyllide to chlorophyll can take place under these conditions when in contact with the gametophyte of a gymnosperm. This experimental assay will allow further investigations on the photostimulus of chlorophyll formation in angiosperms.

As indicated by Shlyk (163) isotopic methods played a major role in determining the biosynthesis of chlorophyll b. The observation that the SA-Chl a was higher than the SA-Chl b supported the biosynthetic sequence of a $\rightarrow$b when newly formed chlorophyll b molecules become associated with previously formed molecules, less labelled or unlabelled, which diluted the isotopic flow. Based on SA data and the different solubility properties of new and old chlorophyll a molecules, Shlyk (163) suggested that young chlorophyll a molecules are specifically converted to chlorophyll b at specific "structural loci". Subsequently young and old chlorophyll a and b molecules were thought to be spacially distinct from each other.

Upon illumination of etiolated material a detectable quantity of chlorophyll a appears from the photoconversion of the accumulated protochlorophyllide while chlorophyll b appears after 1 to 3 hr. (163). With increased sensitivity in analytical techniques employed, etiolated material when exposed for 20 sec. pre-illumination period accumulated chlorophyll b immediately when subject to dark and light conditions. Since the initial rate of chlorophyll a biosynthesis has been shown to be proportional to the amount of chlorophyllide, chlorophyll b formation was suggested to be synthesized through an intermediate even though it

develops without a delay. The identity of this intermediate as chlorophyll a was indicative in the observance of continual synthesis of chlorophyll b after chlorophyllide esterification was completed in the dark and chlorophyll a synthesis had stopped (163).

In mature leaves, the absorption spectrum of chlorophyll extraction has been shown to be composed of several different forms of natural chlorophyll complexes as demonstrated by curve analyses (60). The major chlorophyll a components from spinach chloroplasts have an absorption peak at 662, 670, 677 and 683 nm with minor absorption components at longer wavelengths; the 692 and 705 nm forms. The chlorophyll b components have an absorption peak of 650 nm and a smaller form at 640 nm. The components that absorb around 680 nm contribute more to the absorption spectrum of the System I particles which has System I activity than by System II particles while the chlorophyll b components are associated more so with the System II particles.

Detergent treated chloroplast lamellae have been resolved into seven principal chlorophyll-containing bands (91) which make up the two photosynthetic systems (91) and at least 21 total polypeptides (51). The relationship of chlorophyll a and b complexes and structural membrane polypeptides with Photosystem I and II activity are under intensive investigation. Cobb and Wellburn (36) have demonstrated significant changes during plastid morphogenesis:

SDS-extractable polypeptides differ quantitatively during greening indicating a reorganization of existing membranal proteins and qualitative changes during plastid senescence. In contrast, Henriques and Park (89) used a pulse and chase assay to demonstrate that during greening the lamellae proteins undergo a constant turnover at different rates. From studies of Chlamydomonas, Hordeum and Pisum mutants which possess little or no capabilities for grana formation, Anderson and Levine (5) have demonstrated that two polypeptides isolated from the chlorophyll-protein complex of Photosystem II are involved in the stacking process of plastid lamellae and active in structural organization of the chlorophyll-protein complex. In comparison two polypeptides which are associated with the Photosystem I protein-complex were shown not to be involved with grana formation but the possible function of orientating chlorophyll a in Photosystem I remains to be verified. In 1966 Anderson and Boardman (4,23) characterized the Photosystems I and II particles which were isolated from spinach chloroplast by digitonin treatment and separated by differential centrifugation. This evidence and subsequent studies (9,67,104,111, 141) of these two types of particles whose properties are consistent with their photosynthetic activity has demonstrated the concept of two photosystems existing in higher plant photosynthesis. Several review articles have been published on the recent models for photosynthetic electron transport between these particles (9,19,116,141,187).

Biosynthesis of carotenoids. The schematic pathway for carotene biosynthesis was originally postulated by Porter and Lincoln (145) in 1950. This pathway was based on the structures of the acyclic carotenes (colorless polyenes) and the existence and inheritance of both cyclic and acyclic carotenes in tomato fruits (113). Following a modification of this pathway in 1962 (145) substantial evidences for this revised scheme was obtained from labelled studies of mevalonic acid and terpenyl pyrophosphates into each of the cyclic and acyclic carotenes in ripening tomatoes (1) and isolated tomato plastids (2). Currently it is generally believed that the more unsaturated carotenes are formed from phytoene by a stepwise dehydrogenation and that lycopene is then converted to mono- and dicyclic carotenes (113,114). A comprehensive discussion of these reactions and others have been reported by Porter and Anderson (146) and partially illustrated in Figs. 2 and 3. Verification of most of these metabolites as direct intermediates for carotenoid biosynthesis has been accomplished by enzymatic interconversion preparation obtained from tomato fruit plastids (113,114,146).

According to Porter and Anderson (146), the oxygenated carotenoids in higher plants arise late in the biosynthetic pathway of carotenoid formation. However the direct conversion of α and β-carotene to lutein and zeaxanthin respectively, and other xanthophylls as illustrated in Fig. 3

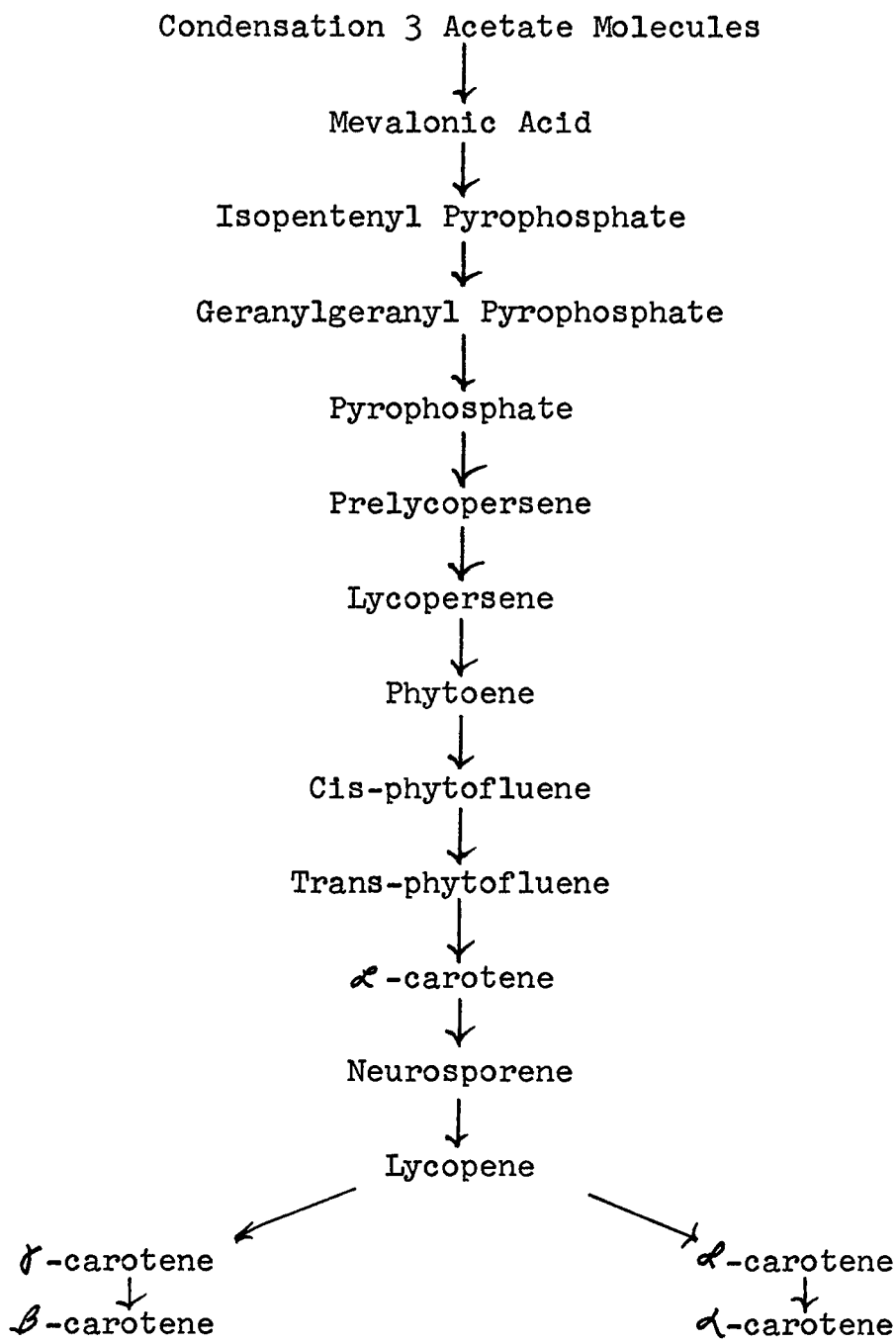


Figure 2. Biosynthesis of Carotenes as postulated by Kushwaha, S. C., et al. (113) and modified by Barnes, F. J., et al. (14).

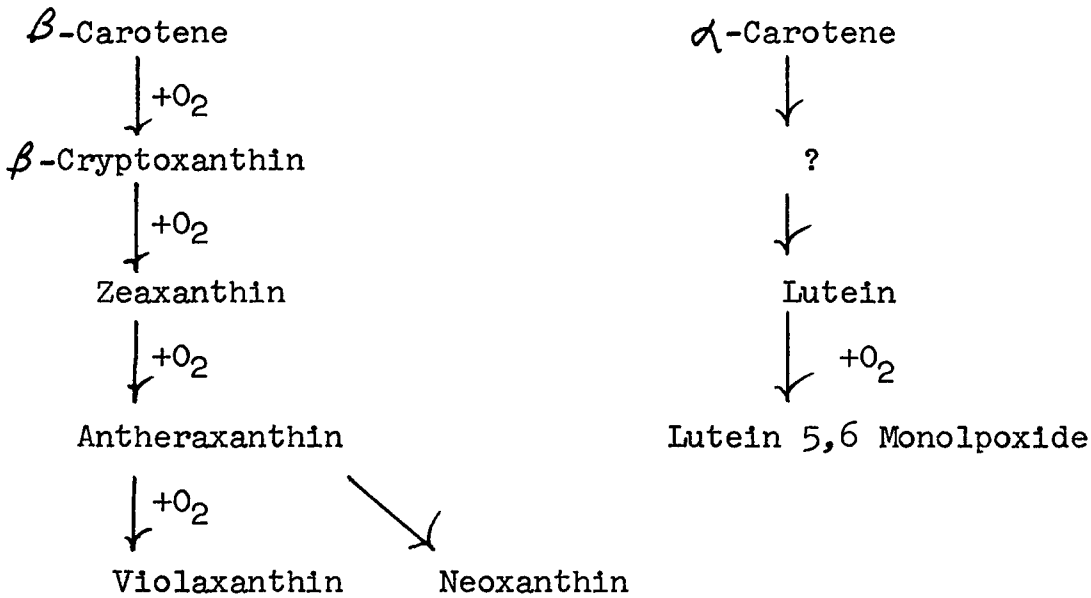


Figure 3. Biosynthesis of the oxygenated carotenoids as postulated by Porter and Anderson (146).

may be valid but remains in doubt since several interconversions of xanthophylls have been reported. The incorporation of oxygen into these compounds has for a long time been recognized as a requirement for biosynthesis of xanthophylls and has been known to occur through two processes, hydroxylation and epoxidation (146).

Several photosynthetic functions for carotenoids have been postulated: energy transfer to chlorophyll (50, 68), electron transport (216), and protection of chlorophyll against photo-oxidation (73). Goedheer (68) demonstrated that β-carotene possessed the ability to absorb and transfer light energy at near 100% efficiency to chlorophyll in both Photosystem I and II whereas xanthophylls lacked this capability. In comparison Photosystem I particles isolated from carotenoid mutants which accumulated lycopene and β-carotene possessed comparable energy transfer efficiencies with particles isolated from normal maize (56). Therefore these results demonstrated a role in energy transfer for β-carotenes but lessens the essentiality of this carotenoid for normal photosynthetic activity and chloroplast development (56).

Yamashita, et al. (216) suggested that xanthophylls as well as carotenes are involved with oxygen evolution and electron transport in photosynthesis. The authors postulated a coupling mechanism between the electron transfer of photolysis and Photosystem II and the oxygenation of

xanthophylls. Supposedly the light-induced epoxidation of zeaxanthin and antheraxanthin to give violaxanthin which occurs in the presence of molecular oxygen was considered to be related to the photo-bleaching of carotenoids and oxygen production in photosynthesis. However more recent evidence on the evolution of oxygen in photosynthesis being involved in the conversion of xanthophylls to their epoxides failed to demonstrate this interrelationship (151).

The ability of carotenoids (75) and, to be more specific, β-carotene (39) to protect chlorophyll from photo-oxidation has been considered as a classical role for these pigments in photosynthesis. The resistance to the photodestruction of chlorophyll molecules appears to develop about the same time as the development of the ability for energy transfer between β-carotene and chlorophyll (31). Over and above this role, β-carotene has been demonstrated by several laboratories to be able to stimulate the rate of O_2 evolution at low light intensities (39, 140). This role was not obligatory but when present this metabolite has been shown to restore redox potential of C-550 and the primary photochemical activity of Photosystem II in hexane extracted spinach chloroplasts (39, 140).

The orientation or environment of carotenoids in chloroplast membrane remains to be clarified (104). Evidence obtained by selective extraction and spectral analysis, Ji, et al. (97) have postulated an in vivo environment for

β-carotene as being neither purely hydrophobic nor extremely polar and being closely associated with the phytol chains of chlorophyll. The β-carotene-protein and other carotenoid complexes isolated from chloroplast lamellae have been postulated to be products of the isolation procedure used as indicated by the modified absorption spectrum of the complexes (103) even though evidence indicates to the contrary (97). A more comprehensive discussion of molecular orientation of carotenoids and other membrane components in chloroplast lamellae are available in several publications (19,111,141,208).

Valadon and Mummery (191) have demonstrated that light regulates carotenoid synthesis in mung bean seedlings. Changes in pigment concentration of dark and light grown seedlings indicated a 13 fold increase in total carotenoid synthesis with a greater increase occurring in the formation of xanthophylls than carotenes; lutein increased more than β-carotene. Under dark grown conditions, the bean seedlings lacked the ability to synthesize α-carotene, violaxanthin and neoxanthin but these metabolites appeared by the fourth day when the etiolated seedlings were illuminated. The other carotenoids identified appeared after 24 hr. under light conditions and increased throughout the growth period, especially lutein and β-carotene. High levels of neoxanthin and violaxanthin also appeared after 8 days of illumination. In dark-grown barley seedlings,

Lichlenthaler (121) demonstrated that the light-stimulated synthesis of carotenoids and other terpenoids parallel the formation of chlorophyll and thylakoids. The dark-grown carotenoids were postulated to be stored in the numerous osmiophilic plastoglobuli located in the etioplasts along with the prolamellar body and protochlorophyllide. Upon transformation the number of the osmiophilic bodies decrease and their content (carotenoids and other lipid compounds) evidently are being used to form a photoactive thylakoid system.

Until recently the photoreceptor of this light-stimulated process had not been elucidated (139). The action spectrum for the formation of chlorophyll a, b and β-carotene agreed with the absorption band of protochlorophyllide, in vivo. Since a rapid synthesis of β-carotene upon illumination does not exist, the light stimulus on carotenoid biosynthesis must regulate a pre-carotene precursor other than protochlorophyllide. Also keeping in mind that the pigment-protein complexes, especially Photosystem I, contain high levels of β-carotene the authors (139) proposed a mechanism interrelating the rate of formation of β-carotene upon the rate of formation of chlorophyll a and b. Consequently it was suggested that there may not be any other regulatory reaction other than the photoconversion of protochlorophyllide in the process of forming initially chlorophyll a (transformation) and then chlorophyll b and β-carotene (lamellae development and photosynthetic activity).

MATERIALS AND METHODS

Tissue Culture

Seeds of Glycine max (L). Merrill which possess a nuclear gene that influences the development for chlorophyll synthesis (205) were purchased from the Carolina Biological Supply Company. Surface contaminants were controlled by soaking the seeds in 10% NaOCl (commercial Clorox, autoclaved) for 5 minutes followed by washing 3 times in sterile, double distilled water. The seeds were allowed to imbibe water for 2 hr. in a Laminar Flow transfer chamber and then germinated in autoclaved, moistened vermiculite under controlled environmental conditions. After 16 days following germination the progenies from the heterozygous parent (Y_{11} y_{11}) demonstrated a segregation of a phenotypic expression for chlorophyll synthesis in a Mendilian ratio of 1(NG):2(LG):1(Y, lethal).

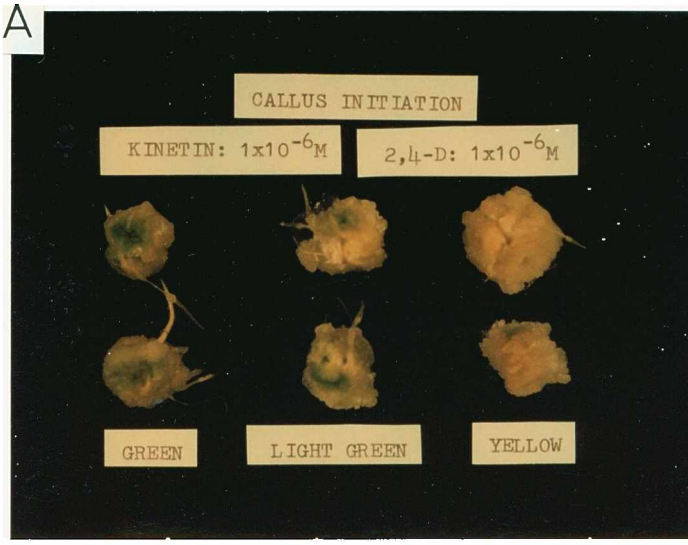
Callus cultures in the reported experiments were initiated from excised hypocotyls of each phenotype. The primary explants were surface sterilized with 5% NaOCl (commercial Clorox, autoclaved) for 15 minutes, washed 3 times with sterile, double distilled water and then incubated on a modification of Miller's media (127) which was supplemented with $1x10^{-6}$M 2,4-D and $1x10^{-6}$M kinetin (Fig. 4). The initiated callus (without the roots) was subdivided and routinely maintained with subcultures on the basal medium containing $1x10^{-6}$M NAA and $1x10^{-6}$M kinetin for several months. After completion of the growth hormonal studies, this callus system was maintained on 1 mg/l NAA and 1 mg/l kinetin for

Figure 4.

THE INITIATION, MAINTENANCE AND CYTODIFFERENTIATION OF THREE SOYBEAN PHENOTYPES.

A. The initiation of the cultured variants on 2,4-D ($1x10^{-6}M$) and kinetin ($1x10^{-6}M$). B. The maintenance of the cultured variants on NAA ($1x10^{-6}M$) and kinetin ($1x10^{-6}M$) after 30-days of growth. C. The maintenance of the cultured variants on NAA (1 mg/l) and kinetin (1 mg/l) after 26 months in culture. D. The cytodifferentiation of the normal green phenotype when maintained on coconut milk and yeast extract (Table No. 2).

A


CALLUS INITIATION
KINETIN: 1x10^{-6}M
2,4-D: 1x10^{-6}M
GREEN
LIGHT GREEN
YELLOW

B

C

D

the duration of these studies.

The basal media as originally described by Miller (127) was used as modified (Table No. 1). The salts and organic components employed were the same whereas the Fe-EDTA complex described by Murashige and Skoog (130) was incorporated in these studies. The optimal concentrations of ascorbic acid and ALA were determined by appropriate experimentation and then added to the basal media. The vitamins and agar were added to the medium before autoclaving at 15 lbs/in^2 for 15 minutes.

In all experiments reported here the tissues were taken from stock cultures which were maintained at 27°C±1 under continuous fluorescent lights (Sylvania Gro-Lux) at low light intensity (700 lux). Small pieces (90-110 mg) were transferred to experimental media contained in glass jars and then covered with sterile polypropylene.[1] The experimental cultures were incubated under similar environmental conditions as the stock cultures with the exception of being subjected to a high light intensity (3000 lux).

Fresh and dry weight determinations were employed as growth parameters throughout these studies. Unless otherwise indicated in the legend at least 8 replicates of 5 pieces each were made in all growth studies. All pieces

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[1] The polypropylene was prepared by placing square pieces of the appropriate size into a magazine which was wrapped and sealed in aluminum foil and then sterilized by autoclaving at 15 lbs/in^2 for 20 minutes.

Table No. 1. Composition of the basal media as modified from Miller's media (127). The pH was adjusted to 5.8 with 1N NaOH and 0.1 N HCl.

Media Components	Stock Solution Final Conc. (mg/l)	Stock Solution ml/liter of media	Final Conc. (gm/l)
A. Major and minor salts.			
KNO_3	1,000	100	10
NH_4NO_3	1,000		10
$Ca(NO_3)_2 \cdot 4H_2O$	500	40	12.5
KH_2PO_4	300	100	3
$MgSO_4 \cdot 7H_2O$	71.5		.72
KCl	65	5	13
$MnSO_4 \cdot 4H_2O$	14		2.80
$ZnSO_4 \cdot 7H_2O$	3.8		.76
H_3BO_3	1.6		.32
$Cu(NO_3)_2 \cdot 3H_2O$	0.35		.07
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1		.02
KI	0.8	5	0.16
Na_2EDTA	37.3	10	3.73
$FeSO_4 \cdot 7H_2O$	27.8		2.78
B. Organic constituents.			
Sucrose	30,000		30
Myo-inositol	100	20	5
Nicotinic acid	2	1	2
Pyridoxine-HCl	.4		0.4
Thiamine-HCl	.4		0.4
Ascorbic acid*	75	15	5
ALA*	4	4	1
NAA*	1	2.5	0.4
Kinetin*	1	2	0.5
Agar	8,000		8

* Other components added to the basal media following experimentation.

were pre-weighed except in experiments on the growth hormonal studies. Initial weights for these investigations were determined by averaging representative replicates of each phenotype. The growth parameters were determined after a 30-day growth period. The growth values were calculated by the following formulas:

Fresh Weight (gm) Growth Value =

Final Weight - Initial Weight/Piece

Dry Weight (mg) Growth Value =

Final Weight - Initial Weight/Piece

These data points were plotted on semilogarithmic paper (45). The dry weight determinations were obtained by lyophilizing frozen callus tissue for 36-48 hr.

In the carbon source studies each sugar solution was sterilized separately using a 0.45 μ disposable membrane in a Millipore filterstand. The aseptic energy source was then added slowly to cooled medium which had been autoclaved at 15 lbs/in^2 for 15 minutes. After mixing the experimental media were dispensed into autoclaved jars which were allowed to cool before use. The experimental media were incubated at 37°C for 3 days to ensure a germ free condition before transferring the experimental cultures.

