PREVENTION OF FAR RED LIGHT DESENSITIZATION OF GIBBERELLIN STIMULATED GERMINATION OF GRAND RAPIDS VARIETY LETTUCE SEEDS

WILLIAM RONALD MILLS

PREVENTION OF FAR RED LIGHT DESENSITIZATION OF GIBBERELLIN STIMULATED GERMINATION OF GRAND RAPIDS VARIETY LETTUCE SEEDS

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

William Ronald Mills

May 1973

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ABSTRACT

The objective of this research was to determine the following: (1) if far red irradiation of Grand Rapids variety lettuce seeds in chloramphenicol or actinomycin D prevents the loss of sensitivity to GA_3, (2) if chloramphenicol and actinomycin D increase the permeability of the far red treated seeds to GA_3, and (3) if the presence of chloramphenicol and actinomycin D during far red treatment inhibits protein synthesis in lettuce seeds during a subsequent dark germination phase.

Results from these experiments indicate that Grand Rapids lettuce treated with continuous far red light in chloramphenicol and actinomycin D are sensitive to gibberellic acid stimulation of dark germination. Seeds far red irradiated in water are inhibited when germinated in gibberellic acid. Seeds presoaked in gibberellic acid for 3 hours prior to 24 or 48 hours continuous far red light are not inhibited in their germination response. Treatment of the seeds with far red light in actinomycin D appears to decrease the permeability of the seeds to ^{14}C-leucine supplied with gibberellic acid. Far red irradiation of Grand Rapids lettuce seeds in chloramphenicol appears to enhance the permeability of the seeds to ^{14}C-leucine in

gibberellic acid during the period of dark germination. Incorporation of ^{14}C-leucine into protein is inhibited in seeds exposed to continuous far red light in the presence of actinomycin D. However, seeds irradiated in the presence of chloramphenicol have a two-fold increase in ^{14}C-leucine incorporation into protein. The greater amounts of ^{14}C-leucine incorporation into protein in chloramphenicol treated seeds may be a reflection of increased permeability of the seeds and not a true index of protein synthesis. Seeds far red treated in water exhibited the highest extractable exogenously supplied gibberellin as determined by the lettuce hypocotyl bioassay, even though these seeds were inhibited in their germination response by far red light. Seeds that were far red treated in chloramphenicol had lower extractable exogenously supplied gibberellin than those irradiated in water. Exposure of seeds to far red light in the presence of actinomycin D preceding treatment with exogenous gibberellic acid showed extremely low extractable gibberellin. Even though low gibberellin levels were found for seeds far red treated in chloramphenicol and actinomycin D before treatment with exogenous gibberellic acid, these seeds exhibited high germination in darkness following the far red treatment.

PREVENTION OF FAR RED LIGHT DESENSITIZATION OF GIBBERELLIN STIMULATED GERMINATION OF GRAND RAPIDS VARIETY LETTUCE SEEDS

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by

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May 1973

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To the Graduate Council:

I am submitting herewith a Thesis written by William Ronald Mills entitled "Prevention of Far Red Light Desensitization of Gibberellin Stimulated Germination of Grand Rapids Variety Lettuce Seeds." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Benj. A. Stone
Major Professor

We have read this thesis and recommend its acceptance:

Dan L. Findley
Second Committee Member

Floyd M. Ford
Third Committee Member

Accepted for the Council:

Wayne E. Stamp
Dean of the Graduate School

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CHAPTER I

INTRODUCTION

Flint and McAlister (1935, 1937) found that lettuce seeds are regulated in their germination response by light. Borthwick et al. (1952a) reported that Grand Rapids variety lettuce seeds are stimulated to germinate by red light and are inhibited by far red light. Several physical conditions, surgical operations, and a variety of chemicals have been shown to substitute for the light requirement for germination. Germination in Grand Rapids lettuce is totally stimulated by gibberellic acid (Kahn, Gross, and Smith, 1957). Certain inhibitors of RNA and protein synthesis are capable of stimulating germination in unirradiated lettuce seeds. These antimetabolites include D-chloramphenicol, L-thero-chloramphenicol, and actinomycin D (Black and Richardson, 1965, 1967, 1968). When the duration of far red light treatment is several hours or more, gibberellin stimulated germination of Grand Rapids lettuce is markedly inhibited. Burdett (1972) found that far red stimulation of desensitization of lettuce seeds to gibberellic acid could be overcome by various methods which suggest that far red light reduces lettuce endosperm permeability to exogenous gibberellin.

This study was undertaken to determine the following: (1) if far red irradiation of Grand Rapids lettuce seeds in the presence of chloramphenicol or actinomycin D prevents the loss of sensitivity to gibberellic acid, (2) if chloramphenicol and actinomycin D increase the permeability of the far red treated seeds to gibberellic acid, and (3) if the presence of chloramphenicol and actinomycin D during far red treatment inhibits protein synthesis in lettuce seeds during a subsequent dark germination phase.

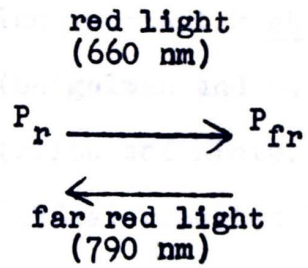
CHAPTER II

REVIEW OF LITERATURE

Flint and McAlister (1935, 1937) first reported that the germination of light requiring lettuce, Lactuca sativa, is promoted by red light and suppressed by far red light. Borthwick et al. (1952a) found that lettuce seed germination is promoted by radiation in the region of 525 nanometers (nm) to 700 nm. The greatest promotion resulting from a given irradiance was in the region of 660 nm. Germination was inhibited by radiation in the region of 700 nm to 820 nm, with the maximum inhibition between 710 and 750 nm.

Although the majority of seeds seem to be insensitive to light, many are stimulated or inhibited by exposure to continuous or short periods of illumination (Black, 1969). In addition to Grand Rapids variety lettuce, Lepidium virginicum (Toole, et al., 1955), Rheum rhaponticum, Nicotiana tabacum, Agrostis alba (Boucher, 1956), Arabidopsis thaliana (Shropshire, Klein, and Elstad, 1961), and Hypericum japonicum and Epilobium cephalostigma (Isikawa and Yokohama, 1962) are photoblastic, i.e. they are stimulated to germinate by white light. Lamium amplexicaule is inhibited in its germination response by red and stimulated by far red light (Jones and Bailey, 1956).

Borthwick et al. (1952a, 1954) and Butler, Hendricks, and Siegelman (1965) reported that repeated reversibility of the physiological responses regulated by red and far red light, clearly indicates that the actions are mediated by a photoreceptor pigment system which exists in two interconvertible forms. One form, commonly called phytochrome (P_r), absorbs maximally near 660 nm. The other form also known as phytochrome (P_{fr}), absorbs maximally near 730 nm. The following reaction scheme has been proposed by Borthwick et al. (1952a).



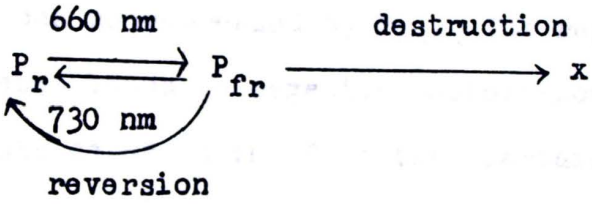
The fore mentioned photoreversible pigment was initially purified and assayed by Butler et al. (1959) and termed phytochrome by Butler, Hendricks, and Siegelman (1960). Hillman (1967) described phytochrome as a blue-green biliprotein readily soluble under alkaline conditions and bearing as a chromatophore one or more bilitrene moieties closely related to the chromatophores of the algal pigments phycocyanin and allophycocyanin. Mumford and Jenner (1966) using gel filtration and ultracentrifugation estimated the molecular weight of phytochrome to be about 60,000. Recent evidence indicates that the 60,000 molecular

weight phytochrome is an artifact resulting from the proteolysis of a larger phytochrome (Gardner, et al., 1971). Pratt (1973) and Cundiff and Pratt (1973) have estimated the molecular weight of phytochrome to be at least 240,000 and possibly close to 440,000.

Many different physiological responses to light can be partially understood on the basis of the various properties of phytochrome (Black, 1969). Induction of many responses has been shown to be photoreversible. Some of these responses are the following: seed germination (Borthwick et al., 1952a), flowering (Borthwick et al., 1952b), anthocyanin synthesis (Seigelman and Hendricks, 1957), and chlorophyll formation (Price and Klein, 1961).

Borthwick et al. (1952a) reported that lettuce seed germination was enhanced by the pigment which absorbed light maximally in the far red region of the spectrum. He also reported that seed germination was inhibited by the pigment which had its absorption maximum in the red region of the spectrum. Red light causes the formation of the active form of phytochrome P_{fr} which then induces germination. The conversion of P_r to P_{fr} requires about one-quarter the amount of energy as does the reverse reaction, which is why white light has the same action as red (Black, 1969). Reversal by far red of red light induction gradually diminishes as the period of darkness between red and far red

exposure increases. During this time, the seeds escape from the phytochrome control as P_{fr} initiates the reactions leading to germination (Borthwick et al., 1954). Mancinelli and Borthwick (1964) noted that in darkness P_{fr} reverts slowly to the inactive form of phytochrome. Butler, Lane, and Seigelman (1963) and Butler et al. (1965) indicated that both reversion of P_{fr} to P_r and destruction of P_{fr} have been measured. One does not necessarily find both reactions in any given tissue. Pratt and Briggs (1966) proposed the following scheme for the non-photochemical reactions of phytochrome:



Shropshire (1972) reported that measurement of the physiological responses and in vivo spectrophotometry have led to a variety of hypotheses as to the cellular mechanism by which phytochrome functions. Black (1969) gave five possible mechanisms for the action of phytochrome in plants. Two principal hypotheses appear to be generally accepted. One is that phytochrome operates at the level of gene expression resulting in the control of the synthesis of specific enzymes (Mohr, 1966, 1969). Since Stewart et al. (1964) reported that all plant cells are totipotent, Mohr (1969) suggested that the total genes of each particular

cell of a dark-grown seedling which is able to respond to P_{fr} must be divided into at least four functional types. These are active, inactive, potentially active, and repressible genes. According to his hypothesis, active genes are those which function the same way in an etiolated plant as they do in the light-grown plant. Inactive genes are active neither in the dark-grown seedling nor the seedling exposed to light. Potentially active genes are those which are ready to function and whose activity can be started or increased in some way by P_{fr}. The activation of potentially active genes leads to positive photoresponses. Repressible genes are those which can be repressed by P_{fr}. The repression of repressible genes leads to negative photoresponses.

A second hypothesis based on the fact that certain phytochrome regulated responses can be measured in 5 minutes or less suggests that phytochrome exerts control at the level of membrane permeability (Fondeville, Borthwick, and Hendricks, 1966); Hillman and Koukkari, 1967; and Hendricks and Borthwick, 1967). The work of Tanada (1968) lends support to this hypothesis. The root tips of barley or mung bean were excised and swirled in a liquid medium in a glass beaker. The glass surface had been previously charged with phosphate ions. Tips treated with red light adhere to the glass surface. The tips do not adhere, or adhere very little, to the beaker surface in the presence of far red

light. The above discussed phenomenon is called the Tanada effect (Shropshire, 1972). The Tanada effect is photoreversible and can take place within 30 seconds. The response requires indoleacetic acid, adenosine triphosphate, ascorbic acid, and manganese, magnesium and potassium ions (Tanada, 1968). Changes in permeability might possibly lead to the occurrence of reactions when enzymes and substrate are allowed to come into contact (Black, 1969).

At least three other possible mechanisms of phytochrome action in seed germination have been discussed by Black (1969). One is that P_{fr} is a key enzyme possibly involved in fat metabolism. Tietz (1953) reported charged lipolytic activity in seeds following illumination with white light, but Nyman (1966) found no indication of any effect of light on lipolytic activity before visible germination had taken place. A second possible mechanism is that changes in respiration occur in response to light. Nyman (1966) noted that in Pinus sylvestris anaerobic respiration increases after irradiation; albeit, Black (1969) reported that in many other seeds respiration is unaffected by light. Since gibberellin is known to replace the action of phytochrome in lettuce germination (Kahn, Gross, and Smith, 1957; and Kahn, 1960), it has been suggested that P_{fr} stimulates gibberellin biosynthesis (Brian, 1955). The work of some authors (Kohler, 1966a,b; and Reid, Clements, and Car, 1968)

tends to support this hypothesis, while others (Ikuma and Thimann, 1963); and Scheibe and Lang, 1965) have rejected this proposal as far as germination is concerned. Negbi, Black, and Bewley (1968) reported a strong synergism between P_{fr} action and exogenously supplied gibberellin.

Grand Rapids variety lettuce seeds require light for germination to occur (Borthwick et al., 1954). As previously mentioned, gibberellin can substitute for light in stimulation of germination of Grand Rapids variety lettuce (Kahn, et al., 1957; and Kahn, 1960). Several other chemicals have been shown to circumvent the light requirement for the germination of photosensitive lettuce. A group of antimetabolites including D-chloramphenicol, L-thero-chloramphenicol and actinomycin D have been shown by Black and Richardson (1965, 1967, 1968) to stimulate lettuce germination. Thompson and Kosar (1938) reported stimulation of lettuce germination by thiourea. Germination in darkness of photosensitive lettuce has been shown to be stimulated by kinetin, (Miller, 1956, 1958; and Ikuma and Thimann, 1963). Miller (1958) did report that P_{fr} is required for the kinetin stimulation of germination of Grand Rapids lettuce seeds.