Pigment Determination

Chlorophyll and carotenoid content were determined quantitatively in 4 replicates of 10 pieces each (combination of 2 jars) by grinding lyophilized callus tissue in

a mortar and pestle with 90% acetone and a small quantity of $CaCO_3$. All operations were performed under diffused light at 4°C. The homogenate was incubated at -20°C for 4 hr. before clearing the pigment-acetone solution by centrifugation (13,000xg-10 min.). The optical density of the supernatant was read at 663, 645 and 480 nm against 90% acetone in a Hitachi Perkin-Elmer UV-V15 Spetophotometer. The levels of chlorophyll a, b, and total chlorophyll for the phenotypic variants were determined by Kirk's nomogram (110). The chlorophyll concentration can be easily read on the nomogram to within $\pm$ 0.1 μg/ml. Extinction coefficients (124) for pure chlorophyll a and b in 90% acetone were used to construct this nomogram. To estimate the carotenoid content, the absorbance at 480 nm was corrected for the contributions of chlorophyll a and b by equations derived by Kirk and Allen (108).

$$\delta A_{480}^{CAR} = \delta A_{480} + (0.114 \times \delta A_{663}) - (0.638 \times \delta A_{645})$$

Chlorophyll determinations of primary leaves from each phenotypic class were made on fresh material. Cold acetone was added to 0.5 gm of tissue and a small quantity of $CaCO_3$ and then homogenized as previously described for callus tissue.

Growth curves were obtained by taking fresh and dry weight measurements of samples (20 pieces-5 pieces/jar) taken periodically during a 30-day incubation period.

Doubling times, based on fresh weights, were determined during the linear phase of growth. The data values were plotted as log fresh weight (gm) growth value/piece versus time.

Column chromatography. This technique which separates the chlorophylls and carotenoids as illustrated in Fig. 19 was devised according to Strain (165,166). Special care was taken in handling of the callus extracts and the solutions of the pigments. The extraction and separation were performed as rapidly as possible and under cold conditions to avoid isomerization of the pigments. The pigments were stored under nitrogen in screw-type Ehrlemeyer flasks until the petroleum ether solutions were fractionated on a powder sugar column. Exposure of the solutions to overhead lights was avoided. The purest organic solvents were employed: acetone and hexane (Pesticide grade - Fisher Scientific or its equivalent), petroleum ether (Ligroine, 63° to 76°C - Eastman) and ethanol (absolute - United States Industrial Company).

Lyophilized callus tissue was subject to pigmental extraction as previously described. Following centrifugation (13,000xg-10 min.) the cleared extract was transferred to a 250 ml separatory funnel (pre-chilled) and diluted gently with cold petroleum ether (Ligroine, 63° to 75°C) and 10% cold aqueous salt solution. The biphasic solution

was gently rotated backwards and forwards before the lower phase was separated and disgarded. The petroleum ether phase was washed 8 times with cold distilled water. The pigments were concentrated to approximately 1 ml under a gentle stream of nitrogen. Moisture was removed from the nitrogen by glass-wool filtration. Several NaCl crystals were added to the dark-green, petroleum ether solution to eliminate remaining water.

The sugar column was packed with powdered sugar (Imperial Sugar containing 3% corn starch to prevent caking) which was dried overnight at 60°C then at 80°C for 1 hr. The glass column (1.8 cm, i.d. x 25 cm) was gently packed in small portions with dried sugar until the column bed reached 15 cm in height.

A small volume (1 ml) of concentrated, pigmental solution was allowed to adsorb onto the column and was washed with hexane containing 0.5% N-propanol until the carotenes (α and β) were eluted from the column. Under these conditions, the pigments separated in the following order: β-carotene (α-carotene), chlorophyll a, lutein (zeaxanthin), chlorophyll b, violaxanthin and neoanthin. The individual pigment zones were removed with a thin, stainless steel spatula and eluted from the adsorbent with their respective solvents: chlorophyll a and b (acetone) and xanthophylls (ethanol). Following centrifugation (13,000xg-10 min.) each pigment was identified by their respective absorption spectrum (43,124).

Cellular Observations and Chromosomal Behavior

Cellular observations and chromosomal behavior were viewed under phase-contrast microscopy. Tissue samples were taken from callus cultures of each phenotype. They were fixed in glacial acetic acid - 95% ethanol (1:3, v/v) for 24 hr. and stored in 70% ethanol. To prepare for cellular observations 30-day old callus tissue was fixed and hydrated through a graded series of ethanol (70%-50%-25%-water), hydrolized (30 min.) in 5N HCl (58), rinsed in water and then processed by the squash technique. A small piece of hydrolized tissue was placed on a glass slide (alcohol washed and stored in a slide box) and covered with 45% acetic acid. The callus tissue was teased with a needle probe to obtain smaller aggregates of cells. A cover slip (22x40 mm) was gently placed over the cells which were dispersed by applying pressure to the cover slip. After absorbing exess acetic acid (blotting between two pieces of filter paper) the slide was passed through a flame and made permanent by the freeze-dry technique (37). After removing the cover slip with a razor blade the slide was allowed to dry for 30-45 minutes and preserved in Peramount.

Chromosomal behavior was observed in 10-day old cultures which were incubated for 10 hr. in basal medium-colcemid (10 μg/ml) at equal volume to arrest the mitotic cells at metaphase and then prepared as previously described. The chromosomes were stained for 1 minute in 1% orcein

(dissolved in 45% acetic acid) before squashing. The stain was refluxed for 24 hr. and allowed to cool before use.

Each squash preparation was observed under a Wild M-20 phase contrast microscope before freezing on dry ice. Photographs of cellular observations and chromosomal behavior were taken with a Wild Photo Tube H attachment and a Wild Camera II with Kodak High Contrast Copy film. The prints were made on Kodak Kodabromide F-3 paper.

RESULTS

Growth Studies

Influence of growth hormones on certain growth characteristics. The initiated callus tissue were evaluated under different hormonal conditions (Fig. 4). In the first series (Fig. 5) small callus inocula were transferred to experimental media supplemented with different concentrations of NAA. After a 30-day incubation period all three cultured phenotypes turned brown with the exception of the cultures maintained on low concentrations of NAA (0.5 mg/l and 1.0 mg/l) in which case only the cells at the base of the callus turned brown. When cultured on intermediate levels of NAA (4 and 6 mg/l), the variants consisted of a very loose, friable mass of cells. The cultures which maintained the highest level of growth yields and chlorophyll development (visual observations) were grown on experimental media which was supplemented with 1 mg/l of NAA.[1]

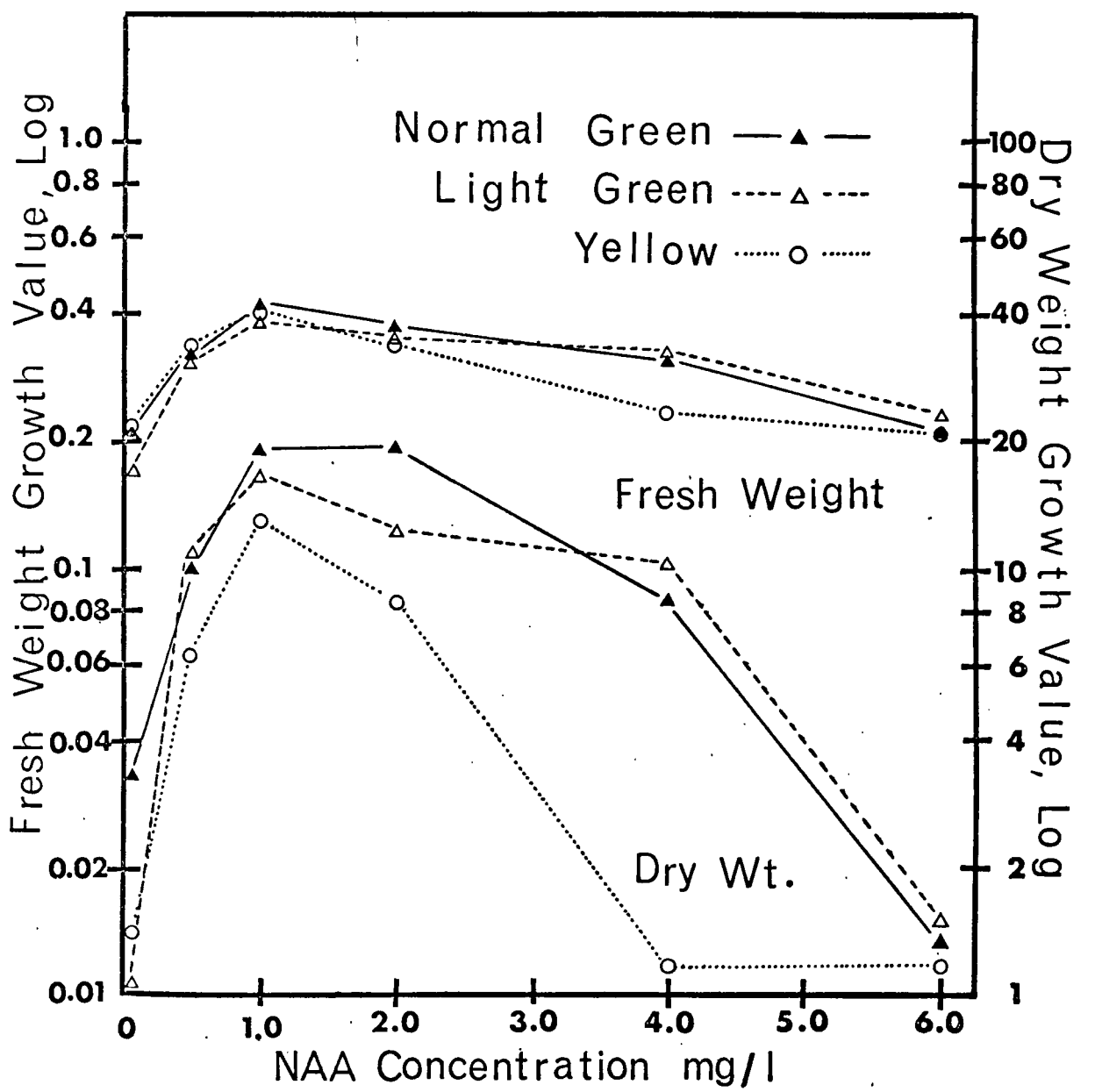
In the kinetin series, the phenotypic variants demonstrated a broad degree of sensitivity in chlorophyll development (visual observations), callus texture, and growth

[1] Preliminary studies on these callus variants indicated that these cultures required an exogenous supply of a cytokinin and/or an auxin. When maintained on the basal media without growth hormones for two successive transfers, these phenotypes turned dark brown and failed to increase in fresh weight. However, when supplemented with either NAA, IAA or 2,4-D and kinetin, the cultures demonstrated a sensitivity (callus growth and chlorophyll formation) corresponding to the combinations and concentrations of growth hormones employed.

Figure 5.

EFFECT OF NAPHTHALENE ACETIC ACID ON GROWTH YIELDS OF THREE SOYBEAN PHENOTYPES.

Each data point represents the mean value of at least 35 pieces of 30-day old callus cultures. The values at the ordinate represents the initial fresh and dry weights. The calculations for the growth values were as follows: Fr. Wt. (gm) Growth Value = Final Wt. - Init. Wt./piece. Dry Wt. (mg) Growth Value = Final Wt. - Init. Wt./piece.


Normal Green
Light Green
Yellow
Fresh Weight
Dry Wt.
Fresh Weight Growth Value, Log
Dry Weight Growth Value, Log
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
100
80
60
40
20
10
8
6
4
2
1
0
1.0
2.0
3.0
4.0
5.0
6.0
NAA Concentration mg/l

yields (Fig. 6). When cultured on intermediate (2, 4 and 6 mg/l) and high levels (10 and 20 mg/l) of kinetin, the chlorophyllous ratio of the phenotypic variants were not as readily distinguishable. In most cases the wild-type and LG variants contained appreciably high levels of chlorophyll while the Y phenotype appeared light green in coloration. In the presence of 10 and 20[1] mg/l of kinetin, the growth value decreased slightly with regional browning occurring in all phenotypic classes. When cultured on 2 mg/l and higher levels of kinetin, the variants demonstrated that the rigid texture of these cultured phenotypes was proportional to the added concentrations of kinetin. At 1 mg/l of kinetin the three soybean phenotypes were made up of a loose, friable mass of cells which maintained the expected chlorophyllous ratio that occurred in the seedling stage (Table No. 7).

As indicated by the growth values in these studies (Figs. 5 and 6) these soybean phenotypes appeared to be more sensitive to a cytokinin than an auxin. Therefore the kinetin series was repeated with the exception of the addition of NAA (1 mg/l) (Fig. 7). When cultured on kinetin (1 mg/l) and NAA (1 mg/l) the chlorophyllous nature of the NG and LG phenotypes were green in coloration while the Y

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[1] Data points at 20 mg/l of kinetin were not included in Fig. 6. The growth yields reflected the decreasing trend illustrated at 10 mg/l with the exception of the wild-type variant.

Figure 6.

EFFECT OF KINETIN ON GROWTH YIELDS OF THREE SOYBEAN PHENOTYPES.

The details are as in Figure 5.

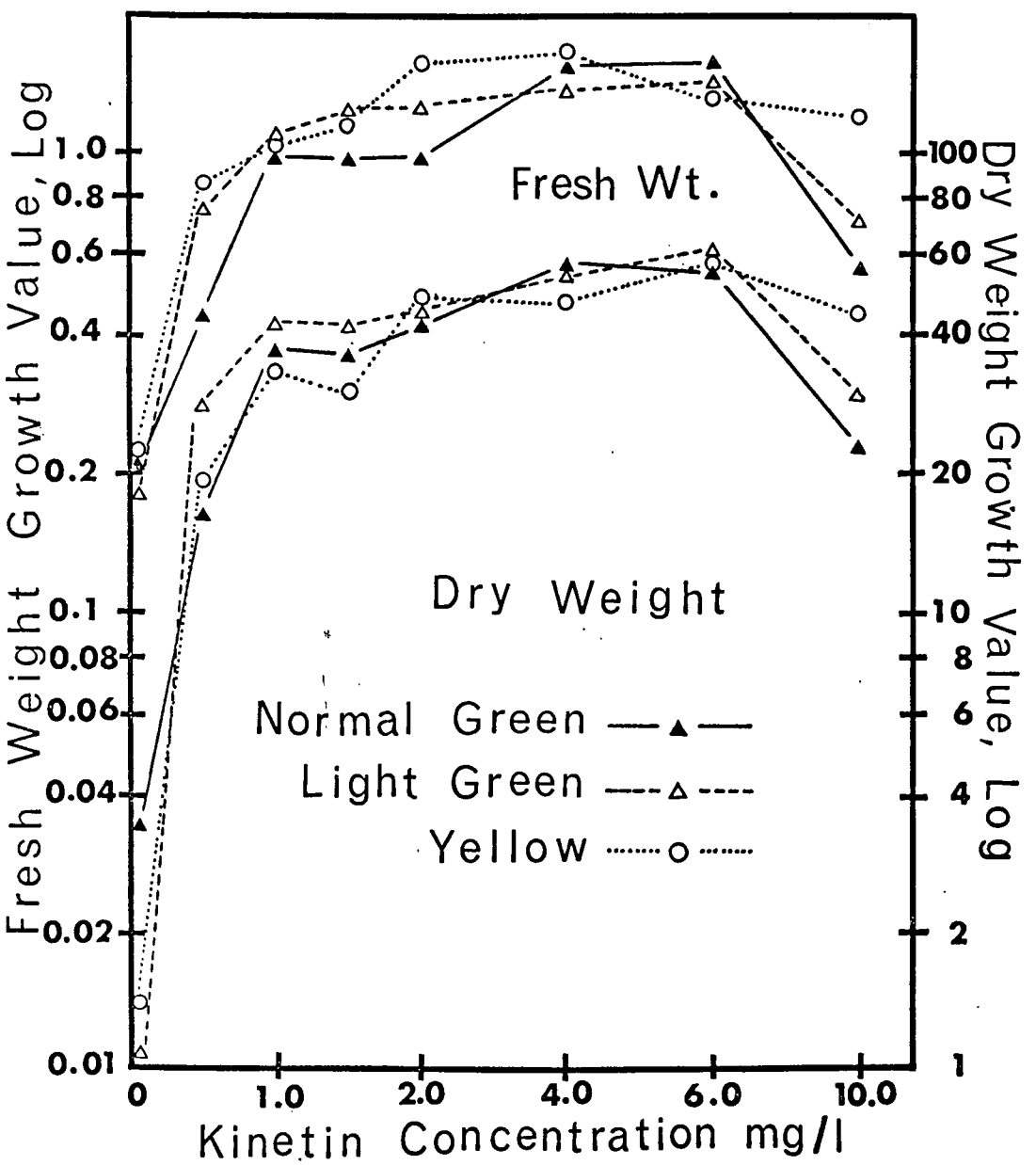
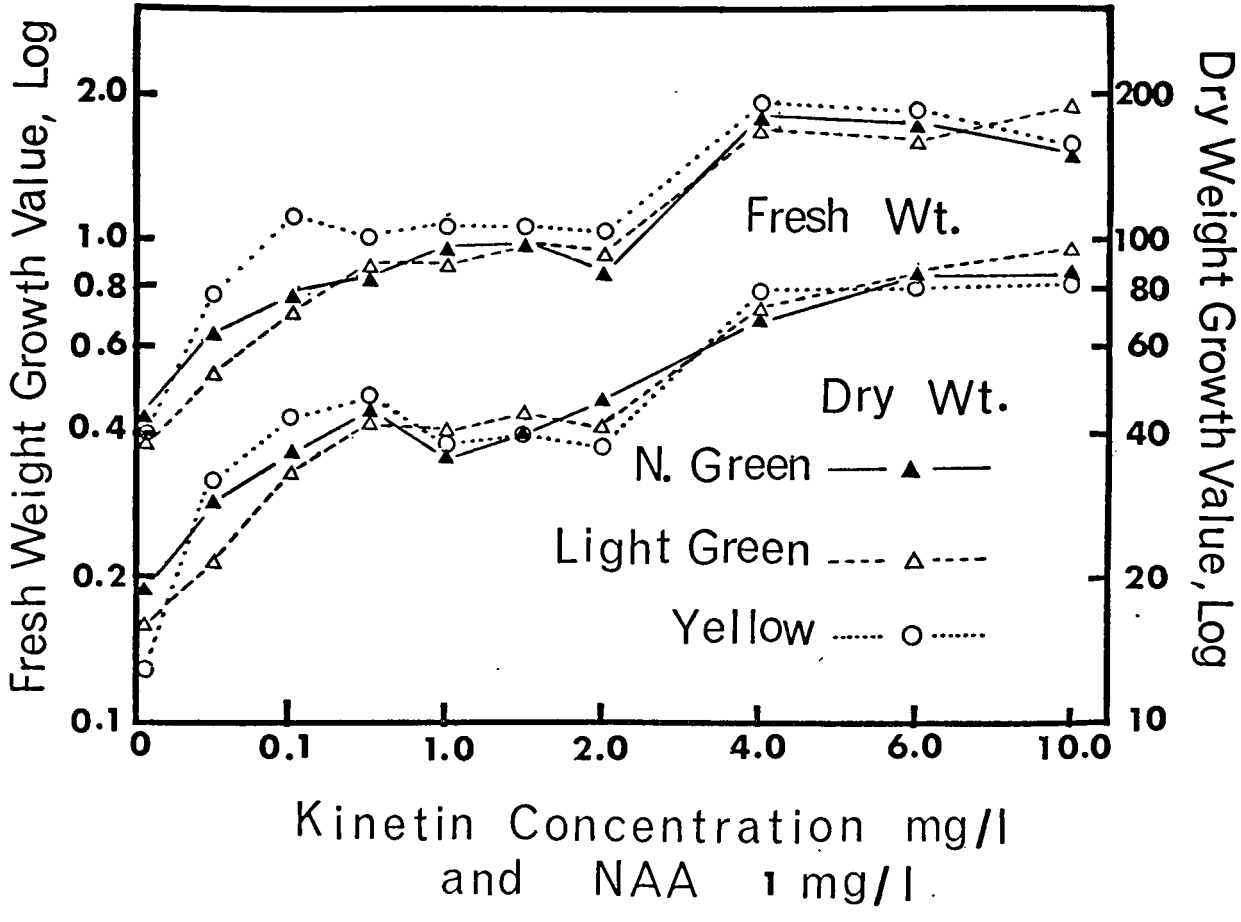

Fresh Weight Growth Value, Log
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Dry Weight Growth Value, Log
100
80
60
40
20
10
8
6
4
2
1
Fresh Wt.
Dry Weight
Normal Green
Light Green
Yellow
0
1.0
2.0
4.0
6.0
10.0
Kinetin Concentration mg/l

Figure 7.

EFFECT OF KINETIN AND NAPHTHALENE ACETIC ACID ON GROWTH YIELDS OF THREE SOYBEAN PHENOTYPES.

The values at the ordinate represents the fresh weight and dry weight determinations of the phenotypes cultured on 1 mg/l NAA. Other details are as in Figure 5.


Fresh Weight Growth Value, Log
Dry Weight Growth Value, Log
2.0
1.0
0.8
0.6
0.4
0.2
0.1
200
100
80
60
40
20
10
Fresh Wt.
Dry Wt.
N. Green
Light Green
Yellow
0
0.1
1.0
2.0
4.0
6.0
10.0
Kinetin Concentration mg/l
and NAA 1 mg/l

variant appeared light green. This hormonal supplement also supported a loose, friable mass of callus cells. The addition of NAA (1 mg/l) to the intermediate levels of kinetin supported less chlorophyll development and a less rigid callus texture in the variants than when a cytokinin was added separately.

In the presence of NAA (1 mg/l) the high levels (10 and 20[1] mg/l) of kinetin also appeared to inhibit the greening ability of all three variants whereas the expected toxicity effect on growth values due to the high cytokinin concentration was not evident as observed in the previous experiment when high levels of kinetin were added separately. The addition of NAA (1 mg/l) also lessened the toxicity effect as indicated by a decrease in browning in all three phenotypic variants. The decrease in callus friability was evident as originally observed in the cytokinin series but not to the same extent. Based on the response of these phenotypes to the cytokinin-NAA series (Fig. 7) the concentration of 1 mg/l for both growth hormones was added routinely to the basal media. It was interesting that the cells at the base of the cultures incubated on high levels of cytokinin turned orange which indicated a possible chloroplast to chromoplast transformation.

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[1] Data points at 20 mg/l kinetin + 1 mg/l NAA was not included in Fig. 7. The hormonal effect at this level supported similar growth values as noted at 10 mg/l kinetin plus 1 mg/l NAA.

Influence of several supplements on certain growth characteristics. The addition of growth hormones in various combinations and specific supplements to the basal media revealed a broad sensitivity to growth values, chlorophyll development (visual observation), callus texture, browning and organ development (Table No. 2 and Fig. 4). The addition of coconut milk and yeast extract had opposite effects on growth values and chlorophyll development. Alone or in combination with growth hormones coconut milk supported substantial growth values and chlorophyll development whereas yeast extract inhibited these growth characteristics. When coconut milk was added in combination with yeast extract, with or without growth hormones, the detrimental condition (browning) due to the presence of yeast extract was minimal. Organ development in the form of fine root-hair differentiation was observed in callus cultures which were incubated under the following conditions: NAA-coconut milk and coconut milk-yeast extract (Table No. 2 and Fig. 4).

When coconut milk was added to an intermediate kinetin concentration (6 mg/l) comparable growth values and high chlorophyllous levels were maintained in the phenotypic classes. The addition of NAA (1 mg/l) to these growth stimulants resulted in an increase in growth values and a decrease in a greening ability in all three phenotypes. The callus texture of this cultured system which were grown under these conditions resulted in considerable increase in a rigid callus condition.