Various physical alterations have also been found to initiate germination of photosensitive lettuce seeds in darkness. Ikuma and Thimann (1964) and Schiebe and Lang

(1965) reported that germination is stimulated by cold treatments. Frankland and Wareing (1960) have presented evidence that chilling increases endogenous gibberellin levels in seedlings. Black (1969) noted that the amount of P_{fr} which is required for the promotion of germination depends strongly on the conditions of stress imposed upon the embryo. Evenari (1965b) reported that an isolated embryo without its fruit and seed coats no longer has any light requirement, is not inhibited by short treatments with far red light, and does not develop thermodormancy. The opening or pricking of the endosperm membrane makes the seed behave like the isolated embryo. Ikuma and Thimann (1963) reported that neither red nor far red light was found to affect the elongation of the radicle of the de-coated seeds, whereas both affected the germination of intact seeds. They hypothesized that in order to account for the ability of red light to initiate germination, the final step in the germination control process is the production of an enzyme whose action enables the tip of the radicle to begin elongation.

Several workers, including Borthwick et al. (1954) have shown that far red light inhibits germination of Grand Rapids variety lettuce. Far red treatment can convert dark germinating seeds into those which display typical red, far red reversibility (Borthwick, et al., 1954). Early work

by Kahn (1960) and Ikuma and Thimann (1960) showed that prolonged far red treatments inhibit the action of gibberellic acid (GA_3) especially at sub-threshold levels. Kahn et al. (1957), and Kahn (1960), Ikuma and Thimann (1960), and Negbi, Black, and Bewley (1968) have reported GA_3 stimulation is not completely inhibited by short durations of far red light. Negbi et al. (1968) further reported that a GA_3 concentration of 50 micrograms per milliliter (µg/ml) is completely inactivated by preceeding far red light treatments of 6 hours. However, with concentrations of 100-500 µg/ml of GA_3, longer durations of far red light are necessary to achieve desensitization. They found that 6 hours of far red is ineffective in preventing the action of 100 µg/ml GA_3, while 18 hours far red was effective.

Black (1969) noted that by giving prolonged far red before supplying GA_3 it is possible to determine if a GA_3 induced process or one independent of GA_3 is inhibited by the far red treatment. He stated that prolonged far red prevents subsequent gibberellin action and therefore cannot act on a gibberellin induced process. He further reported that far red inhibition is irrevocable in darkness, since seeds kept on GA_3 for 72 hours after far red treatment fail to germinate.

Negbi et al. (1968) illustrated that 3-4 hours of far red light are sufficient for full desensitization to GA_3

stimulation if a 50 µg/ml concentration is used. Burdett (1972) also presents data which indicates severe inhibition of GA_3 stimulation of germination in Grand Rapids lettuce. This inhibition was manifested even when a concentration as high as 1 mM (346.4 µg/ml) was used.

Negbi et al. (1968) have investigated the effects of prolonged far red light on gibberellin action. They found that it is not necessary to expose seeds continuously, as far red from the 6th to the 12th hour of imbibition on GA_3 also prevents germination. Since far red inhibition, under most conditions, is irrevocable in darkness, Black (1969) assumed the existence of a far red sensitive pigment which is necessary for GA_3 action. He reported that most of this pigment seems to be present only after approximately the 6th hour of imbibition, and once it is changed by far red light, it apparently does not reform nor is any more synthesized. He further proposed that the pigment is formed by thermal reactions from a precursor, but the equilibrium between the two is very much on the side of the precursor. Far red removes the pigment and more forms from the precursor to restore equilibrium. Prolonged far red finally drains off all the pigment and all the precursor. The precursor is apparently not synthesized and therefore the inhibition permanent.

Burdett (1972) reported that the desensitization of Grand Rapids lettuce seeds by continuous far red light could be overcome by various physical or chemical treatments. He showed that GA_3 sensitivity of seeds having their endosperms punctured by a fine needle is not affected by prior far red exposure. He also reported a similar increase in GA_3 sensitivity of seeds having the hormone injected underneath the endosperm. He further noted that water imbibed seeds irradiated for 48 hours with far red light exhibited only 1% germination for 36 hours in the presence of 1 mM GA_3. When the same irradiation procedures were followed and the seeds were germinated in darkness in a 1 mM GA_3 solution buffered at pH 2.5, near maximum germination was found. Seeds germinated in the acetic buffer alone showed only 2% germination. From these data, Burdett (1972) proposed that the persistent loss of GA_3 sensitivity is due to an effect of the far red irradiation on the permeability of the endosperm to GA_3.

As reported earlier, Kahn et al. (1957) noted that gibberellin is capable of substituting for light in breaking seed dormancy. Donoho and Walker (1957) stated that gibberellin could circumvent the cold required for peach germination. Eagles and Wareing (1964) have shown that GA_3 is capable of substituting for cold or light treatments in breaking dormancy of buds in birch and sycamore.