Table No. 2. Growth yields and observed metabolic events of three soybean phenotypes.*

Additives to Basal Media**	Growth Values			Metabolic Events		
	NG	LG	Y	Normal Green	Light Green	Yellow
Coconut Milk	0.63	0.85	0.82	Green	Green	Light Green
Yeast Extract	0.10	0.11	0.11	Dark Brown	Dark Brown	Dark Brown
Coconut Milk + Yeast Extract	0.65	0.59	0.66	Green Fine Root Hairs	Green Fine Root Hairs	Yellow Green Fine Root Hairs
NAA + Coconut Milk	0.77	0.88	0.86	Dark Green Some Fine Root Hairs	Green Some Fine Root Hairs	Yellow Green Some Fine Root Hairs
NAA + Yeast Extract	0.15	0.13	0.16	Dark Brown	Dark Brown	Dark Brown
NAA + Coconut Milk + Yeast Extract	0.49	1.01	1.09	Explant Brown	Explant Brown Light Green In New Tissue	Explant Brown Yellow Green In New Tissue
Kinetin + Coconut Milk	0.87	0.84	1.08	Green	Green	Light Green
Kinetin + Yeast Extract	0.40	0.49	1.02	Explant Brown Green In New Tissue	Explant Brown Green In New Tissue	Explant Brown Yellow Green In New Tissue

*,** For details, see the end of Table No. 2.

Table No. 2 Continued. Growth yields and observed metabolic events of three soybean phenotypes.*

Additives to Basal Media**	Growth Values			Observations		
	NG	LG	Y	Normal Green	Light Green	Yellow
Kinetin + Coconut Milk + Yeast Extract	1.04	1.60	1.42	Green	Green	Light Green
NAA + Kinetin + Coconut Milk	0.94	1.14	1.23	Dark Green	Dark Green	Yellow Green
NAA + Kinetin + Yeast Extract	0.91	0.92	0.87	Dark Green (R)	Light Green (R)	Light Green (R)
NAA + Kinetin + Coconut Milk + Yeast Extract	1.43	1.22	1.23	Dark Green (R)	Dark Green (R)	Dark Green (R)
Kinetin (6 mg/l) + Coconut Milk	1.16	1.27	1.23	Dark Green (R)	Dark Green (R)	Light Green (R)
Kinetin (6 mg/l) + NAA + Coconut Milk	1.29	1.38	1.69	Green (R)	Green (R)	Light Green (R)
IAA + Kinetin	1.09	1.09	0.98	Green (R)	Brownish Green	Light Green
IAA + Kinetin (2 mg/l)	1.07	1.02	1.10	Brownish Green	Brownish Green	Brownish Green (R)

*,** For details, see the end of Table No. 2.

Table No. 2 Continued. Growth yields and observed metabolic events of three soybean phenotypes.*

Additives to Basal Media**	Growth Values			Metabolic Events		
	NG	LG	Y	Normal Green	Light Green	Yellow
NAA + Kinetin + Casein Hydrol.	0.99	1.06	1.34	Brownish Green (R)	Brownish Green (R)	Light Green
NAA + Kinetin + Amino Acid Mix.	1.13	1.47	1.18	Dark Green	Light Green	Light Green

* Concentrations of various additives unless already noted: NAA (1 mg/l), IAA (1 mg/l), Kinetin (1 mg/l), Coconut Milk (10%), Yeast Extract (0.1%), Casein Hydrolysate (0.01%), and Amino Acid Mixture; L-Glutamate (3 mg/l), L-Glycine (3 mg/l), L-Methionine (1 mg/l), L-Aspartate (100 mg/l) and L-Phenalalanine (1 mg/l). The texture of the callus cultures was of a flacid nature unless otherwise noted (rigid - R). Growth parameter (fresh weight (gm) growth value/piece) and observations were made of 30-day old callus cultures.

** The basal media employed was a modification of Miller's media (127) as described in Table No. 1.

The addition of IAA (1 mg/l) and kinetin (1 and 2 mg/l) did not improve the growth characteristics of the phenotypic variants (Table No. 2). The addition of caesin hydrolysate or an amino acid mixture in combination with growth hormones supported approximately similar growth values but had an opposite effect on chlorophyll development and browning. The amino acid mixture stimulated a friable, chlorophyllous callus tissue whereas the caesin hydrolysate supported a rigid callus condition with regional browning in the wild-type and LG strains.

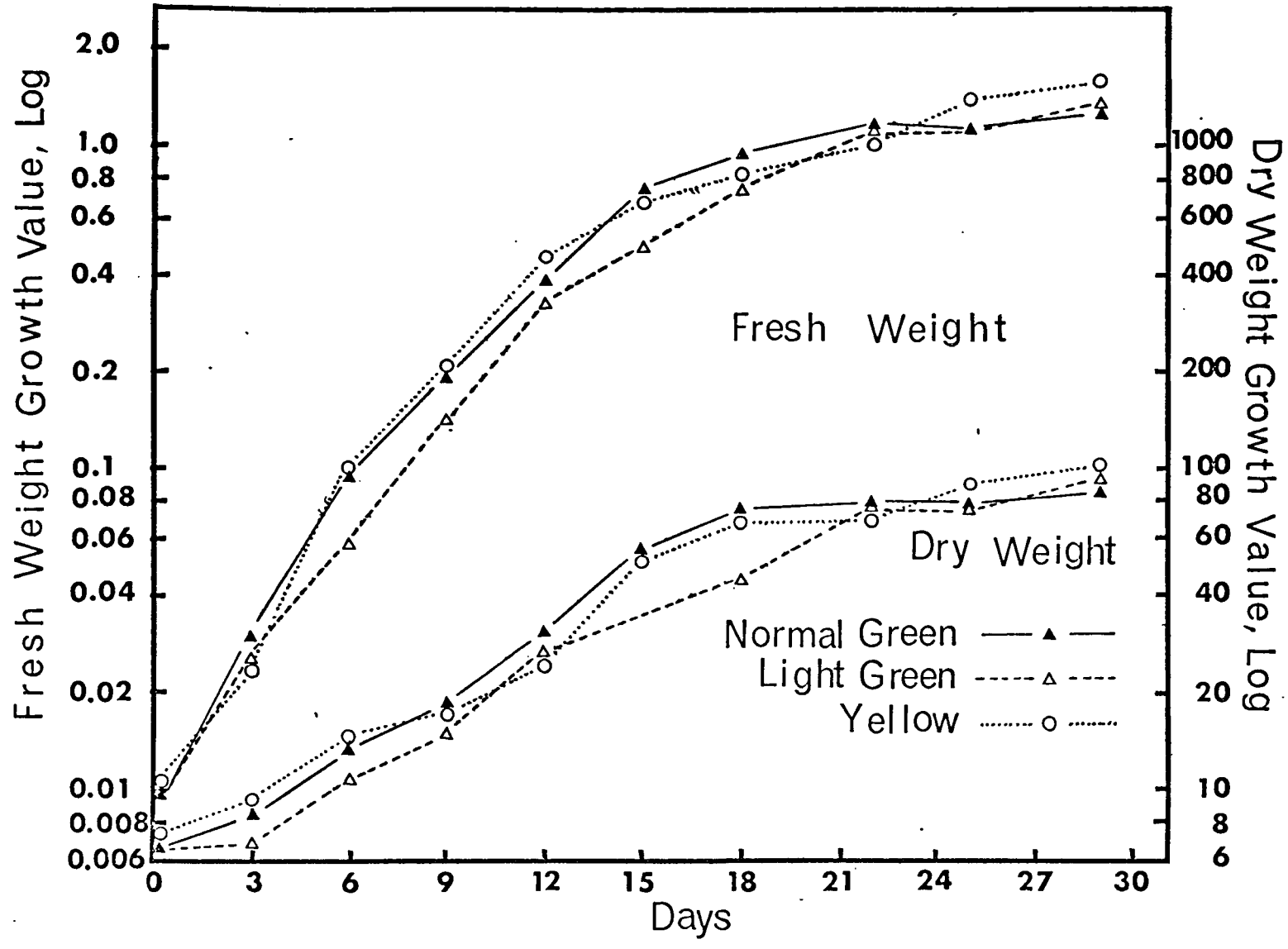
Growth curve of light grown tissues. The growth curves (Fig. 8) obtained from callus tissue grown on basal media containing kinetin (1 mg/l), NAA (1 mg/l) and ascorbic acid (50 mg/l) revealed a typical sigmoid pattern of growth which has been demonstrated in several cultured systems of higher plants (42,62,90,120). As indicated by growth values the lag phase was not present while the exponential phase was very short; if not, non-existant. The highest growth rate (linear phase) occurred from 3 to 12 days with a subsequent decelerated growth rate following to 22 days and then the cells entered the stationary phase. Under the standard conditions of culture employed, the cell doubling time during the phase of linear growth was 41 hr.

The growth curves of the soybean phenotypes were similar in respect to two growth patters. No statistically differential growth rates were observed. Secondly a growth

Figure 8.

GROWTH CURVE OF THREE CHLOROPHYLLOUS SOYBEAN PHENOTYPES

Each point represents the mean of 4 replicates of 5 pieces each. The values at the ordinate represents the initial fresh and dry weight of a representative sample. S.E.M. for each fresh weight data point was omitted from the the growth curve in order to sustain clarity of the figure. No significant difference was observed. Other details are as in Figure 5.


Fresh Weight Growth Value, Log
2.0
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
0.008
0.006
Dry Weight Growth Value, Log
1000
800
600
400
200
100
80
60
40
20
10
8
6
0
3
6
9
12
15
18
21
24
27
30
Days
Fresh Weight
Dry Weight
Normal Green
Light Green
Yellow

values was observed which has been demonstrated in callus cultures of bush bean (120). The authors attributed this increase (fresh weight over dry weight) to a greater uptake of water content in response to cell expansion.

Carbon source studies. The three cultured phenotypes were incubated on basal media containing different carbon sources (Table No. 3). When added to the test media, alone or in combination with glucose or fructose, exogenous sucrose provided substantial carbon and energy sources to support an appropriate amount of growth. The substitution of either glucose or fructose for sucrose as a carbon source produced varied results. When cultured in the presence of glucose, the growth values of all three phenotypes were inhibited (NG 91%; LG 66%; and Y 79%). In the case of the use of fructose as the sole carbon source the growth yield of the wild-type was inhibited (52%) whereas no significantly different growth rates were observed in the other two phenotypes under the experimental conditions employed. Autoclaved sucrose did not improve the growth capacity of these phenotypes.

Cellular observations of 30-day callus tissue. The phenotypic variants when grown under light and dark environmental conditions revealed morphological uniformity with respect to cell size and shape, tissue texture, cytological appearance and degree of organization (Fig. 9). The yellow phenotype consisted of slightly larger and more oval shaped cells

Table No. 3. Comparison of growth yeidls of three soybean phenotypes cultured on various carbon sources (30-days growth).

Basal Media* Plus Carbon Source	Fresh Weight (gm) Growth Value/Piece		
	Normal Green	Light Green	Yellow
3% Sucrose**	1.22*** ±0.11	1.43 ±0.15	1.25 ±0.19
3% Glucose**	0.12 ±0.01	0.35 ±0.04	0.26 ±0.03
3% Fructose**	0.59 ±0.15	1.19 ±0.15	1.06 ±0.19
1.5% Sucrose + 1.5% Glucose**	1.26 ±0.15	1.39 ±0.21	1.51 ±0.24
1.5% Sucrose + 1.5% Fructose**	1.28 ±0.15	1.59 ±0.05	1.42 ±0.17
3% Sucrose (autoclaved)	1.19 ±0.08	1.25 ±0.08	1.31 ±0.10

* The basal media employed was a modification of Miller's media as described in Table 1.

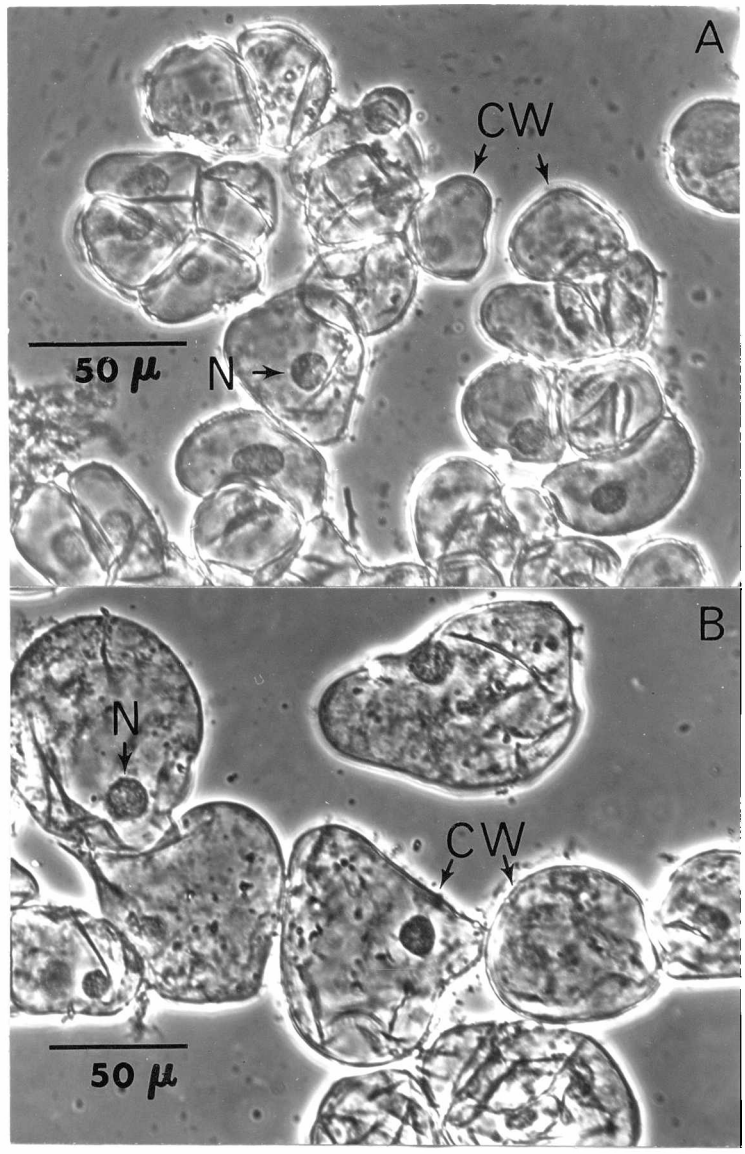
** Each carbon source was filtered separately then added to cooled media as described in Materials and Methods.

*** S.E.M. was determined on 8 replicates of 5 pieces each.

Figure 9.

MORPHOLOGICAL BEHAVIOR OF 30-DAY OLD CALLUS CULTURES OF THREE SOYBEAN PHENOTYPES CULTURED UNDER DARK ENVIRONMENTAL CONDITIONS.

A. Cluster of cells from the normal green phenotype: nucleus (N) and cell wall (CW). The light green phenotype consisted of similar parenchymous-like cells. B. Cluster of cells from the yellow phenotype: details as in Figure 9A.


A
CW
50 μ
N
B
N
CW
50 μ

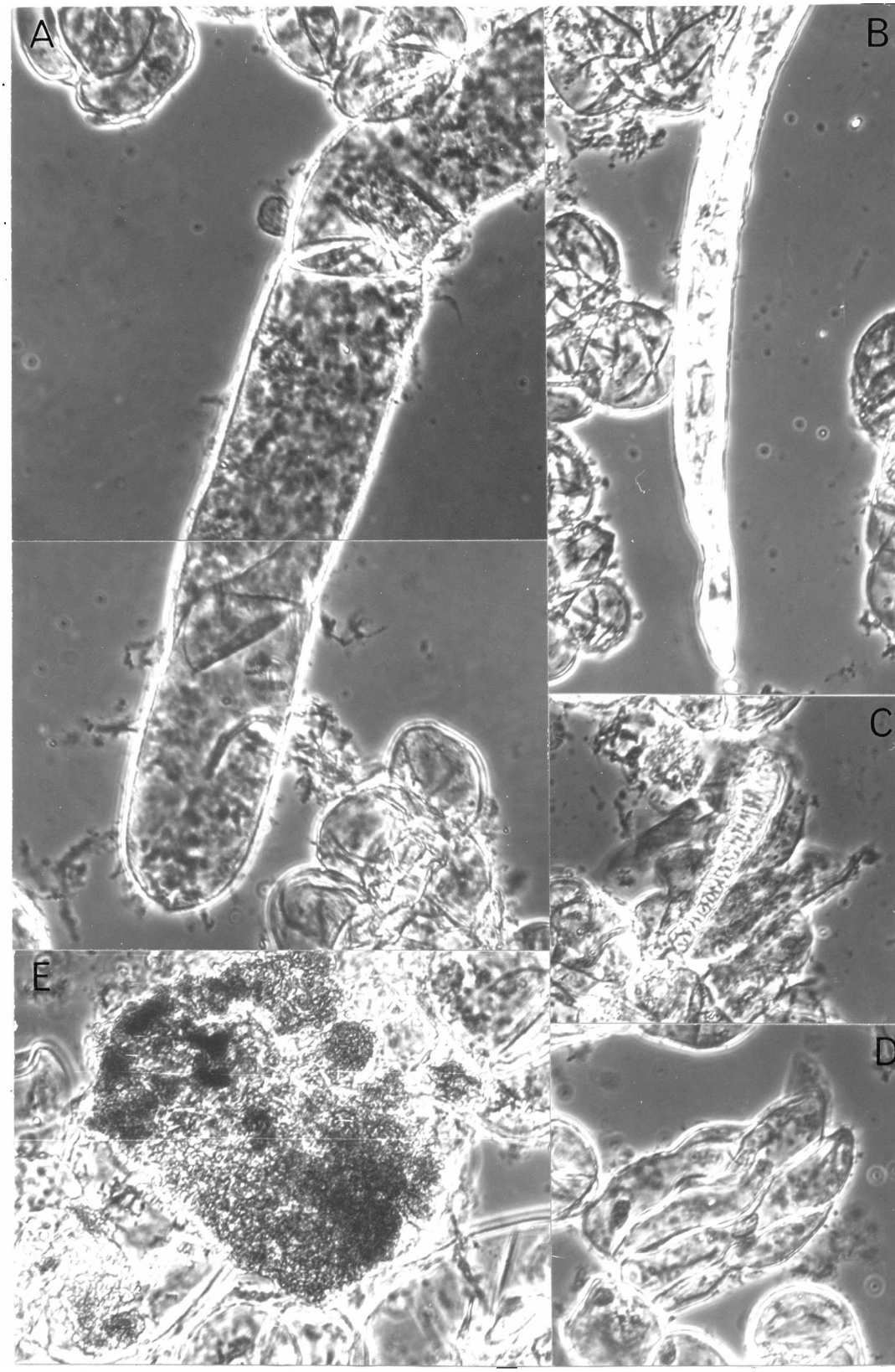
(90x70μ; dimens.) in comparison to the NG and LG variants. The cell types were generally parenchymous-like cells (47x26μ; dimens.) (Fig. 9) with an occasional observation of elongated (120x50μ; dimens.), giant (250x90μ; dimens.), and irregular shaped cells (Fig. 10). A certain degree of differentiation was noted in the form of isolated tracheids with scalariform perforations and an occasional fiber-traeheid (Fig. 10B). Branching tracheid systems were found in some callus fragments which indicated a high degree of cellular organization existed in some callus cultures. No correlation between the callus texture and the number of tracheids was observed. Occasionally an aggregate of orange colored deposits was observed externally and usually associated with a cluster of cells (Fig. 10E). Generally the LG phenotype was not as friable as the wild-type and yellow variants. No morphological difference was distinguishable in light and dark grown cells at the magnification employed (Fig. 9A,B). The three cultured phenotypes under both light and dark conditions consisted of quiescent cells: the nucleus was located along the cell wall possibly due to the presence of a large central vacuole. At this magnification the external cell walls appeared thick and uniform and no cytoplasmic organelles or strands were observed.

Subcellular observations and chromosomal behavior in 10-day callus cultures. Within the limitations of increased manification, the subcellular integrity of the three chlorophyl-

Figure 10.

IRREGULARITIES OBSERVED IN 30-DAY OLD CALLUS CULTURES OF THREE SOYBEAN PHENOTYPES CULTURED UNDER LIGHT AND DARK ENVIRONMENTAL CONDITIONS.

A. Pholem-like cells from the light green phenotype grown under light environmental conditions (400x). B. Fiber tracheid from the normal green phenotype grown under light environmental conditions (400x). C. Tracheid showing a scalariform pattern from the normal green phenotype grown under dark environmental conditions (400x). D. Irregular shaped cells from the light green phenotype grown under light environmental conditions (400x). E. External accumulation of orange-colored deposits found in the light green phenotype grown under dark environmental conditions (400x).


A
B
C
D
E

lous variants became evident in respect to cytoplasmic organelles (Figs. 11 and 12). As early as 10-days the nucleus was located along the cell wall due to the presence of an enlarging vacuole. The nucleolus was situated in the center of the nucleus which was enclosed by the nucleoplasms. The NG and LG phenotypes possessed several chloroplasts within cytoplasmic folds and along the cell wall. Plastids were not distinguishable in the Y phenotype at this manification.

Several irregularities were observed under light conditions: a lobed nucleus, droplets possibly aliphatic in nature, and an elongated cell with an irregular shaped nucleus (Figs. 11A and 12A,B). Under dark conditions, starch grains were observed (Fig. 12D).

The instability of chromosomes is a common occurrence in plant tissue cultures (77,184) and these cultured variants were no exception. Microscopic observations of the chromosomal behavior of 10-day old callus cultures were made of both light and dark grown cells which had been submerged in a liquid media : colcemid solution (composition, see Materials and Methods). In comparison to the number of observations made, relatively few mitotic figures were observed. However the chromosomal complement was noted in several mitotic cells which appeared to be tetraploid (4 N = 80) (Fig. 12C). The remaining cells were arrested in the early prophase which indicates that a longer incubation period in the media : colcemid solution is required

Figure 11.

MORPHOLOGICAL BEHAVIOR OF 10-DAY OLD CALLUS CULTURES OF THREE SOYBEAN PHENOTYPES CULTURED UNDER LIGHT ENVIRONMENTAL CONDITIONS.

A. Normal green phenotype; bilobed nucleus (BN), nucleolus (NUC), chloroplast (C), vacuole (V), cell wall (CW), and cytoplasm (CY) (2100X). B. Light green phenotype; nucleus (N), details as in Figure 11A (2100X). C. Yellow phenotype; details as in Figure 11 B (2100X).

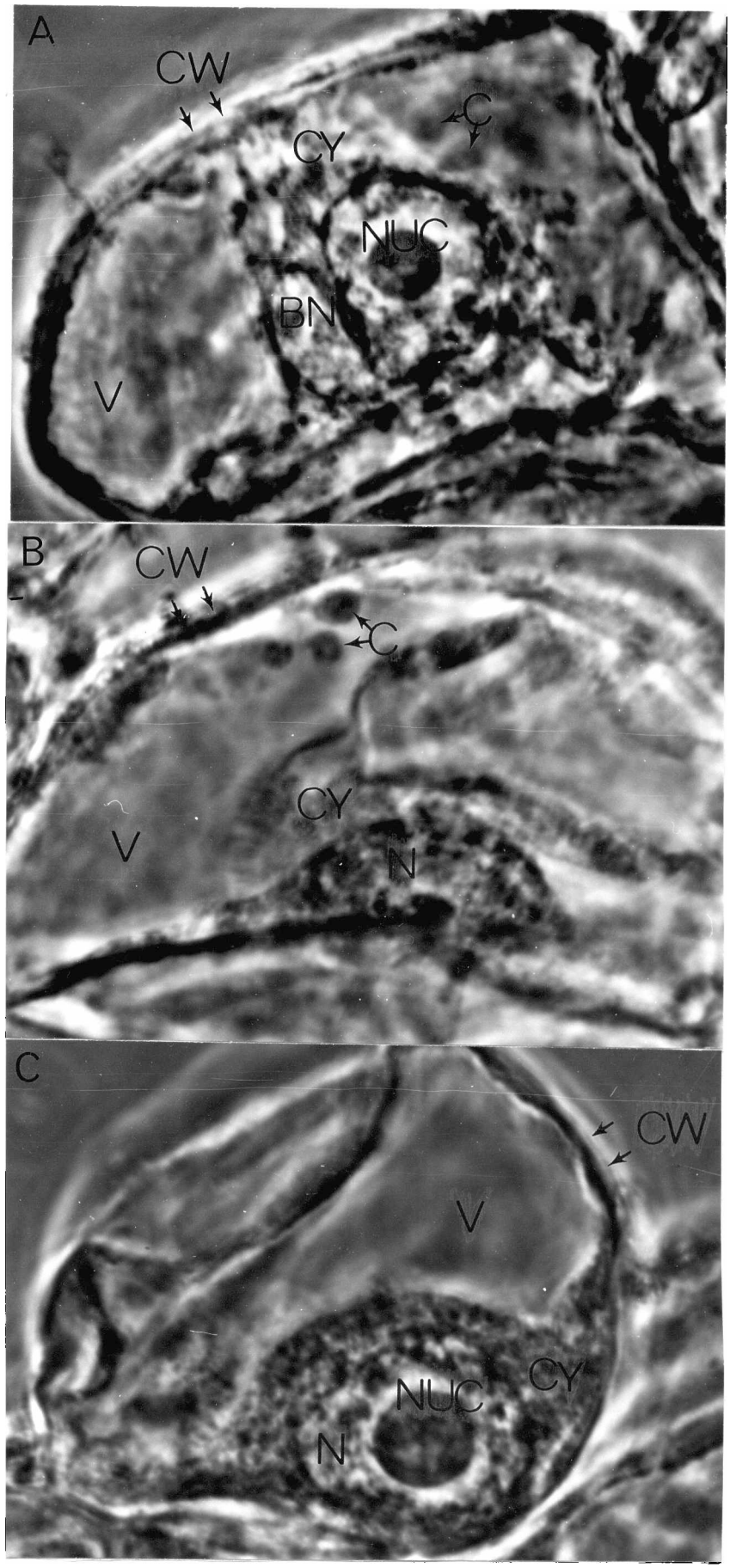
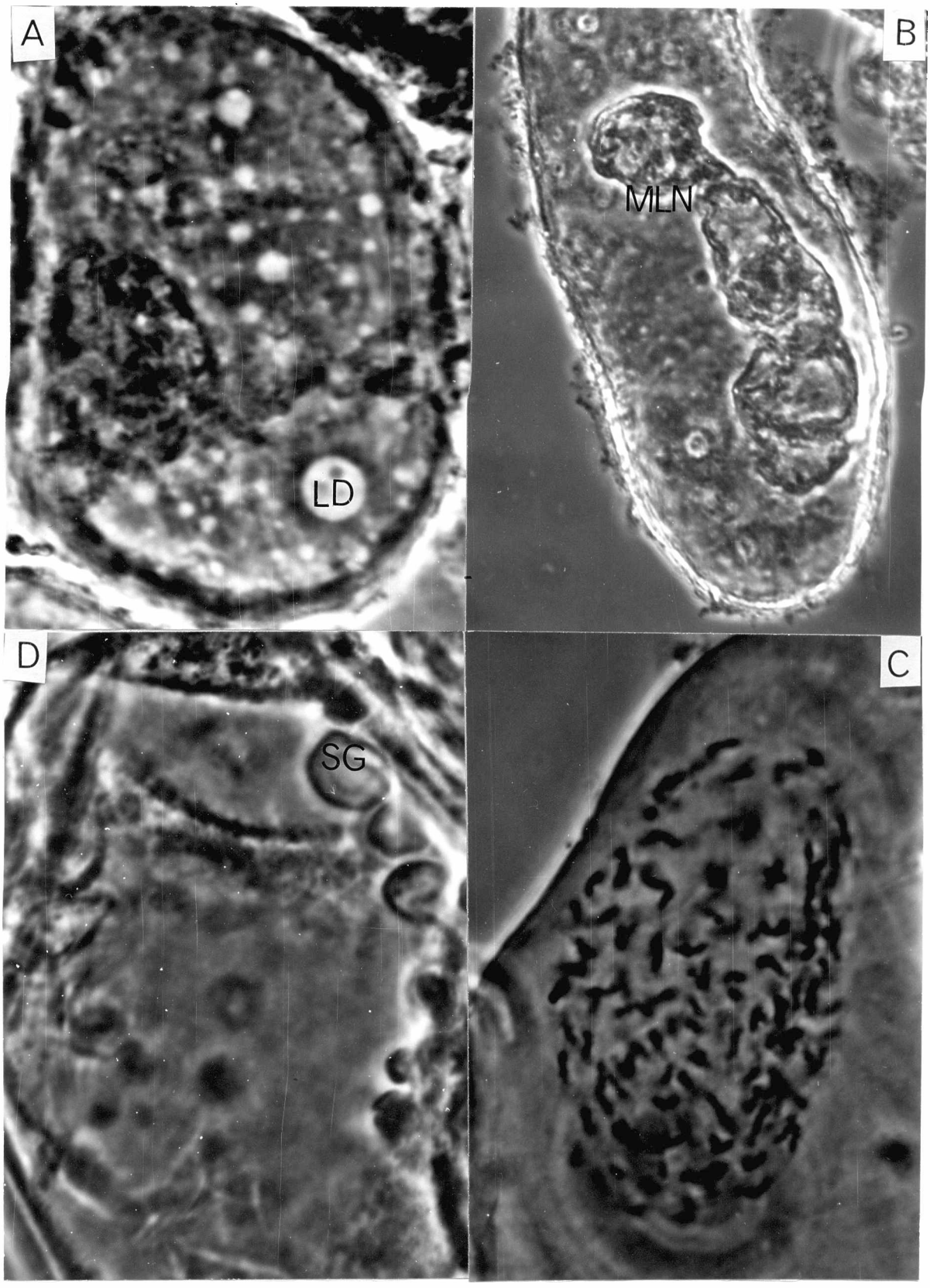

A
CW
C
CY
NUC
BN
V
B
CW
C
CY
V
N
C
CW
V
CY
NUC
N

Figure 12.

MORPHOLOGICAL BEHAVIOR OF 10-DAY OLD CALLUS CULTURES OF THREE SOYBEAN PHENOTYPES CULTURED UNDER LIGHT AND DARK ENVIRONMENTAL CONDITIONS.

A. Normal green phenotype grown under light conditions; nucleus (N), lipid droplets (LD) (2100X). B. Light green phenotype grown under light conditions, multilobed nucleus (MLN) in a elongated cell (2100X). C. Normal green phenotype grown under dark conditions and treated with media: colcemid as described in Materials and Methods, possible tetraploid complement of chromosomes (2N=40). D. Normal green phenotype grown under dark conditions; starch grains (SG) (2100X).


A
LD
B
MLN
D
SG
C

in order to determine chromosomal frequencies of these soybean strains.

Pigmentation of Three Soybean Callus Phenotypes

Preliminary results on chlorophyll and carotenoid extraction of three soybean phenotypes. During preliminary experimentation it became apparent that specific deviation from the general procedure usually employed for chlorophyll extraction were necessary. First of all the extraction of chlorophyllous tissue with small volumes (15 ml) of acetone facilitated the handling of a large number of samples. This proved to be a difficult situation when fresh callus tissue which contained approximately 90% water was used as source material. As noted in Table No. 4 the first acetone extraction of hydrated tissue was insufficient for complete pigmental solubility as indicated by the large standard deviations and the high levels of chlorophyll found in the remaining callus debri. After lyophilization of frozen tissue complete extraction of chlorophyll was accomplished with one homogenation in 90% acetone.

The effect of various carbon sources on the pigmentation of three soybean phenotypes. The type of carbon source used in the experimental media influenced the chlorophyll content in this cultured system (Table No. 5). The phenotypes differed in sensitivity towards the carbon source employed not only with respect to total chlorophyll accumulation but also to the phenotypic expression for

Table No. 4. Methodological procedure on chlorophyll extraction of three soybean phenotypes.*

Treatment**	Normal Green µg/gm fr. wt.			Light Green µg/gm fr. wt.			Yellow µg/gm fr. wt.		
	Chl a	Chl b	Chl a+b	Chl a	Chl b	Chl a+b	Chl a	Chl b	Chl a+b
	***								
No. 1	8.62 ±0.93	3.25 ±0.20	11.86 ±1.13	16.64 ±1.41	6.36 ±0.55	22.99 ±1.95	8.13 ±3.04	2.97 ±1.04	11.10 ±4.07
No. 2	2.87 ±2.14	1.18 ±0.80	4.05 ±2.69	6.11 ±1.34	3.24 ±0.60	9.45 ±6.54	2.62 ±1.76	1.38 ±0.72	4.00 ±2.49
No. 3	8.47 ±4.05	2.88 ±0.44	11.35 ±2.17	8.62 ±1.21	2.83 ±0.37	11.44 ±1.56	3.18 ±0.70	1.05 ±0.19	4.23 ±3.98

* The three phenotypes were cultured on a modification of Miller's media (127) as previously described in Table No. 1 (addition of 50 mg/l ascorbic acid).

** No. 1. Lypholized tissue was homogenized as previously described in Materials and Methods. No. 2. Fresh weight tissue was homogenized in 15 ml of 100% acetone and stored in the refrigerator for 2 hr. Following centrifugation (13,000x g-10 min.), the supernatant was considered the first extraction. No. 3. The pellet of No. 2 preparation was subjected to a second extraction by adding 10 ml (NG and LG) and 5 ml (Y) of 90% acetone and stored in the refrigerator for an additional 4 hr.

*** Mean ± S.E.M. for 4 replicates of 5 pieces each.

Table No. 5. Comparison of pigmentation of three soybean phenotypes cultured on various carbon sources.

Carbon Source	Normal Green (µg/gm fr. wt.) Chl a+b	Chl a	Chl b	Chl a/b ratio	** CAR 480	Light Green (µg/gm fr. wt.) Chl a+b	Chl a	Chl b	Chl a/b ratio	** CAR 480	Yellow (µg/gm fr. wt.) Chl a+b	Chl a	Chl b	Chl a/b ratio	** CAR 480
3% Sucrose*	*** 24.98 ±2.18	17.75 ±1.65	7.18 ±0.71	2.48	0.60	7.72 ±2.60	5.12 ±1.79	2.60 ±0.82	1.97	0.23	29.42 ±12.27	21.95 ±9.11	7.47 ±3.17	2.94	0.59
3% Glucose*	105.82 ±20.77	71.38 ±15.38	34.38 ±7.56	2.08	1.46	30.49 ±5.58	20.70 ±3.99	9.74 ±1.55	2.13	0.60	64.48 ±18.29	49.10 ±9.21	23.32 ±7.00	2.11	1.08
3% Fructose*	76.75 ±20.78	53.42 ±14.03	23.33 ±6.75	2.29	1.15	26.93 ±3.35	18.85 ±2.36	8.07 ±1.09	2.34	0.50	17.30 ±5.17	12.20 ±3.78	5.10 ±1.39	2.39	0.41
1.5% Sucrose* 1.5% Glucose	21.79 ±2.45	15.20 ±1.83	6.37 ±1.02	2.39	0.42	31.99 ±5.09	22.35 ±3.75	9.65 ±1.36	2.32	0.74	11.67 ±2.94	8.85 ±2.16	3.27 ±0.81	2.71	0.40
1.5% Sucrose* 1.5% Fructose	39.32 ±5.41	24.77 ±4.26	14.55 ±1.74	1.70	0.60	34.14 ±3.33	21.69 ±3.14	12.45 ±0.20	1.74	0.49	5.50 ±1.44	3.93 ±1.02	1.58 ±0.45	2.49	0.16
3% Sucrose (autoclaved)	26.47 ±19.13	17.06 ±6.53	9.46 ±2.20	1.80	0.54	17.77 ±2.79	11.46 ±1.92	6.30 ±0.89	1.82	0.45	17.14 ±2.43	12.25 ±1.76	4.89 ±0.69	2.51	0.36

* Each carbon source was filtered separately and then added to the experimental media as previously described in Materials and Methods.

** Carotenoid determinations: $\overset{CAR}{480}$ = Optical Density (O.D.) x ml acetone/gm fr. wt.

*** Mean ± S.E.M. for replicates of 10 pieces each.

pigmentation. In the presence of sucrose or in combination with glucose or fructose the expected phenotypic ratio for chlorophyll formation was observed in the three phenotypes except when the LG and Y phenotypes were cultured on sucrose (filtered) and the LG phenotype was cultured on sucrose-glucose. The LG phenotype when cultured on sucrose accumulated a lower level than expected whereas in the sucrose-glucose combination the chlorophyll content of this phenotype was higher than what was expected. Also the Y phenotype when cultured in the presence of sucrose (filtered) maintained a higher level of chlorophyll accumulation than both the wild-type and LG phenotypes. The LG phenotype was more sensitive to autoclaved sucrose in comparison to filtered sucrose than the wild-type and Y phenotype.

When glucose and fructose were added separately to the experimental media the phenotypic variants accumulated a high level of chlorophyll as well as maintaining the expected phenotypic ratio with the exception of the LG phenotype when cultured on glucose. In contrast the presence of these carbon sources in combination with sucrose decreased the chlorophyll accumulation in these cultured variants. The increased sensitivity of chlorophyll development in these phenotypes to glucose was accompanied with a very low growth rate. Therefore the chlorophyll concentrations were calculated on the basis of number of pieces instead of fresh weight (Table No. 6).

Table No. 6. Comparison of total chlorophyll of three soybean phenotypes cultured on various carbon sources.*

Carbon Source	Normal Green			Light Green			Yellow		
	Chl µg/10 pieces			Chl µg/10 pieces			Chl µg/10 pieces		
	Chl a+b	Chl a	Chl b	Chl a+b	Chl a	Chl b	Chl a+b	Chl a	Chl b
3% Sucrose**	60.87	43.37	17.50	21.78	14.43	7.35	71.59	53.43	18.17
3% Glucose**	25.56	17.27	8.29	21.01	14.29	6.71	30.75	21.80	8.95
3% Fructose**	98.41	68.31	30.11	66.13	46.27	19.86	36.15	25.48	10.68
1.5% Sucrose** + 1.5% Glucose	54.72	38.72	15.99	88.62	61.85	26.77	45.83	33.13	12.70
1.5% Sucrose** + 1.5% Fructose	100.49	63.17	37.32	82.27	53.30	28.97	19.71	14.01	5.70
3% Sucrose (autoclaved)	62.65	40.36	22.50	44.62	28.78	15.85	45.03	32.18	12.85

* Each data value was determined on the basis of total number (10) of pieces.

** Each carbon source was filtered separately and then added to the experimental media as previously described in Materials and Methods.

In this case the phenotypes that were incubated on glucose supported the lowest level of chlorophyll development. This reversal did not occur when the wild-type was cultured on fructose where the growth value was approximately one-half of the NG sucrose-grown callus cultures (Table No. 6). Also chlorophyll accumulation in the phenotypic variants cultured on sucrose (filtered and autoclaved), fructose and their combinations reflected the same metabolic trend as previously described (Table No. 6).

The amount of chlorophyll formation in this carbon source study was compared favorably to other systems (61, 125,193,198) but the level accumulated in primary leaves of these soybean phenotypes (Table No. 7) was considerably higher than its respective cultured phenotypes. In most cases the phenotypic variants also accumulated representative levels of carotenoids in proportional amounts to the chlorophyll content. Subsequently the carotenoid biosynthesis in these cultured phenotypes appeared to be dependent upon the synthesis of chlorophyll.

Effect of ascorbic acid on the growth rate and pigmentation of three soybean phenotypes. With the intent to improve the autotrophic capabilities of these variants small inoculum were incubated on the basal media which was supplemented with kinetin (1 mg/l), NAA (1 mg/l) and various levels of

Table No. 7. Pigmentation in primary leaves of germinated seedlings of three phenotypes.

Time (days)	Normal Green				Light Green				Yellow			
	mg/gm fr. wt.				mg/gm fr. wt.				mg/gm fr. wt.			
	Chl a+b	Chl a	Chl b	CAR* 480	Chl a+b	Chl a	Chl b	CAR* 480	Chl a+b	Chl a	Chl b	CAR* 480
12	1.97	1.42	.55	50.4	1.36	1.02	.34	36.8	.16	.14	.02	12.00
17	2.64	1.97	.67	72.0	1.54	1.23	.30	48.8	.30	.22	.08	21.60
19	3.22	2.67	.65	93.6	2.24	1.73	.51	40.0	.38	.32	.06	16.40

* Carotenoid determinations: $\frac{CAR}{480}$ = Optical Density (O.D.) x ml acetone/gm fr.wt.

ascorbic acid*. Callus tissue of each phenotype grew well on all experimental media employed as indicated by the growth values (Table No. 8) but supported different degrees of total chlorophyll accumulation (Fig. 13). The addition of 75 mg/l ascorbic acid to the basal media stimulated the highest level of chlorophyll synthesis in all three phenotypic variants and at this concentration the variants maintained their phenotypic expression for chlorophyll development. This was not the case for the remaining levels of ascorbic acid. The LG phenotype consistantly contained higher levels of chlorophyll formation than the NG and Y phenotypes indicating that this variant possessed a broader range of sensitivity to ascorbic acid with respect to pigmentation. With this in mind 75 mg/l of ascorbic acid was routinely added to the basal media for stock and experimental cultures.

The chlorophyll a/b ratios of the phenotypes were effected when ascorbic acid was added to the basal media (Table No. 8). High ratios occurred in these variants at different levels of ascorbic acid: NG phenotype at 25 mg/l, LG phenotype at 25-100 mg/l and Y phenotype at 50 mg/l. The Y phenotype maintained higher chlorophyll

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* The stock cultures for this experiment were maintained on the basal media as previously described (Table No. 1) without the addition of ascorbic acid for one month before use.

Table No. 8. Growth yields and pigmentation of three soybean phenotypes cultured on various concentrations of ascorbic acid

Additive	Normal Green					Light Green					Yellow				
	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480
Control	1.24 ±.08***	9.66 ±2.61	4.02 ±.72	2.40	1.39	1.04 ±.12	17.46 ±.74	8.15 ±1.29	2.14	1.66	1.32 ±.08	3.49 ±.63	1.21 ±.12	2.87	.88
Ascorbic Acid (25 mg/l)	1.40 ±.07	6.21 ±.43	2.25 ±.59	2.76	1.46	0.93 ±.04	20.07 ±2.18	8.26 ±.79	2.43	2.03	1.21 ±.15	3.49 ±2.96	1.49 ±.27	2.35	.95
Ascorbic Acid (50 mg/l)	1.20 ±.07	11.05 ±3.22	4.38 ±.96	2.52	1.03	0.94 ±.07	20.04 ±4.14	8.11 ±1.82	2.47	2.19	1.25 ±.13	4.84 ±1.50	1.49 ±.53	3.26	.88
Ascorbic Acid (75 mg/l)	0.91 ±.07	25.90 ±4.90	9.67 ±1.83	2.68	3.23	0.84 ±.06	24.71 ±4.43	9.88 ±1.67	2.50	3.00	1.09 ±.12	7.80 ±3.09	2.69 ±1.02	2.90	1.33
Ascorbic Acid (100 mg/l)	1.10 ±.09	12.40 ±2.17	4.95 ±.94	2.51	1.94	0.79 ±.09	28.75 ±2.51	11.45 ±1.19	2.51	2.98	1.13 ±.16	6.48 ±1.45	2.22 ±.43	2.92	1.45

* Fresh weight (gm) growth value/piece

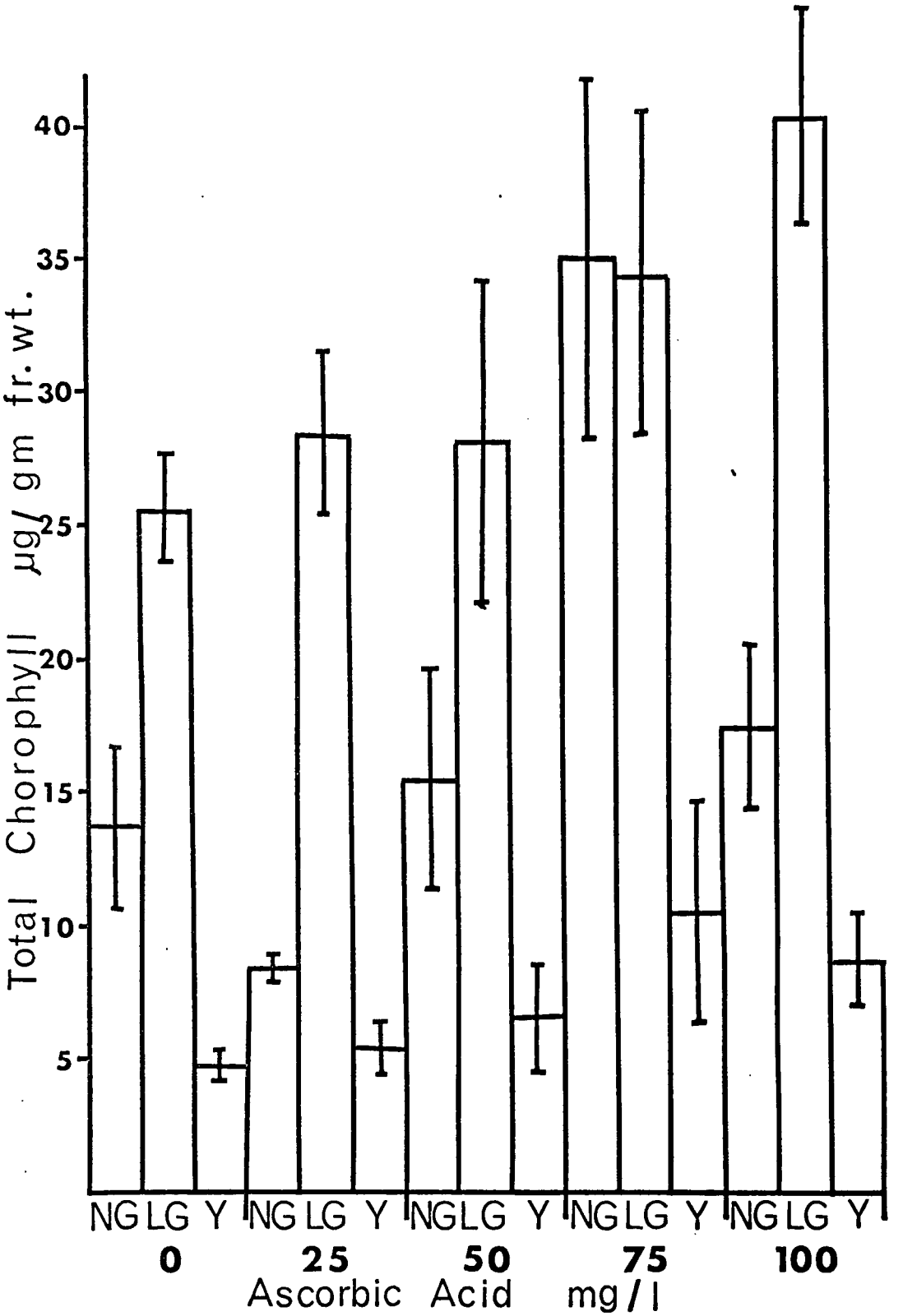
** Carotenoid determination: $\frac{CAR}{480}$ = O.D. x ml acetone/gm fr. wt.