One of the most striking effects of gibberellin is its ability to stimulate stem growth. Dwarf peas have been brought to grow at the rate of standard peas through the application of less than a microgram of GA_3 per plant. Phinney (1956) has shown that certain single-gene dwarf mutants of maize will grow to normal height with gibberellin application. Since the response is remarkably sensitive and as little as 0.001 ug GA_3 per plant is sufficient for a detectable reaction, maize may be used for gibberellin bioassay. Stem growth in cucumber (Brian and Hemming, 1961) and lettuce (Frankland and Wareing, 1960) are also stimulated by gibberellin. Robbins (1957) reported that gibberellin stimulated internode elongation can be so great that bushy plants grow like vines. Stowe and Yamaki (1959) found the number of internodes is unchanged by gibberellin treatment, although elongation occurs. They also noted that usually growth promotion is restricted to young tissue with mature tissue not influenced by gibberellin.

Several other morphological changes may be stimulated by gibberellin. Lang (1957) found induction of flowering in a wide variety of annual plants. Some of these are Crepis leontodoides, Lapsana communis, Amethum graveolens, Spinacia oleraces, and Raphanus sativus. He obtained similar results for several biennial plants including Hyoscyamus niger, Daucus carota, Brassica napus, and Petrosilenum

sativum. He further stated that in plants treated with gibberellin, stem elongation in most cases precedes flower initiation in a conspicuous manner. Lang (1957) noted that the primary effect of gibberellin is on stem elongation and that flower formation is induced indirectly. The bolting plant becomes capable of forming inflorescence. Stoddart (1962, 1966) noted that gibberellins do not have a direct effect upon floral initiation in red clover but the presence of a minimum level is apparently necessary for the successful completion of the process. Other morphological manifestations brought about by gibberellin are leaf expansion (Stowe and Yamake, 1957), parthenocarpic growth of fruit (Crane, 1964), and the ability to increase maleness (stamens) in flowers (Galun, 1959).

There are conflicting reports as to whether gibberellin stimulates cell division or cell elongation or both. Lockhart (1960), Stowe and Yamaki (1957), and Feucht and Watson (1959) reported that gibberellin stimulates cell elongation. Greulach and Haesloop (1958) noted that gibberellin stimulation is due to cell division. Sachs, Bretz, and Lang (1959) reported that stimulation of bolting in rosette plants is due to an activation of cell division in the normally inactive subapical meristem. Growth is due to an increase in the number of cells. Many authors give evidence that gibberellin stimulates both cell division and

cell elongation (Guttridge and Thompson, 1959; Cleland, 1964; and Arney and Mancinelli, 1966).

The mechanism of action of gibberellin has not been unequivocally elucidated. One possibility that has received wide attention is that gibberellin exerts its physiological effect by altering the auxin status of the tissue. Kuraishi and Muir (1964) found that treatment of rosette Hyascyamus plants caused a forty-fold increase in auxin level. Auxin clearly will not replace gibberellin in the production of α-amylase in barley (Paleg, 1960). Many gibberellin responses occur even when the action of endogenous auxin is prevented by antiauxin (Cleland and McCombs, 1965). Another possibility that is extremely interesting is that gibberellin acts at the gene level to cause de-repression of specific genes. The activated genes would, in turn, through the production of new enzymes bring about the observed morphogenic changes (Cleland, 1969). Paleg (1960) reported that gibberellin increases α-amylase production in barley. Studies by Varner and Chandra (1964) on the incorporation in vivo of labeled amino acids into proteins of aleurone layers of barley suggest de novo synthesis of α-amylase in response to added gibberellic acid. In the absence of gibberellin, the aleurone cells of barley endosperm contain only trace elements of α-amylase. The evidence that the enzyme has been synthesized de novo comes from the fact that

several labeled amino acids are found to be synthesized into the α-amylase molecule in response to the addition of gibberellic acid. Another is that the appearance of α-amylase requires the synthesis of new RNA, presumably messenger-RNA (m-RNA) which codes for the α-amylase protein. Varner and Chandra (1964) by the use of actinomycin D have shown the timing of the events leading to α-amylase production. The period when the synthesis of α-amylase can be blocked by actinomycin D is the first 7 hours after treatment with gibberellin. This is before any increase in α-amylase can be detected. Thereafter the process is insensitive to actinomycin D. Apparently all of the necessary m-RNA has been formed in this initial period and the synthesis of α-amylase can then proceed, using m-RNA as a template (Cleland, 1969).