*** Mean ± S.E.M. for 4 replicates of 10 pieces each.

Figure 13.

TOTAL CHLOROPHYLL BIOSYNTHESIS IN THREE SOYBEAN PHENOTYPES CULTURED ON VARIOUS CONCENTRATIONS OF ASCORBIC ACID.

Mean ± S.E.M. for 4 replicates of 10 pieces each.


Total Chorophyll µg/ gm fr. wt.
40
35
30
25
20
15
10
5
NG LG Y NG LG Y NG LG Y NG LG Y NG LG Y
0
25
50
75
100
Ascorbic Acid mg/l

a/b ratios at all concentrations employed than the other two phenotypic variants. As observed in the carbon source study, the dependency of carotenoid synthesis for chlorophyll formation was evident when this reductant was added to the basal media. Under the chemical conditions employed the NG and LG phenotypes consistantly maintained higher levels of carotenoids than the Y phenotype.

The presence of 75 mg/l of ascorbic acid suppressed the growth value of these phenotypes in comparison to other concentrations which indicated a negative correlation between chlorophyll formation and growth yield (Table No. 8). This inverse relationship suggested that the nutrients which favored chlorophyll formation does so at the expense of an increased growth rate.

Effect of ALA on the growth values and pigmentation of three soybean phenotypes. ALA and other chlorophyll precursors are known to effect the greening capacity of numerous etiolated systems. Therefore these components were incorporated into experimental media to determine their effect on the growth yields and in particular chlorophyll formation. Small inoculum from each cultured phenotype were incubated on the basal media containing 1 mg/l of both kinetin and NAA, 75 mg/l of ascorbic acid and specific concentrations of known chlorophyll precursors.

These callus variants grew well as indicated by their respective growth values (Table No. 9) when cultured on various levels of ALA but the production of total chlorophyll was not as favorable (Fig. 14). Even different concentrations of ALA and ascorbic acid supported lower total chlorophyll levels (Fig. 15). No significant difference existed in the total chlorophyll content of these variants when cultured on experimental media containing 2 and 4 mg/l of ALA and 75 mg/l of ascorbic acid (Fig. 14).

The fact that low chlorophyll formation occurred in this study when these variants were cultured on media without ALA (+ 75 mg/l ascorbic acid) supported the requirement to deplete the endogenous level of ascorbic acid in order to demonstrate the sensitivity of these cultured variants to this reductant. The same rationale could possibly explain why ALA failed to stimulate higher levels of chlorophyll formation. Along the same lines callus cultures generally require an initial adjustment period when maintained under different cultural conditions. This concept was evident when the pigmentation in these variants was shown to increase with subculturing following a component change in the basal media (Table No. 10).

As previously observed in the ascorbic acid experiment the addition of ALA to the experimental media supported higher total chlorophyll development in the LG phenotype than the NG phenotype (Figs. 13 and 14). Since the

Table No. 9. Growth yields and pigmentation of three soybean phenotypes cultured on various concentrations of δ-aminolevulinic acid.

Additive	Normal Green					Light Green					Yellow				
	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**
Control	1.16*** ±0.06	10.13 ±0.50	4.11 ±0.67	2.46	1.35	1.04 ±0.04	15.76 ±0.79	5.97 ±0.29	2.64	1.64	1.09 ±0.05	7.93 ±0.76	3.00 ±0.36	2.64	1.07
ALA (1 mg/l)	1.29 ±0.78	8.80 ±0.17	3.66 ±0.13	2.40	1.31	1.07 ±0.10	14.09 ±1.92	5.71 ±1.01	2.47	1.89	1.09 ±0.08	7.93 ±1.38	2.85 ±0.42	2.78	1.36
ALA (2 mg/l)	1.11 ±0.06	14.20 ±1.37	5.72 ±0.24	2.48	1.89	1.01 ±0.09	18.22 ±1.98	7.18 ±0.88	2.54	2.63	1.13 ±0.09	9.77 ±0.72	3.28 ±0.48	2.98	1.24
ALA (4 mg/l)	1.20 ±0.16	14.66 ±3.12	6.11 ±1.12	2.40	2.00	0.93 ±0.08	20.65 ±1.66	8.29 ±0.62	2.49	2.81	0.95 ±0.07	8.89 ±1.71	3.02 ±0.57	2.94	1.29
Glycine (3 mg/l) Succinic Acid (1 mg/l)	1.01 ±0.08	18.11 ±1.04	7.13 ±0.98	2.54	1.53	0.98 ±0.05	18.05 ±2.68	7.57 ±0.67	2.38	2.35	1.13 ±0.11	5.72 ±1.13	2.13 ±0.35	2.68	1.04
Glutamic Acid (2 mg/l)	1.50 ±0.14	6.71 ±1.04	2.79 ±0.48	2.40	1.77	0.96 ±0.07	20.24 ±1.95	7.78 ±0.90	2.60	2.45	1.08 ±0.07	6.73 ±0.09	2.28 ±0.14	2.95	1.10

* Fresh weight (gm) growth value/piece.

** Carotenoid determination: $\frac{CAR}{480}$ = O.D. x ml acetone/gm fr. wt.

*** Mean ± S.E.M. for 4 replicates of 10 pieces each.

Figure 14.

TOTAL CHLOROPHYLL BIOSYNTHESIS IN THREE SOYBEAN PHENOTYPES CULTURED ON VARIOUS CONCENTRATIONS OF δ-AMINOLEVULINIC ACID.

Mean $\pm$ S.E.M. for 4 replicates of 10 pieces each.

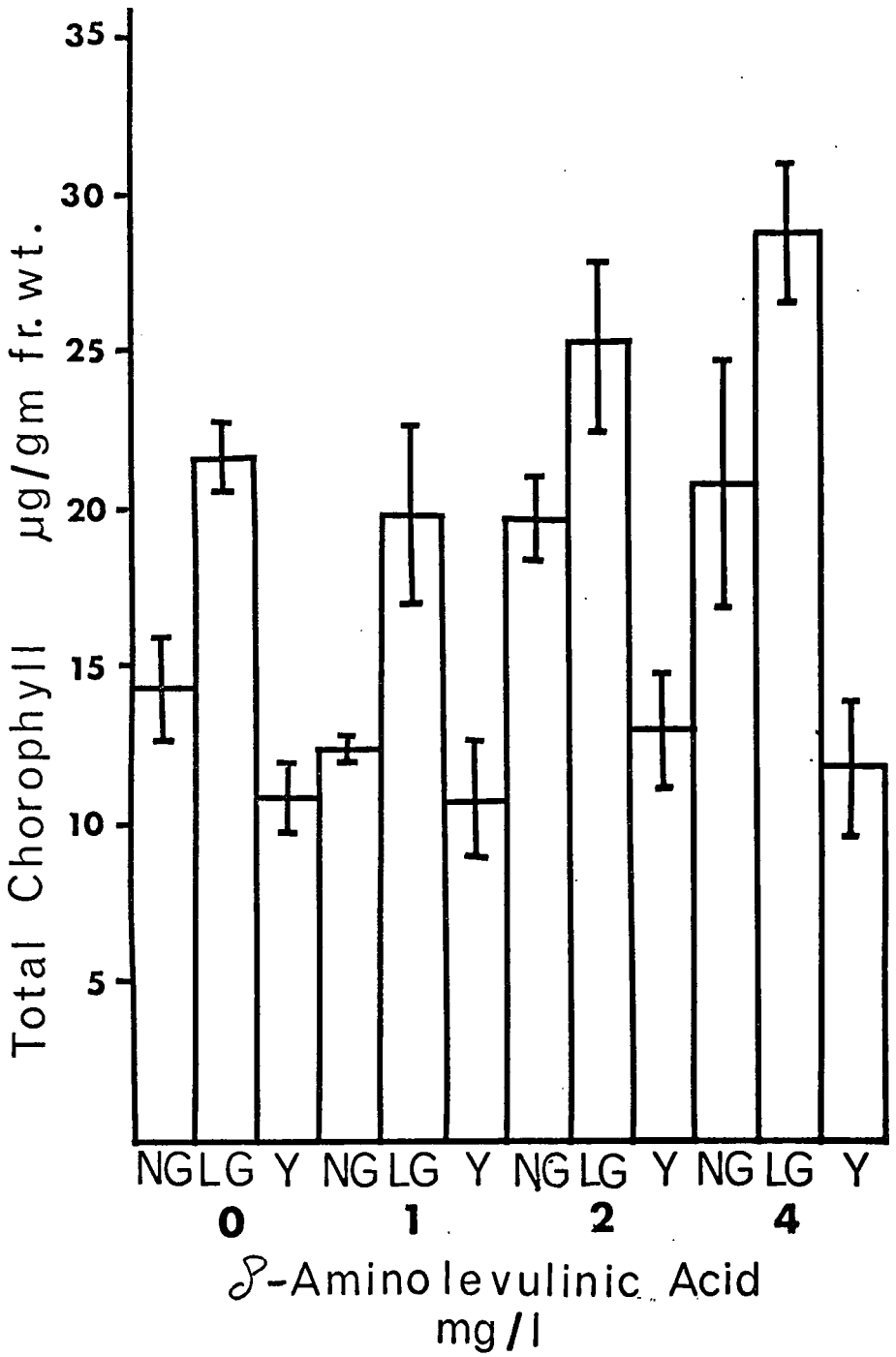

35
30
25
20
15
10
5
Total Chorophyll µg/gm fr. wt.
NG LG Y NG LG Y NG LG Y NG LG Y
0
1
2
4
δ-Amino levulinic Acid
mg/l

Figure 15.

TOTAL CHLOROPHYLL BIOSYNTHESIS IN THREE SOYBEAN PHENOTYPES CULTURED ON VARIOUS CONCENTRATIONS OF δ-AMINOLEVULINIC ACID AND ASCORBIC ACID.

Mean $\pm$ S.E.M. for 4 replicates of 10 pieces each.

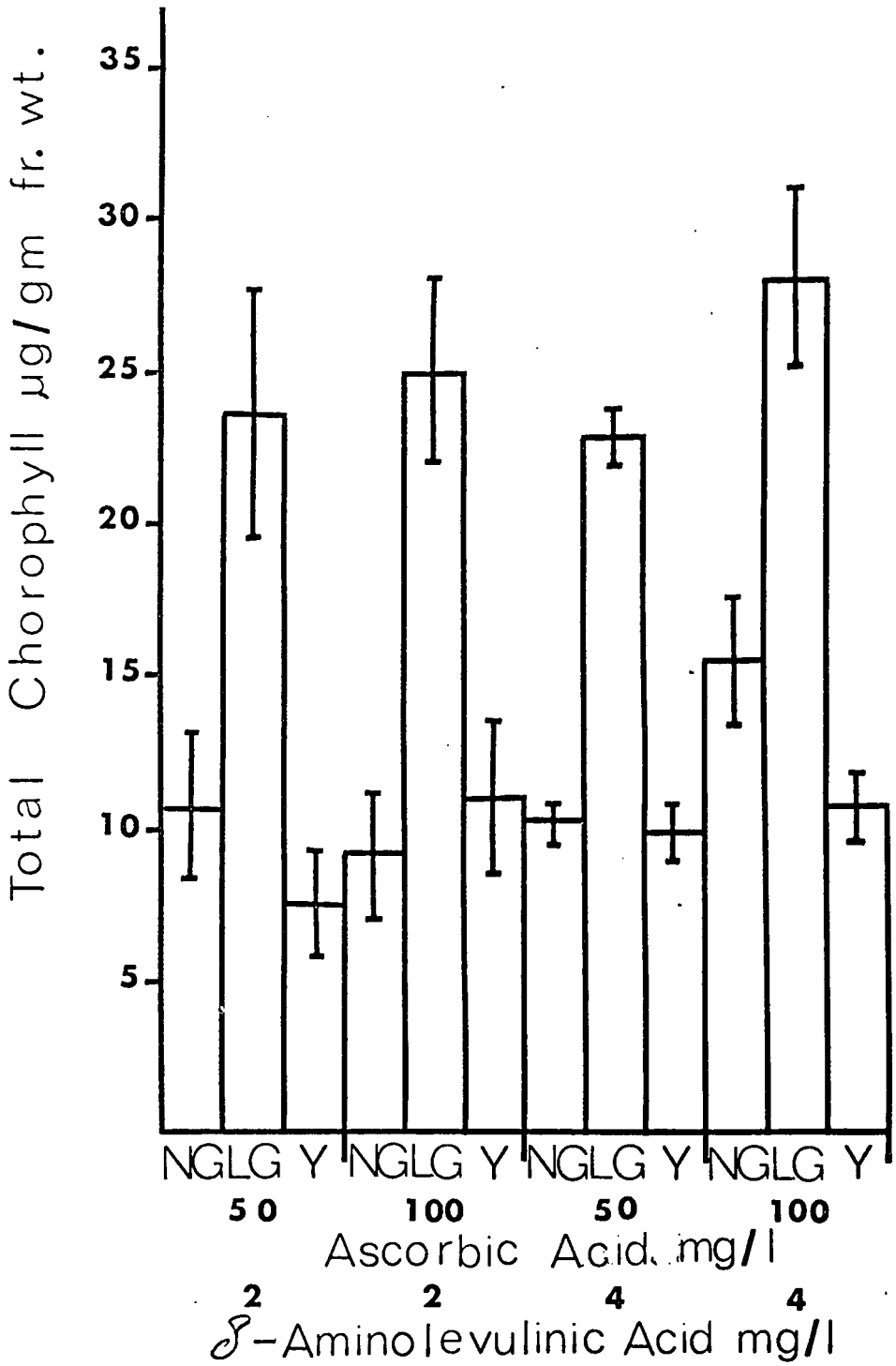

Total Chorophyll µg/gm fr. wt.
35
30
25
20
15
10
5
NGLG Y NGLG Y NGLG Y NGLG Y
50 100 50 100
Ascorbic Acid mg/l
2 2 4 4
δ-Aminolevulinic Acid mg/l

Table No. 10. The effect of subculturing on the pigmentation of three soybean phenotypes following a change in the media composition.

Additive	Normal Green		Light Green		Yellow	
	Chl* a+b	CAR** 480	Chl* a+b	CAR** 480	Chl* a+b	CAR** 480
March, 1974 Ascorbic Acid (75 mg/l)	35.09 ±6.92	1.26	34.59 ±6.09	1.07	10.49 ±4.12	0.66
April, 1974 Ascorbic Acid (75 mg/l) ALA (4 mg/l)	20.76 ±4.23	1.01	28.94 ±2.27	1.13	11.91 ±2.27	0.53
July, 1974 Ascorbic Acid (75 mg/l) ALA (4 mg/l)	32.15 ±8.79	1.32	29.39 ±5.08	1.30	6.18 ±.63	0.61

* Chlorophyll determination: Chl a+b µg/gm fr. wt.

** Carotenoid determination: $\frac{CAR}{480}$ = O.D. x ml acetone/gm fr. wt.

addition of 4 mg/l ALA supported a slightly higher total chlorophyll production than 2 mg/l, the former concentration was added to the basal media for stock and experimental cultures.

The chlorophyll a/b ratios of these variants were not significantly affected by the addition of various concentrations of ALA to the basal media (Table No. 9). The NG and LG phenotypes maintained similar ratios under these culture conditions while the Y strain supported a higher chlorophyll a/b ratio. A similar growth characteristic occurred when the variants were cultured on different concentration combinations of ALA and ascorbic acid (Table No. 11).

Other chlorophyll precursors were studied to determine their effect on the greening ability of these phenotypic variants. Since the synthesis of ALA has been demonstrated to occur by either of two methods: condensation of succinyl CoA and glycine (162) or the transamination of α-ketoglutaraldehyde (64), the combination of succinic acid and glycine and L-glutamate were separately supplemented to the basal media (Table No. 9). Both chlorophyll precursors supported similar total chlorophyll content and chlorophyll a/b ratios in the phenotypic classes except for the NG variant when cultured on L-glutamate. This amino acid stimulated a high growth rate and subsequently a bleached condition for pigmentation occurred in the NG phenotype.

Table No. 11. Growth yields and pigmentation of three soybean phenotypes cultured on various concentrations of ascorbic acid and δ-aminolevulinic acid.

Additive	Normal Green					Light Green					Yellow				
	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**
ALA (2 mg/l) As.Ad. (50 mg/l)	1.39*** ±0.12	7.39 ±1.77	3.05 ±0.71	2.42	1.16	1.03 ±0.11	17.15 ±2.87	6.50 ±1.18	2.64	2.31	1.27 ±0.08	5.49 ±1.37	1.91 ±0.48	2.87	1.09
ALA (2 mg/l) As.Ad. (100 mg/l)	1.36 ±0.14	6.49 ±0.78	2.70 ±0.31	2.40	1.09	0.92 ±0.09	17.87 ±2.27	7.08 ±0.74	2.52	2.44	1.12 ±0.12	8.11 ±0.96	2.88 ±0.45	2.88	1.52
ALA (4 mg/l) As.Ad. (50 mg/l)	1.44 ±0.10	7.24 ±0.42	2.90 ±0.14	2.50	1.31	0.99 ±0.05	17.35 ±0.71	6.52 ±0.28	2.66	2.52	1.15 ±0.09	7.38 ±0.82	2.51 ±0.27	2.94	1.48
ALA (4 mg/l) As.Ad. (100 mg/l)	1.27 ±0.06	11.15 ±1.63	4.31 ±0.57	2.59	1.72	0.80 ±0.08	20.07 ±2.61	8.03 ±0.57	2.50	2.74	1.09 ±0.07	7.99 ±0.87	2.71 ±0.32	2.96	1.59

* Fresh weight (gm) growth value/piece.

** Carotenoid determination: $\frac{CAR}{480}$ = O.D.xml acetone/gm fr. wt.

*** Mean ± S.E.M. for 4 replicates of 10 pieces each.

The inverse relationship between chlorophyll formation and growth rate was not as evident in these phenotype classes when incubated under the cultural conditions employed (Tables 9 and 11). This occurrence could possibly be related to the low level of chlorophyll formation which was supported by this combination of organic nutrients.

The autotrophic capacity of three soybean phenotypes. The cultured variants were incubated on the basal media supplemented with various levels of sucrose. Callus tissue from each phenotype grew well when maintained on 10 gm or more of sucrose but at lower levels a dependency between the growth yields and an exogenous carbon source was evident (Table No. 12). As indicated by the growth value (Table No. 12) the Y phenotype possessed the highest capacity for autotrophic growth. However total chlorophyll formation in the Y variant was not as appreciably affected by the addition of exogenous sucrose to the basal media as the other two phenotypes (Fig. 16). The LG variant demonstrated a broad sensitivity range to sucrose concentration whereas the wild-type required a specific level for chlorophyll synthesis. Similarly the fact that the phenotypes supported higher chlorophyll a/b ratios with increasing carbon source indicated that these variants were effected to some degree by the availability of an exogenous source of carbon. No appreciable difference in the increase of chlorophyll a/b ratios between the wild-type and LG pheno-

Table No. 12. Growth yields and pigmentation of three soybean phenotypes cultured on various concentrations of sucrose.

Additive	Normal Green					Light Green					Yellow				
	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**	GV*	(µg/gm fr. wt.) Chl a	Chl b	Chl a/b	CAR 480**
Control	0.015 ±0.01	3.82 ±0.98	1.78 ±0.44	2.15	2.13	0.019 ±0.01	4.45 ±1.12	2.15 ±0.42	2.07	2.02	0.034 ±0.01	3.74 ±1.08	1.76 ±0.38	2.13	1.77
Sucrose (5 gm/l)	0.79 ±0.08	5.00 ±1.04	2.31 ±0.47	2.16	0.83	0.70 ±0.05	10.76 ±0.39	4.13 ±0.14	2.61	1.55	0.65 ±0.06	6.17 ±1.92	2.87 ±1.64	2.15	1.11
Sucrose (10 gm/l)	1.38 ±0.12	4.29 ±1.44	1.93 ±0.57	2.22	0.78	1.24 ±0.08	13.77 ±0.97	5.65 ±0.46	2.44	1.82	1.12 ±0.08	6.72 ±0.75	2.48 ±0.22	2.71	1.20
Sucrose (15 gm/l)	1.65 ±0.13	3.55 ±1.28	1.44 ±0.44	2.47	0.65	1.12 ±0.18	13.46 ±4.13	5.43 ±1.86	2.48	1.84	1.30 ±0.06	6.93 ±1.34	2.49 ±0.61	2.78	1.12
Sucrose (20 gm/l)	1.77 ±0.07	3.86 ±0.32	1.58 ±0.17	2.44	0.71	1.04 ±0.07	14.43 ±2.06	5.85 ±0.98	2.47	2.05	1.38 ±0.20	4.10 ±0.17	1.43 ±0.14	2.89	0.81
Sucrose (25 gm/l)	1.30 ±0.13	6.89 ±0.33	2.57 ±0.14	2.68	1.33	1.11 ±0.09	15.60 ±0.32	5.40 ±0.09	2.89	2.31	1.11 ±0.10	7.68 ±0.93	2.51 ±0.93	3.06	1.58
Sucrose (30 gm/l)	1.04 ±0.24	23.66 ±6.50	8.49 ±2.30	2.79	2.97	1.02 ±0.09	21.81 ±3.95	7.58 ±1.13	2.88	2.95	1.31 ±0.13	4.72 ±0.45	1.47 ±0.20	3.32	1.07

* Fresh weight (gm) growth value/piece.

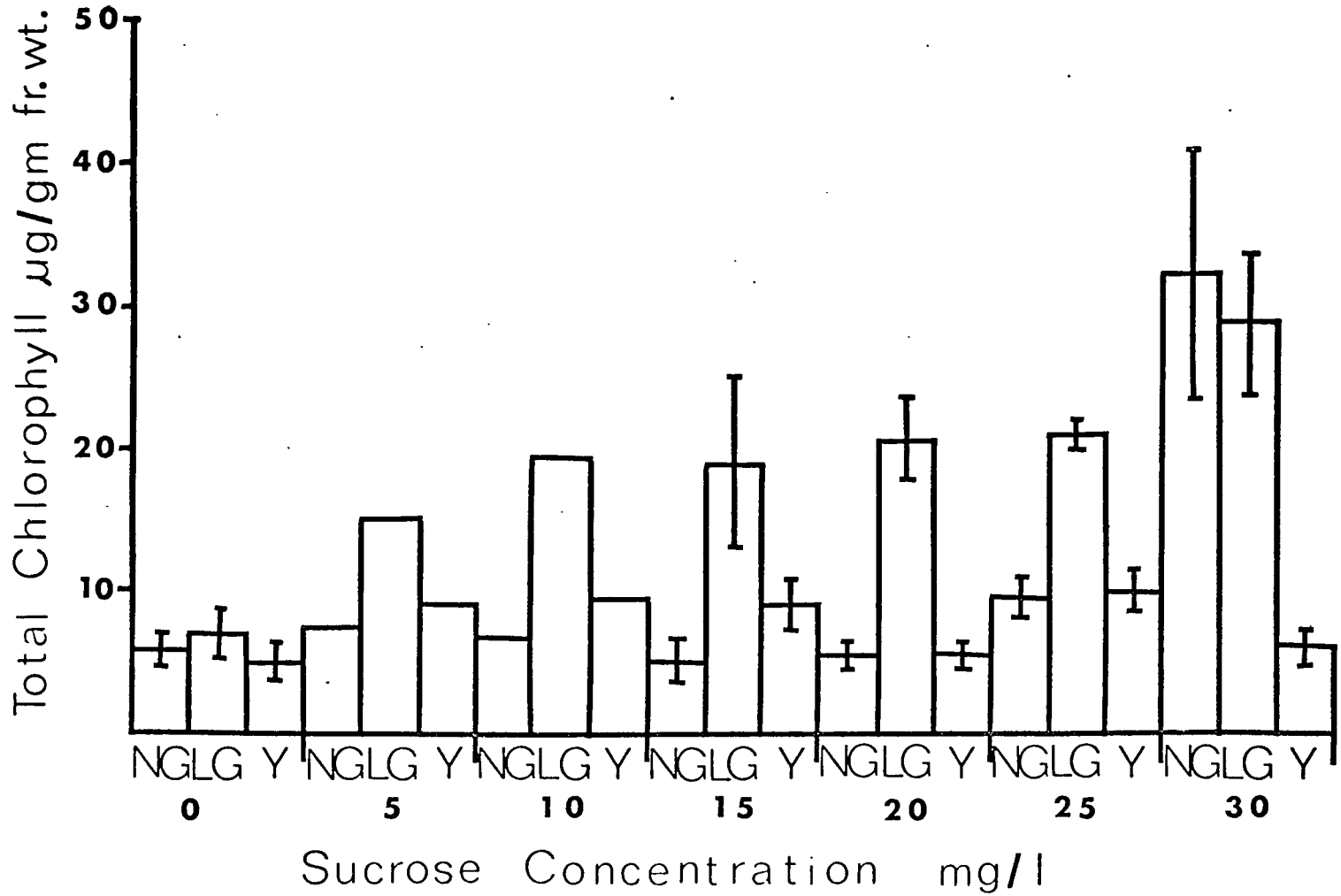
** Carotenoid determination: $\frac{CAR}{480}$ = O.D. x ml acetone/gm fr. wt.

*** Mean ± S.E.M. for 4 replicates of 10 pieces each.

Figure 16.

TOTAL PIGMENTATION OF THREE SOYBEAN PHENOTYPES CULTURED ON VARIOUS CONCENTRATIONS OF SUCROSE

Mean ± S.E.M. for 4 replicates of 10 pieces each.


Total Chlorophyll µg/gm fr. wt.
50
40
30
20
10
NGLG Y NGLG Y NGLG Y NGLG Y NGLG Y NGLG Y NGLG Y
0
5
10
15
20
25
30
Sucrose Concentration mg/l

type was evident whereas the ratios in the Y variant increased at a faster rate and reached a higher level than the other two phenotypes (Table No. 12).

The inverse correlation between growth rate and chlorophyll formation was evident in the NG and LG phenotypes and to a lesser extent in the Y variant (Table No. 12). The sucrose level of 30 gm/l supported the lowest growth value and the highest level of chlorophyll development.

Effect of light intensity on growth values and pigmentation of three soybean phenotypes. Increased illumination of these callus variants had a stimulatory effect on both the growth yields and chlorophyll development (Table No. 13). Callus growth of the NG and Y phenotype increased whereas a decrease in growth value was observed in the LG phenotype. Correspondently the chlorophyll content of the three phenotypic variants (NG: 37%, LG: 47%, Y: 9%) increased when incubated under higher light conditions. Subsequently the negative correlation of the growth value: chlorophyll biosynthesis was evident only in the LG phenotype which supports the observations made in the ascorbic acid and ALA experiments in that a certain level of chlorophyll accumulation NG and LG phenotype (20 µg/gm fr.wt.) and Y phenotype (10 µg/gm fr.wt.) must be obtained before this growth characteristic becomes evident.

Table No. 13. The effect of light intensity on growth yields and pigmentation of three soybean phenotypes.

Light Intensity (lux)	Normal Green				Light Green				Yellow			
	GV*	(µg/gm fr.wt.) Chl a	Chl b	CAR** 480	GV*	(µg/gm fr.wt.) Chl a	Chl b	CAR** 480	GV*	(µg/gm fr.wt.) Chl a	Chl b	CAR** 480
700	1.29*** ±0.16	6.43 ±0.95	2.87 ±0.46	0.95	1.22 ±0.12	8.22 ±1.54	3.22 ±0.57	1.12	1.29 ±0.05	4.68 ±0.47	1.69 ±0.16	0.76
3000	1.38 ±0.14	10.39 ±0.43	4.33 ±0.18	1.55	1.09 ±0.42	15.43 ±1.08	6.25 ±0.26	2.05	1.35 ±0.10	5.18 ±0.40	1.84 ±0.18	1.12

* Fresh Weight (gm) Growth Value/Piece.

** Carotenoid determination: $\frac{CAR}{480}$ = O.D.xml acetone/gm fr. wt.

*** Mean ± S.E.M. for four replicates of 5 pieces.

Callus cultures of the Y phenotype was the least affected of these variants when cultured under low light intensity (Table No. 13 and Fig. 18). On numerous occasions the stock cultures of this strain which were routinely incubated under low-light intensities appeared to accumulate higher levels of chlorophyll (visual observation) than experimental cultures that were maintained on similar cultural conditions with the exception of being incubated under high light intensities. Therefore this bleaching effect at high light intensities which occurred in the Y phenotype could possibly be explained by a defective mechanism for carotenoids synthesis as found in other chlorophyll mutants (56,188,204).

Identification of pigments of three soybean phenotypes. The total absorption spectra of acetone extracts prepared from three soybean phenotypes are shown in Fig. 17. From 400 to 700 nm the spectra of the NG and LG variants showed characteristic absorption maxima for chlorophyll a at 663 nm and about 430 nm, and for carotenoids at 460-470 nm. One variation was noted in the normalized absorption spectrum of the Y phenotype at approximately 465 to 470 nm. Where the wild-type and LG phenotype showed a slight shoulder the Y variant demonstrated a distinct absorption peak. The absorption spectrum of the Y phenotype which was cultured under low light intensities (700 lux) demonstrated only an increase in synthesis of chlorophylls and carotenoids that are characteristic of this variant (Fig. 18).

Figure 17.

TOTAL ABSORPTION SPECTRA OF ACETONE EXTRACTS OF THREE SOYBEAN PHENOTYPES CULTURED UNDER HIGH LIGHT INTENSITY (3000 lux).

The absorption curves were standardized on the bases of total acetone and dry weight. (Absorbance/300 mg dry wt.)

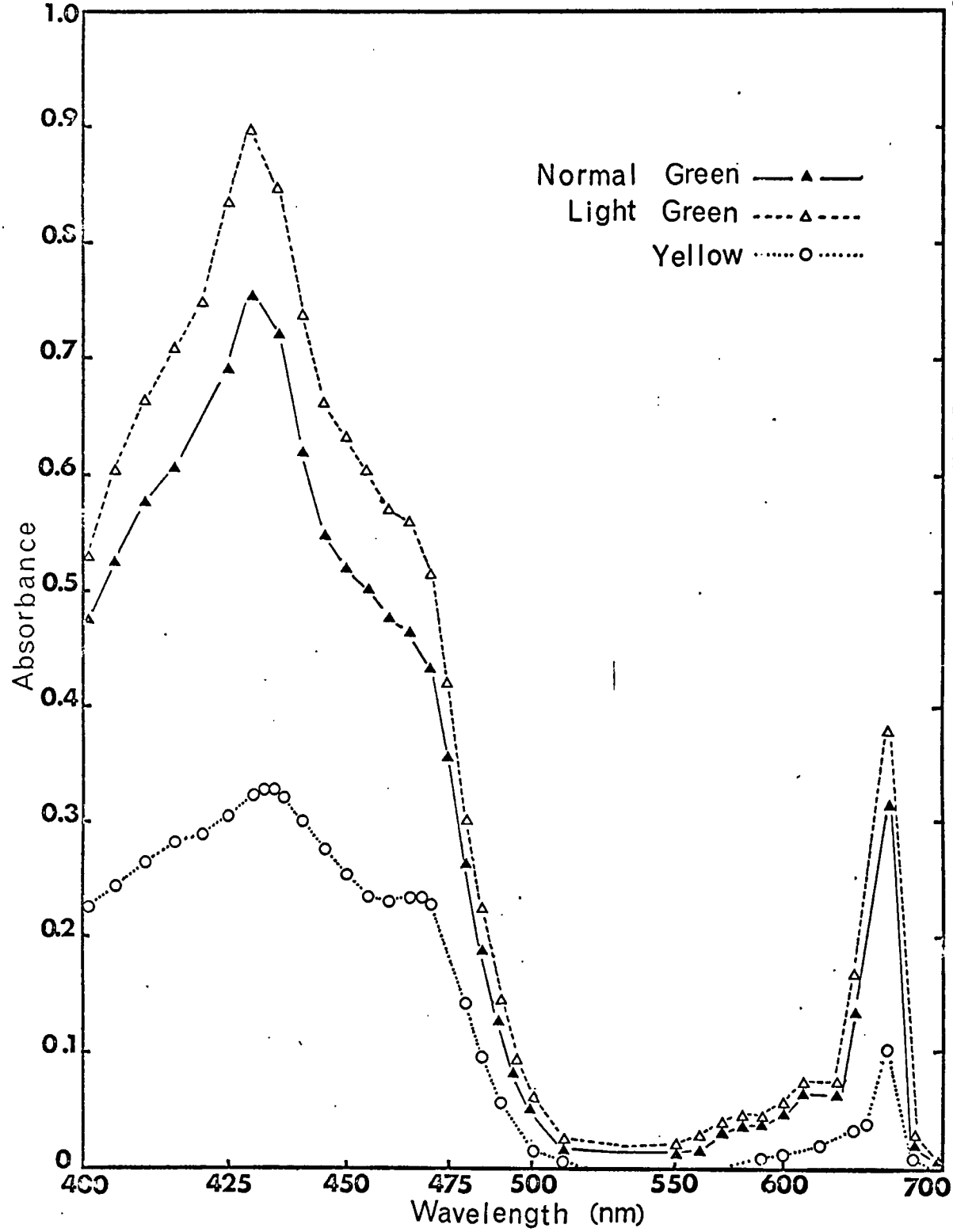
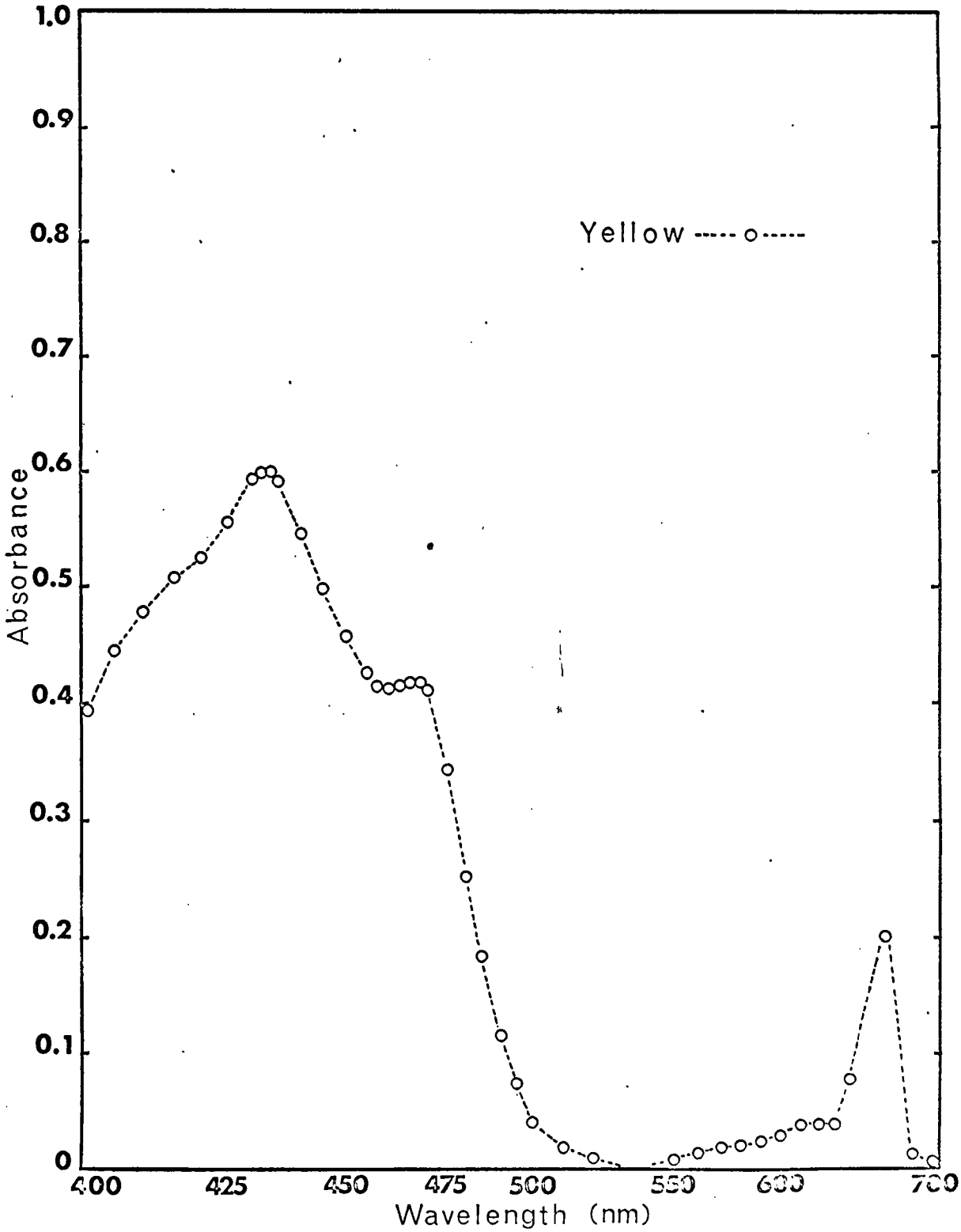

Normal Green
Light Green
Yellow
Absorbance
Wavelength (nm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
400
425
450
475
500
550
600
700

Figure 18.

TOTAL ABSORPTION SPECTRUM OF AN ACETONE EXTRACT OF THE YELLOW PHENOTYPE CULTURED UNDER LOW LIGHT INTENSITY (700 lux).

The absorption curve was standardized on the bases of total acetone and dry weight. (Absorbance/300 mg dry wt.)


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
Yellow
400
425
450
475
500
550
600
700
Wavelength (nm)

The callus pigments of each variant were separated by column chromatography of powdered sugar and hexane plus 0.5% N-propanol as the wash liquid. The discrete zones were resolved in the following sequence (top to bottom): neoxanthin, violaxanthin, chlorophyll b, lutein plus zeaxanthin, chlorophyll a and carotenes (Fig. 19). The chromatographic behavior of the individual pigment was identical with that found in the primary leaves of this soybean system (105) and in other seed plants (171). Subsequently the identification of the individual pigments was based not only on their chromatographic behavior but also on their absorption maxima (Table No. 14). In most cases the absorption maxima of the respective pigments in each variant are similar and comparable to the published maxima with the exception of the carotene fraction of the NG and LG phenotypes and the combined absorption of lutein and zeaxanthin of all three phenotypes. The spectra of the carotene (α and β) fraction of the Y phenotype demonstrated characteristic absorption maxima (418, 443.5, and 469 nm) which corresponds to the absorption peaks of α-carotene more so than β-carotene. However the spectra of carotene (α and β) fraction of the NG and LG phenotypes reveal two additional absorption maxima (near red region-669 nm and violet region-409 nm). These peaks represent absorbing regions which are direct (669 nm) and indirect (409 nm) resultant of chlorophyll a molecules as a contaminant. The absorption spectra of the pre-chlorophyll a region revealed an identical absorption spectrum (408, 440, 468 and 668 nm).

Figure 19.

CHROMATOGRAPHIC BEHAVIOR OF THE CHARACTERISTIC PIGMENTS OF THE THREE SOYBEAN PHENOTYPES.

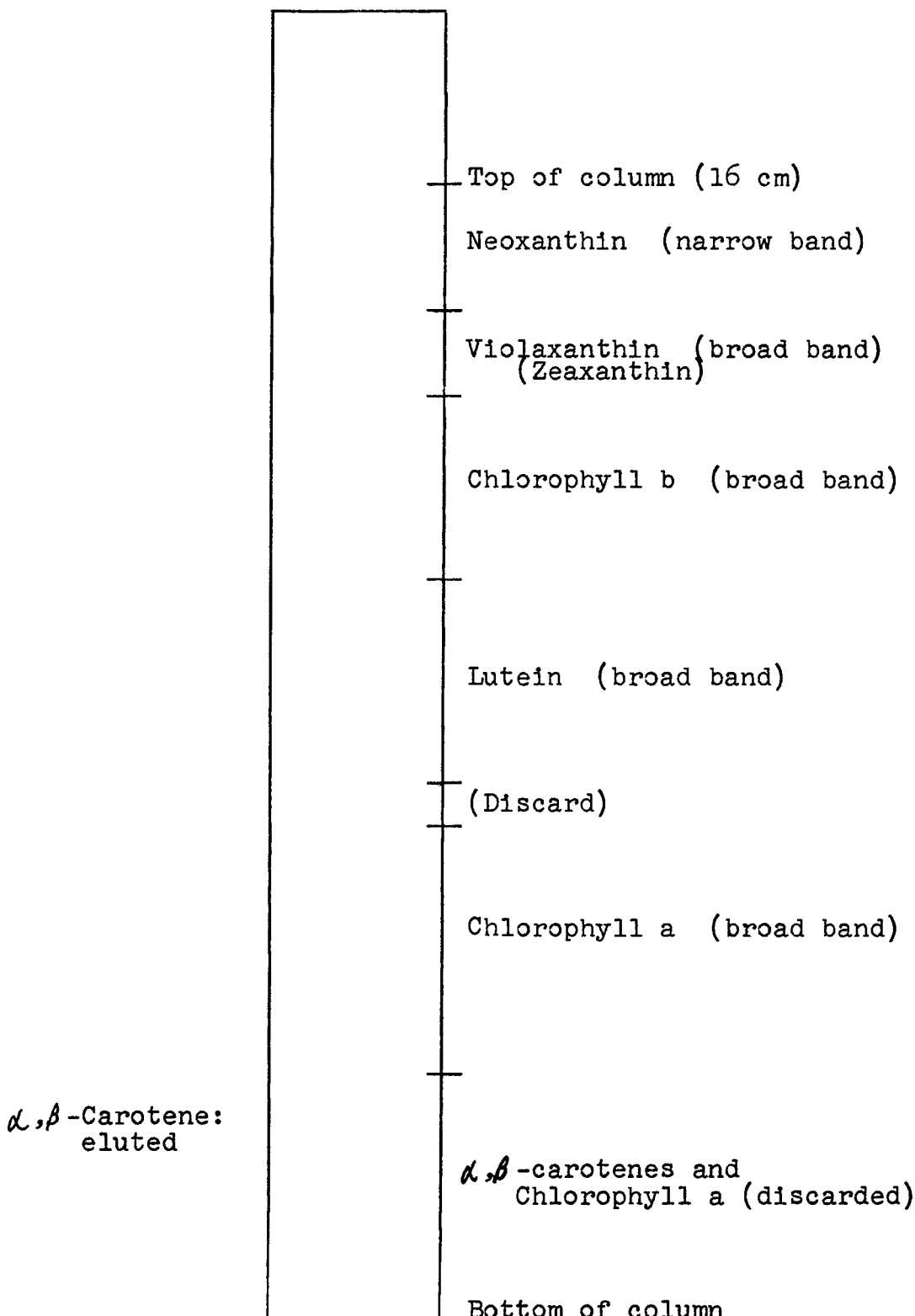

Top of column (16 cm)
Neoxanthin (narrow band)
Violaxanthin (broad band)
(Zeaxanthin)
Chlorophyll b (broad band)
Lutein (broad band)
(Discard)
Chlorophyll a (broad band)
α,β-Carotene: eluted
α,β-carotenes and Chlorophyll a (discarded)
Bottom of column

Table No. 14. Absorption maxima of chlorophylls and carotenoids separated on sucrose column chromatography.

Pigments	Published maxima (nm)	Maxima Found			Solvent
		Normal Green	Light Green	Yellow	
Chlorophyll A	430, 663	429, 663	430, 663	432, 663	Acetone
Chlorophyll B	455, 645	455, 645	456, 645	456, 645	Acetone
α-Carotene	422, 444, 473	408, 440, 468, 668	410, 444, 469, 668	418, 443.5, 469	Petroleum Ether
β-Carotene	425*, 451, 482				
Lutein	420, 446.5, 476	420, 444, 471	418, 443.5, 471	418, 440, 471	Ethanol
Zeaxanthin	423.5, 451, 483				
Violaxanthin	420, 441, 471	418, 437.5, 468.5	418, 440, 470	420*, 440, 470	Ethanol
Neoxanthin	417, 438, 467	415, 438.5, 467	415, 439, 467	410*, 440, 467	Ethanol

* Inflection

** Absorption maxima: chlorophylls (124) and carotenoids (43).

Greening Experimentation In Three Dark Grown Soybean Callus Phenotypes

Effect of ascorbic acid and sucrose on the dark grown variants. Dark grown callus tissue of each phenotype was initiated from the light grown system by serial transfers of the chlorophyllous strains. Routinely the cultures were maintained on the basal media which contained a high concentration of sucrose (6%) and ascorbic acid (100 mg/l). Subsequently the chlorophyll content of these variants was diluted out by cell division and thereafter these dark grown phenotypes were considered to be a non-chlorophyllous callus system.

Under the cultured conditions employed the NG and Y phenotypes maintained adequate growth rates, appeared tan in coloration and consisted of a friable to slightly rigid callus texture. In contrast the LG phenotype demonstrated a browning condition which appeared to have little, if any, effect on callus growth and consisted of a slightly rigid callus texture. In an attempt to find cultural conditions which would support uniformity in callus growth, texture, and minimal browning, the phenotypes were transferred to a basal media containing various concentrations of sucrose and ascorbic acid (Table No. 15). In most cases the amount of carbon source and reductant added to the basal media had little to no effect on the growth characteristics of the NG and Y phenotype. However the browning condition

Table No. 15. General observations of three soybean phenotypes cultured on various concentrations of sucrose and ascorbic acid and maintained under dark environmental conditions (30-days growth).