Antimetabolites which have been found to stimulate germination in Grand Rapids variety lettuce are D-chloramphenicol, L-thero-chloramphenicol, and actinomycin D (Black and Richardson, 1965, 1967, 1968). Black and Richardson (1965, 1967, 1968) observed that the promotive effect of chloramphenicol on germination and the prevention of skotodormancy was accompanied by a reduction of protein synthesis. Other inhibitors of nucleic acid and protein synthesis, including 8-azauracil, 2-thiouracil, and cycloheximide, are strong inhibitors of germination in intact

seeds as well as inhibitors of radicle elongation of half seeds. Chloramphenicol is an inhibitor of protein synthesis in bacteria (Brock, 1961). Chloramphenicol is also known to inhibit chloroplast protein synthesis (Ellis, 1969) and mithchondrial protein synthesis (Freeman, 1969). In bacteria, chloramphenicol was found to bind to the 50 s subunit of the 70 s ribosome (Vasquez, 1966) and prevent the movement of the ribosomes along the messenger ribonucleic acid (Webber and Demoss, 1966). Hanson and Kruger (1966) have suggested that chloramphenicol primarily affects oxidative phosphorylation.

Actinomycin D is a bright red antibiotic containing two peptides (Vinning and Waksman, 1958). It is an inhibitor of DNA-dependent RNA synthesis with a resulting inhibition of protein synthesis (Kirk, 1960; Reich, et al., 1961, 1962; Goldberg and Rabinowitz, 1962). Reich et al. (1962) found that RNA-dependent RNA synthesis is unaffected by actinomycin D. Reich and Goldberg (1964) reported that the binding of actinomycin D to DNA, which requires the presence of guanine in a helical configuration, is responsible for the inhibition of DNA-dependent RNA synthesis by RNA polymerase and accounts for the biological properties of actinomycin D. The susceptibility of RNA synthesis, catalyzed by RNA polymerase, to inhibition by actinomycin D reflects the binding of the antibiotic to the DNA. The authors further reported that

actinomycin D does not directly inhibit the enzyme, nor does it compete with the nucleotide precursors or cofactors, since the inhibition of RNA polymerase by actinomycin D cannot be overcome by increasing the concentration of enzyme, cofactor, or precursors.

Two modes for the binding of actinomycin D have been proposed (Reich and Goldberg, 1964). Hamilton, Fuller, and Reich (1963) proposed that the actinomycin chromophore is hydrogen-bonded to the outside of the DNA helix. Stabilization of the complex is supposedly provided by a hydrogen bond between the actinomycin quinone oxygen and the 2-amino group of guanine and the deoxyribose ring oxygen. The peptide lactones were considered to provide additional hydrogen-bonds with phosphodiester oxygens. Mueller and Crothers (1968) have proposed that the actinomycin chromophore is intercalated into the DNA chain with the peptide lactones projecting into the DNA minor groove. Wells and Larson (1970) have presented evidence concerning the binding of actinomycin D which is inconsistent with the proposal of Hamilton et al. (1963).

Black and Richardson (1967, 1968), as cited earlier, noted a stimulation of germination of Grand Rapids lettuce by D and L-thero-chloramphenicol and actinomycin D. With germination, they reported a concomitant decrease in protein synthesis. If there is a causal relationship between the

suppression of protein synthesis and the stimulation of germination, a plausible explanation is that the protein synthesis which occurs in imbibed seeds in darkness is associated with some inhibitory mechanism. Since Evanari (1965a) stated that the intact endosperm prevents the growth of the embryo in lettuce, Black and Richardson (1968) suggested that whatever the mechanism, chloramphenicol acts as "an inhibitor of an inhibition" by preventing the inhibitory action of the endosperm. Wagner, Bienger, and Mohr (1967) found that chloramphenicol stimulated anthocyanin production in Sinapis. They have attributed the effect of the antibiotic to the prevention of protein synthesis in the chloroplast, thus making phenylalanine available for the synthesis of anthocyanin. A similar mechanism might conceivably operate in lettuce seeds, where inhibition of synthesis of some proteins may make more amino acids available for the synthesis of others, which then causes germination (Black, 1969). Since chloramphenicol has been reported to induce enzyme production in Staphylococcus (Ramsey, 1966), it has been suggested that the stimulation of germination by these antimetabolites might be due to an induction of a specific enzyme (Black and Richardson, 1968).

CHAPTER III

MATERIALS AND METHODS

Seed Germination

Seeds used in this study, Lactuca sativa, variety Grand Rapids, were purchased from Joseph Harris Company, Moreton Farm, Rochester, New York. They were stored in a sealed container at approximately 4°. Seeds were chosen at random and soaked in distilled water in darkness for approximately 1.5 hours, the optimal time (Ikuma and Thimann, 1960), preceding subsequent experimental treatment. Approximately 100 seeds were then transferred to 5 centimeter (cm) Petri dishes supplied with one layer of Whatman No. 1 filter paper. The filter paper was moistened with 1.8 ml of water or chemical solution. To prevent exposure to light, these and subsequent transfers were made in a dark room equipped with a green safety light.