Additives* (Conc.)	Normal Green	Light Green	Yellow
As. Ad. (100 mg/l) Sucrose (6%)	Tan - F**	L. Brown to Brown - R	Tan - R
As. Ad. (50 mg/l) Sucrose (6%)	Tan - R	Brown - F	Tan - R
As. Ad. (100 mg/l) Sucrose (4.5%)	Tan - F	L. Brown to Brown - F	Tan - F
As. Ad. (50 mg/l) Sucrose (4.5%)	Tan - F to L. Brown	Brown - F	Tan - R to L. Brown

* Additives to the basal media (Table No. 1): ascorbic acid (As. Ad.) and sucrose.

** Categories of callus texture: friable (F) and rigid (R).

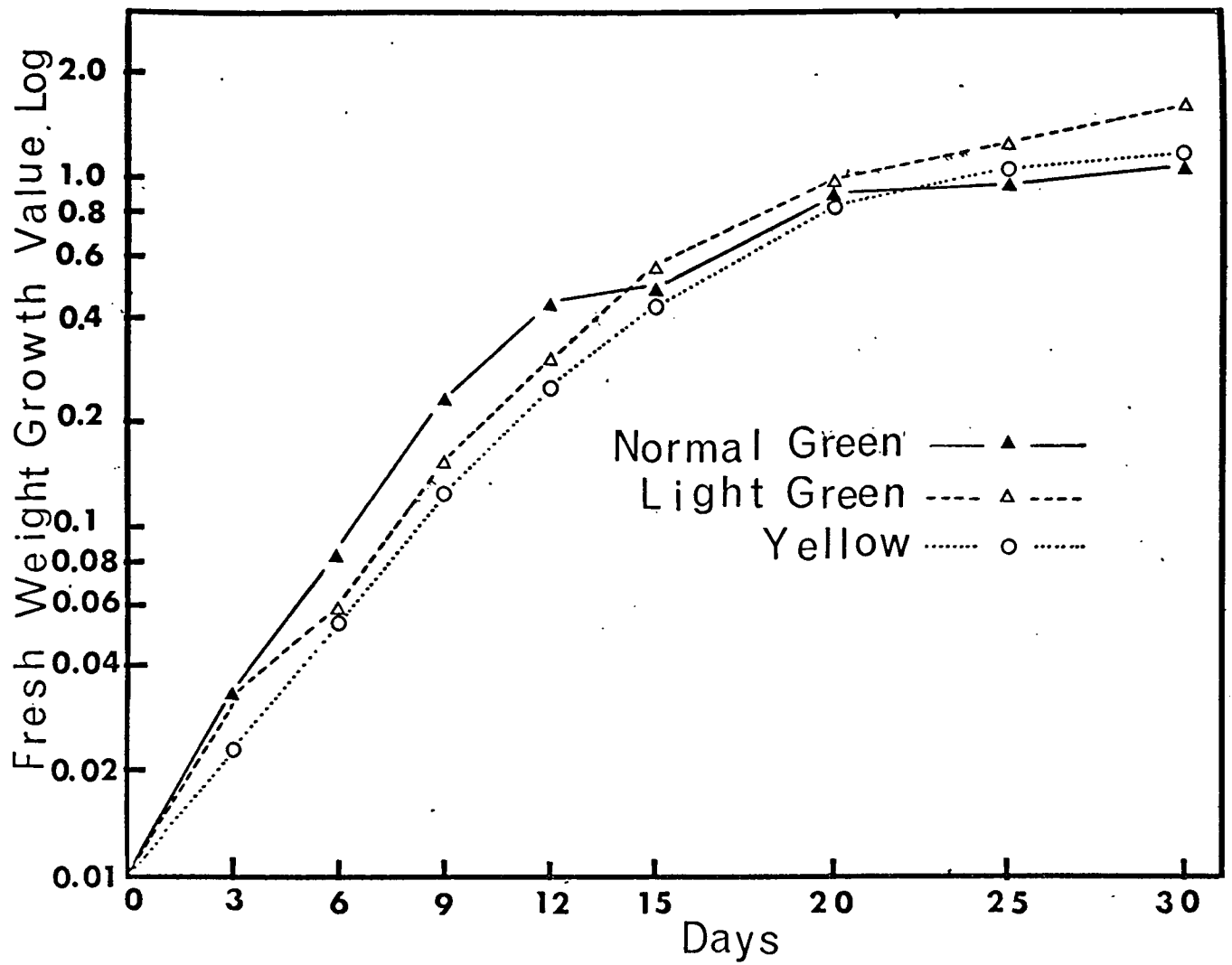
in the LG phenotype was more sensitive to the levels of ascorbic acid than sucrose concentrations. Therefore the dark grown callus system was maintained on the basal media which contained 100 mg/l ascorbic acid and either 4.5 or 6% sucrose as a carbon source. Callus tissue once transferred to these cultural conditions was routinely maintained on its respective medium for the greening studies.

Growth curve of the dark grown cultures when subjected to light conditions. The growth curves (Fig. 20) obtained from dark grown callus cultures revealed a typical sigmoid pattern of growth when incubated on basal media (ascorbic acid 50 mg/l) and illuminated at 3000 lux. This growth pattern was comparable to the chlorophyllous variants with respect to an increase in fresh and dry weights during the 30-day incubation period. As indicated by the fresh weight growth values the lag phase was not present while the exponential phase was very short, if not, non-existant. Maximum growth rate occurred between 3 to 12 days with a subsequent decelerated growth rate to approximately 20 days at which time the callus cells enter the stationary phase. Under the standard cultural conditions employed the cell doubling time of the variants during the phase of linear growth was 50 hr. No significant difference between the growth curves of these cultured phenotypes was evident.

Figure 20.

GROWTH CURVES OF THREE DARK GROWN PHENOTYPES CULTURED UNDER GREEN CONDITIONS.

Each point represents the mean of 4 replicates of 5 pieces each. The calculations for the growth values were as described in Figure 5. S.E.M. for each fresh weight data point was omitted to sustain clarity of the Figure. No significance was observed.


Fresh Weight Growth Value, Log
2.0
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
0
3
6
9
12
15
20
25
30
Days
Normal Green
Light Green
Yellow

Greening of three dark-grown soybean phenotypes. Non-green callus tissue from each variant was transferred to basal media (ascorbic acid 50 mg/l) and illuminated at 3000 lux. Periodically samples were taken and analyzed for their chlorophyll a and b and carotenoid content in each phenotype. Changes in the total amount of chlorophyll (Fig. 21) with respect to time demonstrated a typical greening pattern observed in etiolated plant material (44,181,200). The lag phase occurred during the first 12 days for the NG and LG phenotypes while the Y phenotype demonstrated a requirement for a slightly longer time period (15 days) to initiate the greening mechanisms. Accumulation of total chlorophyll immediately followed the lag phase and paralleled the deceleration of growth (Fig. 21). The accumulation of chlorophyll a and b in each phenotypic strain continued throughout the remainder of the growth period and during this time the chlorophyllous ratios became evident: normal green variant accumulating the higher levels of chlorophyll than the light green and yellow phenotypes. This greening pattern was further demonstrated in the chlorophyll a/b ratios (Table No. 16). Chlorophyll a accumulation (high a/b ratios) occurred during the latter part of the lag phase while its conversion to chlorophyll b occurred at varied times with each phenotype: NG at 9 days, LG and Y at 12 days.

Figure 21.

TOTAL CHLOROPHYLL BIOSYNTHESIS OF THREE DARK GROWN SOYBEAN PHENOTYPES CULTURED UNDER GREENING CONDITIONS.

The experimental media consisted of the basal media as described in Table No. 2 and supplemented with 50 mg/l ascorbic acid. Mean ± S.E.M. for 4 replicates of 5 pieces each. The S.E.M. for days 0 to 12 were omitted from the greening pattern in order to sustain clarity of the Figure. No significant difference was observed.

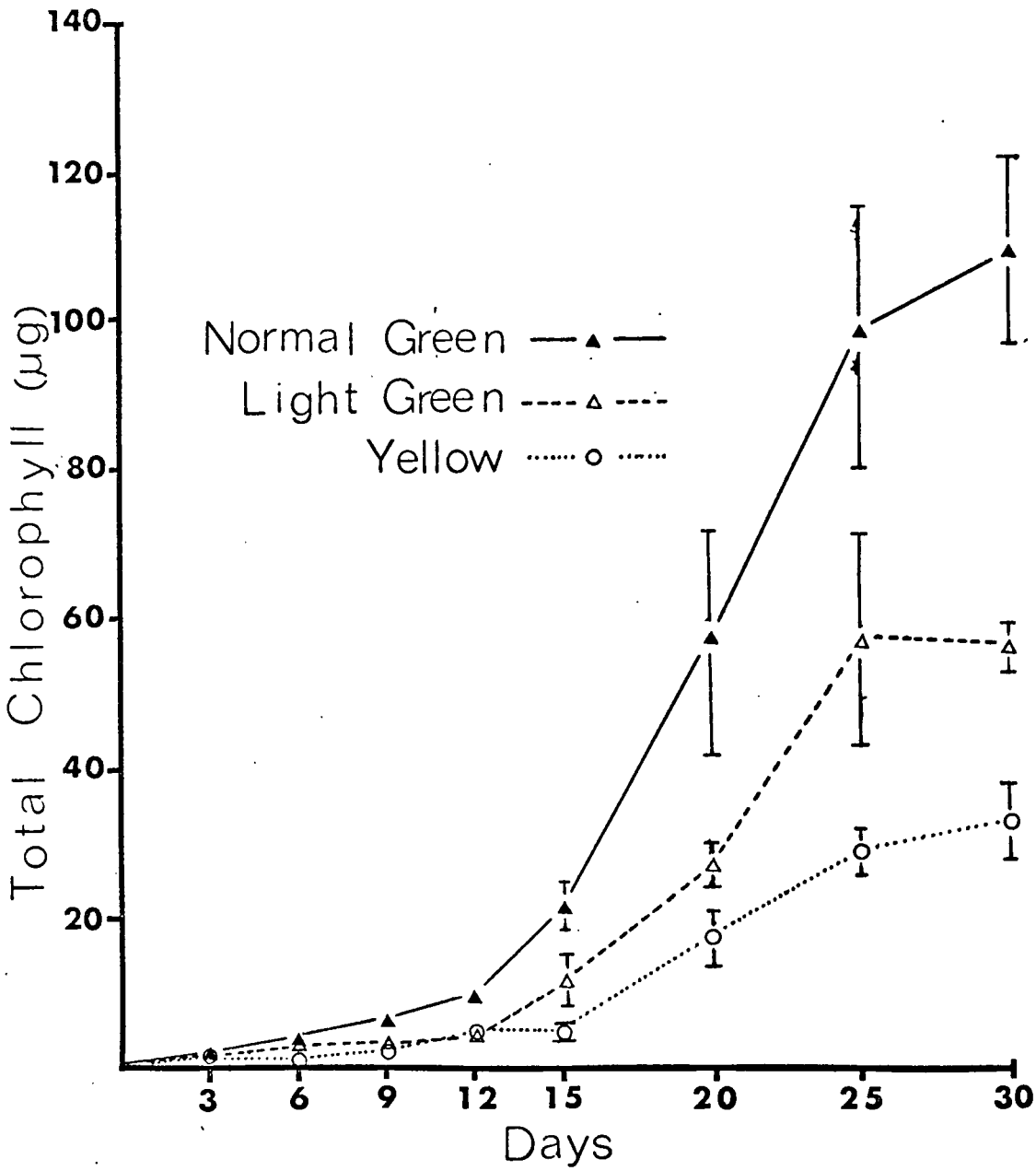

140
120
100
80
60
40
20
Total Chlorophyll (µg)
Normal Green
Light Green
Yellow
3
6
9
12
15
20
25
30
Days

Table No. 16. Chlorophyll a/b ratios and carotenoid content of three etiolated soybean phenotypes cultured on ascorbic acid and incubated under light environmental conditions (lux).

DAYS	NORMAL GREEN		LIGHT GREEN		YELLOW	
	Chl a/b	CAR* 480	Chl a/b	CAR* 480	Chl a/b	CAR* 480
0	-	.29	-	.41	-	.26
3	-	.46	-	.47	-	.34
6	3.38	.44	4.36	.37	-	.33
9	2.79	.31	4.59	.29	4.07	.49
12	2.38	.26	2.49	.36	2.51	.33
15	2.56	.49	2.45	.36	1.96	.16
20	2.71	.66	2.53	.44	2.54	.40
25	2.80	1.11	2.83	.69	2.89	.46
30	2.00	.90	1.93	.46	2.04	.42

* Carotenoid determination: $\frac{CAR}{480}$ = O.D. x ml acetone/gm fr.wt.

When calculated on a µg/gm fr.wt. bases (Fig. 22), the greening pattern of total chlorophyll in each variant revealed an initial chlorophyll a and b accumulation during the early part of the growth period. At day 3, chlorophyll a and b accumulated approximately to the same level in all three variants. At day 6, the NG phenotype continued to accumulate total chlorophyll while the remaining two variants demonstrated a decrease in chlorophyll a and b. Subsequently the wild-type demonstrated a brief decrease in chlorophyll a and b synthesis before the second accumulating phase which in turn was characteristic of all phenotypes. This second accumulating phase corresponded with the increase in chlorophyll a and b as observed when total chlorophyll was plotted with time (Fig. 21).

After the initial accumulating phase the pursuant decrease in total chlorophyll/gm fr.wt. was the result of the breakdown of chlorophyll b (Fig. 23 and 24, A, B). All the phenotypes accumulated chlorophyll a and b at approximately the same rate with chlorophyll a at higher levels than chlorophyll b. In the same light all phenotypes demonstrated a decrease in chlorophyll b content while the amount of chlorophyll a remained constant. Then beginning at approximately day 12 the phenotypes start the second chlorophyll biosynthetic phase. A similar metabolic pattern for carotenoid accumulation ($\frac{CAR}{480}$) was observed (Table No. 16).

Figure 22.

RECALCULATION OF TOTAL CHLOROPHYLL OF THREE DARK GROWN SOYBEAN PHENOTYPES CULTURED UNDER GREENING CONDITIONS.

Total chlorophyll concentration: μg/gm fr. wt.). For other details see Figure 21.

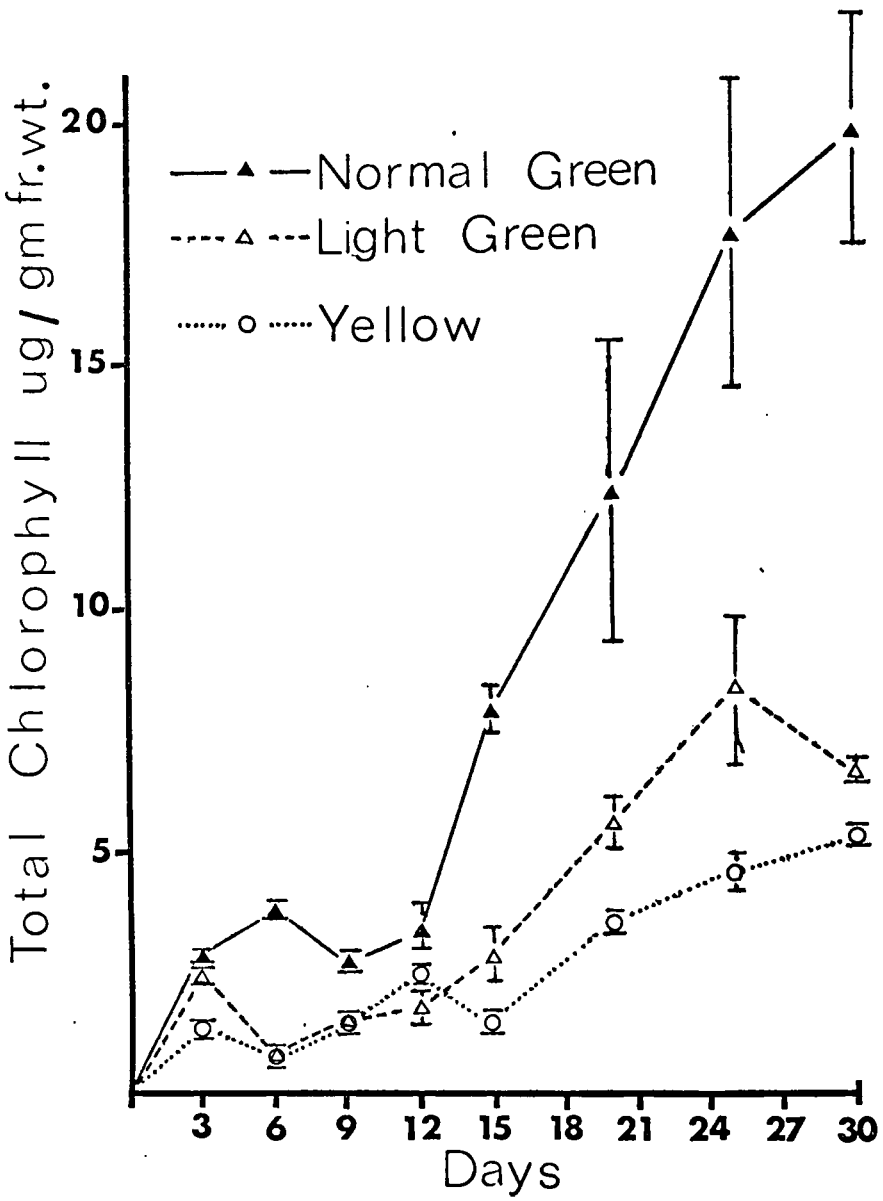

Total Chlorophyll ug/gm fr.wt.
20
15
10
5
Normal Green
Light Green
Yellow
3
6
9
12
15
18
21
24
27
30
Days

Figure 23.

BIOSYNTHESIS OF CHLOROPHYLL a AND b IN THE NORMAL GREEN PHENOTYPE.

Mean ± S.E.M. for 4 replicates of 5 pieces each.

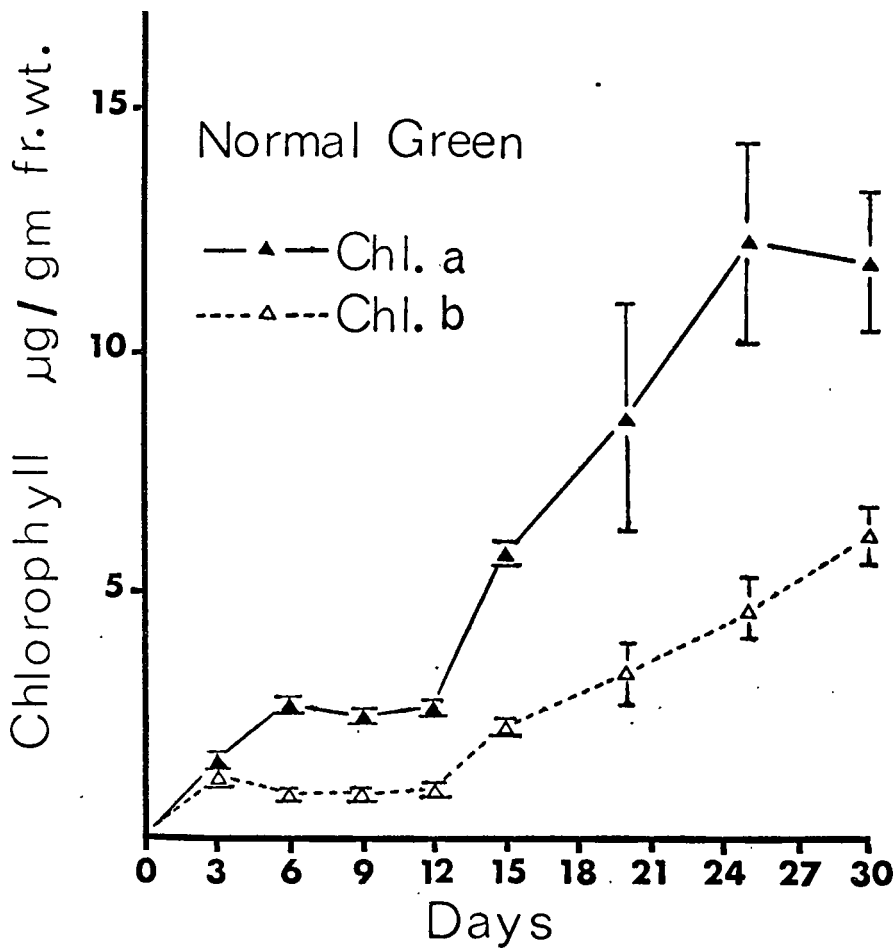
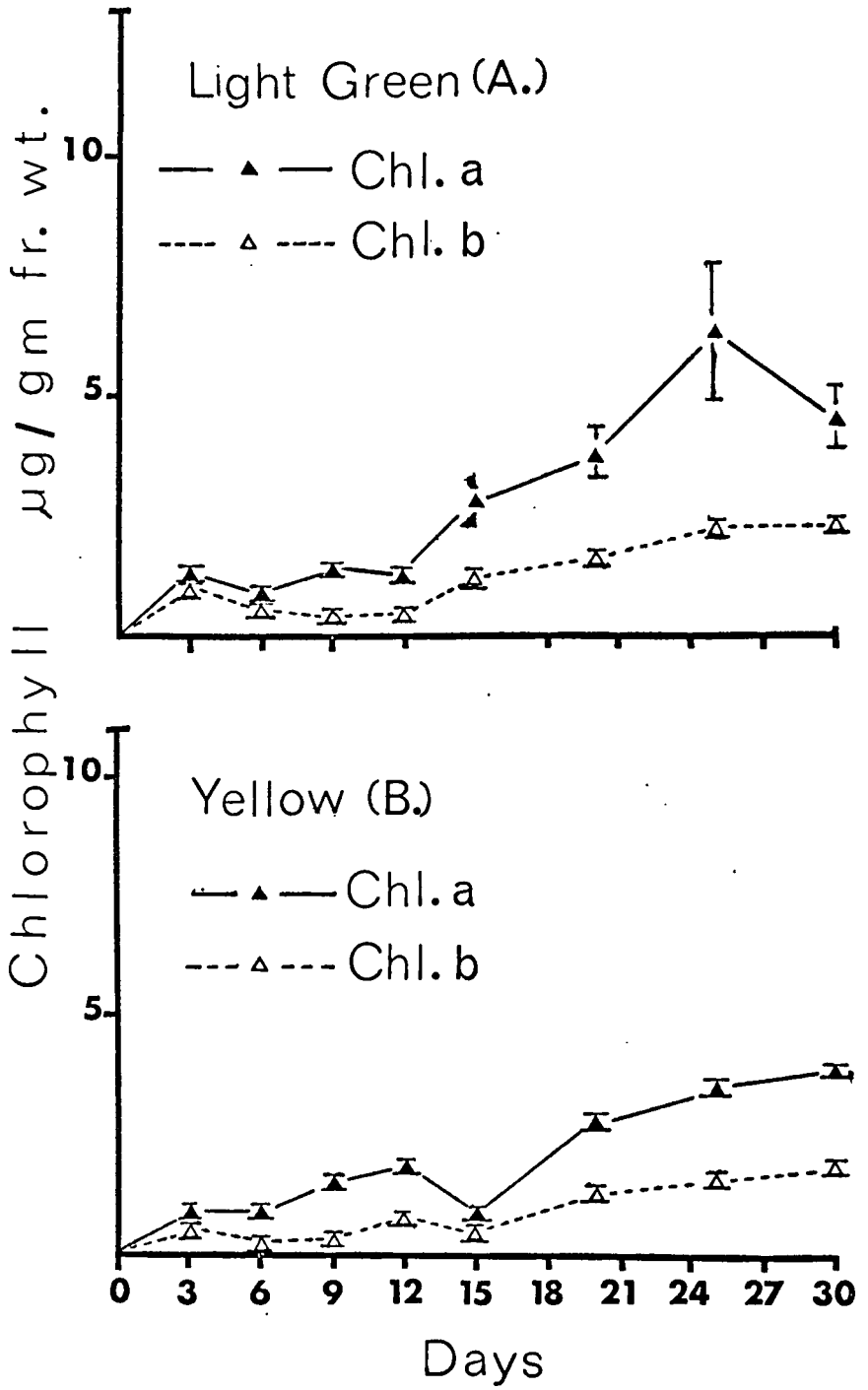

Normal Green
Chl. a
Chl. b
Chlorophyll µg / gm fr. wt.
15
10
5
0
3
6
9
12
15
18
21
24
27
30
Days

Figure 24.

BIOSYNTHESIS OF CHLOROPHYLL a+b IN THE LIGHT GREEN (A) AND YELLOW (B) PHENOTYPES.

Mean ± S.E.M. for 4 replicates of 5 pieces each. For other details see Figure 21.


Light Green (A.)
Chl. a
Chl. b
10
5
Yellow (B.)
Chl. a
Chl. b
10
5
Chlorophyll µg/gm fr. wt.
0 3 6 9 12 15 18 21 24 27 30
Days

When the callus cells enter the stationary phase the phenotypic variants maintained a uniformity in greening capability which suggested that growth sites are distributed randomly throughout the callus-mass. In some callus pieces new growth appeared to be confined to the periferial regions of the callus mass. Subsequently a cavity was formed underneath and therefore only the edge of the callus remained in contact with the medium. Even with this arrangement the greening capability of the variants remained uniform as indicated by even distribution of the green pigmentation. This pattern of greening occurred not only in the etiolated callus system but also in the chlorophyllous tissue as well.

DISCUSSION

Growth Studies

The response of these cultured strains to growth hormones and other additives demonstrated a dependency for a cytokinin-like factor for cell growth and division (Fig. 6 and Table No. 2). Also the failure of these phenotypes to grow on an auxin containing media (Fig. 5), places this callus system in one of four known categories: tissue which requires only a cytokinin for growth (217). Other groups have been characterized: tissues which growth with (59,63) and without an auxin (175), those which require both a cytokinin and an auxin (166) and tissues which respond only to media containing complex mixtures (135).

The addition of NAA (1 mg/l) and kinetin (1 mg/l) to the basal media maintained a callus tissue which was loose and friable in texture and reached approximately 1 Fresh Weight Growth Value (gm)/piece of callus tissue after a 30-day incubation period. This growth condition not only proved to be advantageous in the morphological and greening studies but also substantiated previous reports on the hormonal effects of callus morphogenesis (7,74,129,150,194).

The choice of growth parameters require the realization of growth and development of plant cells in culture (154). Two of the most widely used parameters were employed in this study, namely, fresh and dry cell weight. Packed

cell volume, cell wall, cell number, and mitotic frequency have also been used but each within itself could be misleading. For example Liau and Boll (120) noted a cell enlargement following an exponential increase in fresh and dry weight. Henshaw, et al (195) demonstrated large increases in dry weight taking place at a time when the mitotic frequencies had declined to a low rate. The use of dry weight as a growth parameter is probably the most commonly employed but it also must be considered with caution. For example, changes in cell type and size (154) and contamination of sugar and agar from the media and the storage of carbohydrates (90) tend to confuse the interpretation of dry weight data. For complete characterization of a growth media numerous parameters are necessary, namely, fresh and dry weight, cell number, size and type, mitotic frequencies, nitrogen and carbohydrate content (42,90,120,154).

The fact that coconut milk and yeast extract had opposite effects on callus growth was indicative of species specificity for initiation of cell division (Table No. 2). Grant and Fuller (74) found that yeast extract in varying concentrations with 2,4-D supported higher growth levels in Vicia faba callus root cultures than coconut milk. This variation could possibly be accounted for by the difference in source material thereby possessing different endogenous requirements for callus growth. Subsequently the success in inducing cell division would depend on the chemical

composition of coconut milk and yeast extract which are believed to differ (183,189,219).

The point underlining this concept of growth induction involves an unknown mechanism which stimulates quiescent cells to undergo cell division. Specific biochemical events have been associated with this transition (82,106, 107,220). In the presence of an auxin and cytokinin, cultured tissue of Nicotiana tabacum L. (var. Wis. No. 38) become active in that DNA synthesis and mitotic figures are evident after 3 days (41,143).

The growth patterns of these variants are similar to those found in other plant cultured tissue (42,90,120,154). The above data indicate two fundamental processes associated in the development of these soybean phenotypes. The first phase involves a rapid cell division with a slow increase in dry weight. The second phase represents the stationary growth and corresponds with cell enlargement and a high level of dry weight per callus culture. Davey, et al. (42) and Henshaw, et al. (90) demonstrated that an increase in cell number/ml culture and packed cell volume corresponds with the increase in dry weight. Rose and Martin (154) found that high peaks of nitrogen and carbohydrate content occurred during the exponential phase which corresponded with rapid cell division. The second metabolic trend was characterized with a high level of dry weight with a decreasing rate of nitrogen and carbohydrate accumu-

lation; more so in the case of nitrogen. Based on the nature of the data analyzed, the authors suggested that the terms "cytoplasmic growth phase" and "maturation phase" be recognized in characterizing callus growth.

The carbon source data (Table No. 3) in this investigation indicate that the mechanism for sucrose breakdown remains intact while the utilization of its monosaccharides, fructose and glucose, remains to be clarified. Sucrose utilization in higher plants has been shown to involve 4 enzymes (85,215). The catabolic process occurs by two different pathyways that involves either hydrolysis by invertase activity to give fructose and glucose or by the reverse reaction of sucrose synthetase to give ADP-glucose or UDP-glucose and fructose (85). The anabolic process occurs by either sucrose synthetase activity or sucrose phosphate synthetase and sucrose phosphatase activity (85).

Within this framework, it is possible that these variants possess the capability to utilize sucrose as a carbon and energy source by simple diffusion, by invertase activity and/or the reverse reation of sucrose synthetase. At the present time a controversy exists on whether the hydrolysis of exogenous sucrose acts as a prerequisite for absorption (38,53,143,155). Since cultured tissues have been shown to contain an acid invertase (cell-wall) and neutral invertase (38,53,182) which are found in most plant root systems (123,153,155), it seems reasonable that some of the

sucrose molecules will be hydrolyzed when absorbed into the cell (182) while the remaining molecules are taken up by diffusion. The reverse reaction of sucrose synthetase activity has been shown to occur in tobacco callus tissue and to reach its highest activity at the same time the cells are undergoing rapid cell division (182). Whether this enzyme occurs in this system is questionable.

The mechanism for glucose utilization by these variants seems to be impaired as indicated by the low growth values (Table No. 3). The ability of Acer pseudoplatanus callus tissue to grow on an exogenous supply of glucose most likely results from the ability of this tissue to produce enzymes to utilize this carbon source (215). In support of this concept was the evidence that labelled sucrose was formed by the callus tissue which had been fed radioactive glucose (215). Along the same lines Ball (13) was the first to demonstrate that sucrose, glucose and fructose were produced in a cultured tissue which was fed any sugar. The inability of these soybean phenotypes to utilize glucose as a substrate for growth could possibly be explained by an inhibition of sucrose synthetase activity. A high level of sucrose formation by sucrose synthetase has been found in some plant tissues and organs which require this carbon source for development (85). Therefore lacking this ability the sustained growth of these variants must be accountable to the direct incorporation of glucose into the glycolytic pathway, following

phosphatase activity. The existence of a specific uridine diphosphatase (pH 8.1) has been described (84) which hydrolyses UDP from nucleotide-sugars and subsequently prevents the activation of a usable form of the sugar. If present in these variants at critical levels, the activity of this enzyme could theoretically interfere with the sucrose synthetase activity and decrease the growth rate of these soybean phenotypes.

The inability of the NG phenotype to grow at comparable rates to the LG and Y variants when cultured on fructose may be accountable to a deficient sucrose synthetase. However, other factors must be involved.

Cytodifferentiation involves specific chemical factors which control vascular tissue differentiation (79). These stimuli are believed to be the interrelationship of hormones and sugars with several other influencing factors (186). In 1963 Wetmore and Rier (210) demonstrated that an agar block containing IAA and sucrose could reproduce a similar cytological pattern in cultured callus tissue as an excised bud. The concentration of the sucrose had a differentiating influence in that 2% sucrose produced xylem tissue within the growth centers, 4% produced only phloem and 3% resulted in a complex of xylem-phloem cells (210). Recently Fosket and Torrey (57) demonstrated that cytokinin, the cell division hormone, appeared to be the limiting factor since NAA did not support xylem differentiation in the absence of cytokinin. The physiological effects of sucrose

and growth hormones on the xylem differentiation process has recently been reviewed (176).

The data presented in this investigation supported the hormonal and nutritional concept for tracheid and vessel element differentiation. Numerous xylem and possible phloem elements were observed in the squash preparations (Fig. 12). It was evident that the basal media as supplemented with specific organic components (Table No. 1) supported good growth in the variants as well as cytodifferentiation and fine root hair formation. The appearance of these root hairs in cultures which were incubated on NAA (1 mg/l): coconut milk (10%) and coconut Milk (10%): yeast extract (0.1%) could possibly be due to the growth hormones that are known to occur in these natural complexes (219,221). However Wright and Northcote could not distinguish an appearance of these organs with the chemical composition of the medium (215).

Polysomatism occurs not only in higher plants (126,138) but also in cultured cells (45,77,100,131,156) and root segments (185,192). Based on the observations made of these soybean phenotypes (2N=40) in culture, the chromosomal instability of these cells is quite evident. This genetic heterogeneity has been considered by several workers to be the cause of the variability in physiological response to different chemical environments (117). The induction of these chromosomal changes is not well understood. Torrey

and Fosket (185) demonstrated that the presence of kinetin (0.1 ppm) initiated cortical cells of pea root segments to undergo a polyploid cell division before forming mature tracheary elements. The on-set of polysomatism results in an increase in chromosomal abnormalities, greater frequency of aneuploidy, and the loss of the capability for organogenesis (79,131,184). To some extent the growth conditions of a specific cultured tissue can influence the chromosomal stability (77) but the most effective method in regulating a genetically homogenous cell population has been shown to be the establishment of clonal lines (46).

Pigmentation and Greening of Three Soybean Callus Phenotypes

The chlorophyllous levels of these variants have been shown to be effected by these supplements: carbon source, coconut milk, amino acids, hormones, ascorbic acid and δ-aminolevulinic acid. It is difficult to determine if these additives have a direct effect on cell growth and/or chlorophyll biosynthesis. As demonstrated in this investigation (Table Nos. 2, 5, and 12) and others (34,61,94,95) the concentration and in some cases the type of sugar influenced the level of pigmentation. The reports by these workers indicate that a possible inverse relationship exists between growth and pigmentation. As previously stated the nutritional conditions that tend to stimulate growth are suppressive to chlorophyll synthesis and vice versa.

Substantiation of this negative correlation was evident as reported in this investigation. Low pigmentation occurred under optimal growth conditions (3% sucrose) while high chlorophyll and carotenoid formation occurred when cultured on suppressed growth conditions (3% glucose) (Table No. 2). The different levels of ascorbic acid also demonstrated a negative correlation of callus growth and chlorophyll content (Table No. 8). Jaspars (95) demonstrated that a green tobacco crown-gall strain which required starch for growth was sensitive to the levels of hydrolyzed glucose for chlorophyll synthesis. When cultured

on a wide range of glucose concentrations, in became evident that low (0.5%) and high (8%) levels of glucose supported increased chlorophyll formation while growth was suboptimal. At 3% glucose, pigmentation was minimal while increased growth values were noted. As demonstrated by Jaspars (95) and the evidence reported in this investigation (Table No. 12) both pigmentation and growth are suppressed under conditions of carbohydrate exhaustion. Since most studies in tissue culture are involved with optimal growth conditions, the conditions which promote chlorophyll synthesis are being overlooked.

Laetsch (115) has demonstrated that sucrose (2% and higher) when employed as a carbon sucrose inhibits chlorophyll synthesis in tobacco tissue cultures. Edelman and Hanson (52) have described a "sucrose effect" in a carrot culture strain (CRT 2) which lacked acid invertase (53). This callus strain maintained similar growth values and high chlorophyll levels when cultured on glucose and fructose (52). A spontaneous mutant (CRT 2) of this strain which possessed acid invertase activity grew and synthesized chlorophyll equally well on sucrose as well as on its monosaccharides (52). This "sucrose effect" was not observed in these soybean variants, however the presence and role of invertase in sucrose metabolism remains to be investigated.

Various supplements such as coconut milk, yeast extract, casein hydrolysate, synthetic amino acids and ascorbic acid have varied effects on the chlorophyll production in these variants (Table No. 2). The addition of coconut milk and a synthetic amino acid mixture to the basal media supported adequate callus growth and high chlorophyll production in these variants. However yeast extract and, to a less extent, casein hydrolysate support low growth values and a browning condition. Under the experimental conditions employed in this investigation it is difficult to assess which physiological condition was emphasized. Nevertheless a "green factor" has been suggested to be present in immature coconut milk which stimulates chlorophyll synthesis to occur in the dark (136). A comparable factor has also been found in the megametophyte of black pine which stimulates the formation of chlorophyll in primary leaves of transplanted wheat embryos in the dark (26). Vasil and Hildebrandt (193) demonstrated that undefined media containing coconut milk supported increases in growth of several chlorophyllous callus genera. The incorporation of yeast extract in the high-salt medium produced the greatest amount of growth as well as chlorophyll formation. Under autotrophic conditions (61), the addition of coconut milk, casein hydrolysate, kinetin, IAA, and ascorbic acid stimulated increased chlorophyll production in the chlorophyllous cultures employed in previous investigations (193). Coconut milk in combination with kinetin supported

good callus growth and chlorophyll formation whereas coconut milk alone supported only callus growth. The addition of ascorbic acid as a reductant favored callus growth in certain species while thiourea stimulated growth in all cultured genera (61).

In contrast, ascorbic acid supported adequate callus growth and high chlorophyll production in these soybean variants when grown on heterotrophic conditions (3% sucrose Table 8). Other cultured tissue routinely require this additive for good callus growth (30). Under the chemical environment employed, high levels (75 mg/l) of ascorbic acid increase the production of chlorophyll in the NG and LG phenotype and to a lesser extent in the Y phenotype. The sensitivity of ascorbate system (ascorbic acid dehydroascorbic acid) to physiological changes is well documented but a mechanism for this system at the molecular level has not been found (54). In many plant systems and more specifically the Brassica family, ascorbic acid, in the presence of its oxidase enzyme, can reduce the o-quinonoid bodies formed by polyphenol oxidase (11). Therefore this redox system prevents the accumulation of o-quinones and in turn prevents the browning condition observed in many cultured genera (11).

Growth hormones as well as nutrition influence chlorophyll synthesis. Several workers have demonstrated that low levels (0.5-1.0 mg/l) of an auxin, NAA or IAA more so

than 2,4-D, provides adequate callus growth and then cytokinins initiates the physiological condition conducive for chlorophyll formation (24, 177, 198, 199) and chloroplast maturation (168). Boasson and Laetsch (24) cultured Nicotiana tabacum L. var. Maryland Mammoth pith callus on starvation media for 3 days. After exhaustion of the endogenous cytokinins the callus cultures grew at a very slow rate on a salt media which indicates that the cytokinin synthesized by the tissue regulated cell growth at the expense of chlorophyll formation. The addition of kinetin to the salt media was not effective in initiating chlorophyll synthesis. Only when sucrose was added in combination with kinetin did the tissue support chlorophyll synthesis. Again these data are indicative of the interrelationship between cell growth and chlorophyll formation in that if callus growth is limited by other factors then the available cytokinins will regular chlorophyll and chloroplast development.

The role of kinetin in chloroplast biochemistry has been clarified by recent investigations of Kaul and Sabharwal involving two genetically similar tobacco clones (101,102). A non-green clone which failed to synthesize chlorophyll under light or darkness was stimulated to synthesize chlorophyll when maintained on low amounts (1%) of sucrose and high levels (2 mg/l or higher) of kinetin (53). A green callus clone which was derived by spontaneous mutation from

the albino failed to synthesize chlorophyll under similar chemical environments. Subsequently the genetic transition in this green clone occurred in its endogenous capacity to synthesize cytokinin. This became evident when the green clone supported chlorophyll formation when cultured on low amounts (1%) of sucrose and no kinetin. The mechanism initiating chlorophyll induction in this system involved δ-aminolevulinic acid dehydratase, kinetin and a low growth rate (1% sucrose) (102). In the non-green clone kinetin stimulated a significant increase in δ-aminolevulinic acid dehydratase activity preceding the greening condition. However kinetin caused a decrease in δ-aminolevulinic acid dehydratase in the green clone indicating an antagonistic effect between the exogenous kinetin and the endogenous cytokinins. It would be of interest to investigate the ALA dehydratase activity and other enzymes (160) in the soybean phenotypes which possess an exogenous cytokinin requirement for growth.

The ability for chlorophyll formation in these variants varied with their chemical environment. In most cases the LG phenotype synthesized higher levels of chlorophyll than NG phenotype while the Y phenotype maintained relatively low levels of pigmentation. However this variant was sensitive to the optimal physiological condition for greening. In contrast the greening ability of the parent material maintained the chlorophyllous ratio which was characteris-

tic of its genotype. There are numerous reports which discuss the variability of phenotypic products in tissue culture in comparison to the parent material (32,42,112,167, 195,213). Also it is believed that cultural selections account for growth patterns that appear in long-term tissue cultures (190). Therefore it is possible that the selection of the greener cultures which possibly occurred by a somatic mutation for the experimental tissues and serial transfers of the stock cultures accounted for this increased potential for chlorophyll synthesis in the LG phenotype. To further substantiate this hypothesis somatic mutation for increased greening capability in callus tissue cultures have been described by several workers (7,80,95,101,102,198). However this rationale does not rule out the possibility that the experimental conditions employed favored the LG phenotype for chlorophyll formation.

The accumulation of total chlorophyll by these phenotypes under greening conditions demonstrated a sigmoid pattern as found in other cultured genera (7,101,160,178,179) and higher plant systems (15,181,200). Although studies of Glycine max (7) and Hypochaeris, Acer and Oxalis (178) are believed to be in conflict, the net formation of chlorophyll took place in these variants during the decelerating phase of growth after division had terminated. Upon close inspection of the data presented by these workers (7,178), it became evident that light grown material was used in the

experimental culture which according to the data a functional chlorophyll synthesizing mechanism exists. Substantiation of this reasoning was the finding that dark-grown Oxalis (179) demonstrated a lag-phase in chlorophyll formation when subjected to continuous illumination. This sequence of cellular events was verified in these soybean phenotypes grown under continuous light and greening conditions. It was interesting that green Haplopappus cultures (178) demonstrated a similar greening pattern as noted in this investigation and non-green Oxalis tissue (179).

When the chlorophyll content was calculated on a fresh weight basis, two accumulation phases were observed which has not been previously reported in the literature. The decline in chlorophyll b formation before the second chlorophyll accumulation phase could possibly be related to proplastids transformation.

Most investigators agree that the undifferentiated protoplast usually found in shoot meristem differs biochemically from etioplast which are formed in seedlings germinated in the dark (109). Numerous reports have been published which describe etioplast (25,76,126,194,207) and proplastid biogenesis (20, 165) in higher plants during greening conditions. Sjolund and Weier (165) found that dark grown Streptanthus cultures contained proplastids that possess a "membrane complex" body which somewhat resembles the organized prolamellar body found in etioplasts.

A similar structure termed "interconnected tubular net" was found in protoplast of dark-grown cultures of Populus tremuloides Michx. by Blackwell et al. (20). Following illumination this network of membranes form a resemblance of a poorly organized prolamellar body with radiating thylakoidal extensions (20). These lamellar membranes subsequently become the granal system characteristic of mature chloroplasts (20).

Since the greening process is a slow development system (15,181,200) and can be controlled by several factors (164,201,202), the decline of chlorophyll b formation in these variants could be accountable to a slow transformation of prolamellar-like bodies to mature chloroplasts. Verification of this hypothesis remains to be documented with fine structural studies of these phenotypes. Added interest in this information could be the role that the level of chlorophyll has on the formation of these membranous structures.

In conclusion, the ability of these phenotypes to undergo callus growth and chlorophyll synthesis was influenced by the hormonal concentrations and supplements which were added to a modification of Miller's media (127). The factors that stimulated chlorophyll synthesis were kinetin, ascorbic acid, δ-aminolevulinic acid, glucose, coconut milk, and a synthetic amino acid mixture, whereas the factors that favored callus growth were casein hydrolysate, fructose and sucrose (except autoclaved sucrose at 30 gm/l). Therefore

the chemical requirements which favor chlorophyll formation suppress callus growth and vice versa. In support of this hypothesis the correlation coefficient (r_{XY}) was calculated in order to determine the degree of association between chlorophyll formation and callus growth of these phenotypes that were maintained on ascorbic acid (75 mg/l, Fig. 13). As indicated by the high negative values (NG: -.92, LG: -.94, and Y: -.99), it was evident that chlorophyll formation and callus growth in this cultured system are negatively correlated (see Appendix A for calculations).

The morphological characteristic and genetic heterogeneity of these phenotypes were found to be similar to other cultured genera (77, 133). Under the cultural conditions employed, the Y phenotype consisted of cells which were slightly larger than the NG and LG phenotypes plus all three variants demonstrated a degree of cytodifferentiation as noted by the observation of numerous tracheids. Even though several tetraploid cells were observed, the chromosome complement remains to be determined.

The pigmentation of these soybean phenotypes varied according to the physical and chemical cultural conditions employed. The heterozygous variant possessed the greater potential for chlorophyll synthesis possibly due to a somatic mutation and cultural selection of the greener cultures for maintenance of the stock cultures. This rationale does not

negate the possibility that the physiological conditions employed were sub-optimal for chlorophyll synthesis in the NG phenotype. This was evident in the high degree of sensitivity that this variant demonstrated (Figs. 13 and 16).

APPENDIX A

Correlation coefficient (r_{XY}) calculation: Raw score method.

$$r_{XY} = \frac{n\,XY-(\,X)(\,Y)}{n\,X^2-(\,X)^2 \cdot\; n\,Y^2-(\,Y)^2}$$

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