Seeds were irradiated with far red (FR) light for 24 or 48 hours at approximately 25°. The light source was a 150 watt reflector flood operating at 120 volts. The lamp was secured to a ring stand 60 cm above the seeds. An 8 cm water screen was placed between the light source and the seeds. The seeds were irradiated in a light secure box

equipped with a Carolina Biological Supply (CBS) No. 750 far red filter between the light source and the seeds. The seeds received an approximate irradiance of 4.8×10^{-3} joules cm^{-2} sec^{-1}.

The seeds were irradiated with far red light in the presence of water or solutions of chloramphenicol (CAP) or actinomycin D (Act. D). After the far red irradiation period, seeds were washed thoroughly with distilled water in a Buchner funnel. The seeds were then transferred to 5 cm Petri dishes containing Whatman No. 1 filter paper moistened with 1.8 ml of water or gibberellic acid (GA_3) solution. These methods are similar to those described by Burdett (1972). The dishes were placed in light proof Petri dish sterilization cans and germinated in darkness for 48 hours at 25°. Two to 6 replicate dishes were employed for each treatment. Emergence of the radicle, determined by inspection with the naked eye, was the criterion for germination.

In most experiments a similar set of treatments was carried out on seeds receiving no far red irradiation. In these cases, seeds were pre-soaked in water, in darkness, for 1.5 hours. They were transferred to dishes containing water or the experimental solutions as indicated in the results. The seeds were then germinated in darkness according to the fore mentioned procedures.

An experiment was conducted in which the effects of pre-soaking of seeds in GA_3 solution were studied. The above procedures were followed except for the following modifications. Seeds were pre-soaked for 3 hours in a 0.5 millimolar (mM) GA_3 solution. They were washed with distilled water preceding dark germination or far red irradiation. One group of seeds was then germinated in darkness in water, CAP, Act. D. or GA_3. Another group of seeds was irradiated with far red light for 48 hours in water, CAP, Act. D., or GA_3 preceding dark germination in water.

The chemicals used were obtained from the following sources: actinomycin D from Merck, Sharp, and Dohme Research Laboratories, Rathway, New Jersey; D-chloramphenicol from Sigma Chemical Company, St. Louis, Missouri; and gibberellic acid from Eastman Organic Chemicals, Rochester, New York.

Light intensity was determined through the use of a YSI-Kettering model 65A Radiometer, Yellow Springs Instrument Company, Yellow Springs, Ohio.

Appearance of Labeled Amino Acid In the Soluble Precursor Pool and Incorporation of Labeled Amino Acid Into Protein

Seeds were pre-soaked in sterile distilled water for 1.5 hours preceding far red irradiation for 48 hours in 1.8 ml or 8.0 ml sterile solution of Act. D., CAP, or water.

One-tenth gram or 1.0 gram of seeds was placed in each sterile Petri dish on sterile Whatman No. 1 filter paper. Following far red irradiation, the seeds were washed with sterile distilled water and transferred to sterile 5 cm Petri dishes containing 1.8 ml of distilled water or GA_3 solution. The distilled water and 1 mM GA_3 solution were sterile and contained penicillin and streptomycin at a concentration of 10^{-4} molar (M). The GA_3 solution and the distilled water were sterilized by filtration through a Swinnex-25 with a Millipore HA 0.45 micron (μ), 25 mm filter into sterile flasks. All transfers were made with sterile instruments. Transfers were made in a dark room with a green safe light. One microcurie (μc) of ^{14}C-L-leucine was added to each dish. The seeds were allowed to germinate in darkness at 25° for 15 hours.

Determination of ^{14}C-L-leucine in the total precursor pool and incorporation into protein was assayed according to a modification of the procedures of Holleman and Key (1967). After 48 hours far red irradiation in the presence of ^{14}C-L-leucine, the seeds were rinsed 4 times with distilled water. The seeds were then homogenized in a cold mortar and pestle or a VirTis homogenizer in 10 ml of 0.01 normal (N) Tris buffer pH 7.4. A 4 ml fraction of the homogenate was filtered through Miracloth (Miracloth, Chicopee Mills Incorporated, New York). The mortar or homogenization flask

were rinsed twice with 2.5 ml of Tris buffer with this solution then being poured through the Miracloth. A 4.5 ml portion of the filtrate was made to 10% trichloroacetic acid (TCA). The TCA treated filtrate was kept at 2° for 1 hour and then centrifuged for 10 minutes at 1000 g. A 0.2 ml aliquot from the supernatant from the preceding centrifugation was added to the filter paper and the paper placed under a 250 watt infrared lamp. This lamp was employed to facilitate drying. The pellet from the 1000g centrifugation was dissolved in 3 ml of 0.5 N solium hydroxide. Cold TCA was added until the final concentration was 10% and allowed to stand for 1 hour at 2°. The solution was centrifuged at 1000 g for 10 minutes and the precipitate washed twice with 5% TCA. The pellet was then dissolved in 4 ml of 2 N ammonium hydroxide (NH_4OH). A 0.2 ml aliquot of the NH_4OH solution was added to 1 inch squares of filter paper. The papers were dried under a 250 watt infrared lamp. Radioactivity of the original supernatant and of the NH_4OH pellet solution was determined by placing the filter paper squares in scintillation vials. Each vial contained 15 ml of scintillation solution (4 grams of 2,5-diphenyl-oxazolyl and 50 milligrams of 1,4-bis-2 (5-phenyloxazolyl) benzene/ liter of toluene). The vials were placed in a Nuclear Chicago Unilux III liquid scintillation counter and counted for 10 minutes. Protein was assayed according to the methods of Lowry, et al. (1951).

The chemicals used were obtained from the following sources: ^{14}C-L-leucine, specific activity 253 millicuries (mc)/mM was purchased from New England Nuclear Corporation, Boston, Massachusetts; streptomycin sulfate and penicillin G from Nutritional Biochemical Corporation, Cleveland, Ohio.

Bioassay of Gibberellin in Experimentally Treated Lettuce Seeds

Seeds were selected at random and pre-soaked in distilled water for 1.5 hours. Three grams (dry weight) of seeds were placed in 5 inch Petri dishes on Whatman No. 1 filter paper. The filter paper had been previously moistened with 8 ml of water, or solutions of Act. D. or CAP. The seeds were irradiated with FR light in a manner similar to that previously described. After irradiation, the seeds were transferred in a darkroom equipped with a green safe light to the 5 inch Petri dishes containing filter paper treated with distilled water or 1 mM GA_3 solution. The seeds were germinated in darkness for 15 hours at 25°.

Following the 15 hour dark germination period, the seeds were washed 3 times with distilled water. Seeds were then heated for 10 minutes at 85°-95° to inactivate the enzymes. The seeds were homogenized in 20 ml cold water in a VirTis 45 homogenizer. The homogenate was centrifuged at 3800 g for 10 minutes. The precipitate was homogenized

twice more in 20 ml cold water. A syringe was used to remove the supernatant to exclude the upper lipid layer. Enough ammonium chloride (NH_4Cl) was added to make the extract to 0.2 M NH_4Cl. The extract was centrifuged for 10 minutes at 3800 g and the supernatant removed.

Sephadex G-15 was slurried in 2% sodium chloride (NaCl) solution and poured into a vertically mounted glass column. The column had been partially filled with 2% solution of NaCl to insure correct packing of the gel. Two liters of water were passed through the column prior to addition of the extract. Twenty-five ml of the extract containing added NH_4Cl was placed on a 2 x 35 inch column of Sephadex G-15. The column was eluted with water with about 25 fractions of approximately 10 ml each being collected in 25 minutes. The preceding methods are similar to those of Renyolds (1970).

A bioassay for gibberellin similar to that described by Frankland and Wareing (1960) was carried out. Grand Rapids variety lettuce seeds were germinated in 5 cm Petri dishes on Whatman No. 1 filter paper. The filter paper had previously been moistened with 4 ml from each 10 ml Sephadex G-15 fraction. The seedlings were placed 15 cm below a flourescent light source and the temperature maintained at 28°. The hypocotyl length was recorded after 5 days, each hypocotyl being measured to the nearest millimeter (mm).

The chemicals used were obtained from the following sources: Sephadex G-15 from Pharmacia Fine Chemicals, Piscataway, New Jersey; gibberellic acid from Eastman Organic Chemicals, Rochester, New York.

CHAPTER IV

RESULTS

Effect of Chloramphenicol and Actinomycin D Treatment During Continuous Far Red Irradiation of Grand Rapids Lettuce on Subsequent Dark Germination in Gibberellic Acid

Black and Richardson (1965, 1967, 1968) reported that the isomers of chloramphenicol and actinomycin D could stimulate dark germination of Grand Rapids variety lettuce. They also noted that chloramphenicol could prevent the onset of skotodormancy.

Experiments were undertaken to determine the effect of continuous far red irradiation in the presence or absence of CAP or Act. D on gibberellin stimulated dark germination of Grand Rapids lettuce seeds. Unirradiated seeds germinated in water, actinomycin D and GA_3 (Figure 1) showed the typical germination responses (Borthwick et al., 1952a; Kahn, 1960; and Black and Richardson, 1967). Seeds irradiated in the presence of water and Act. D and then germinated in water in darkness were inhibited in their germination response (Figure 1). Seeds irradiated in the presence of Act. D before germination in GA_3 exhibited a significantly higher germination percentage than seeds irradiated in water preceding GA_3 treatment. It is interesting to note that

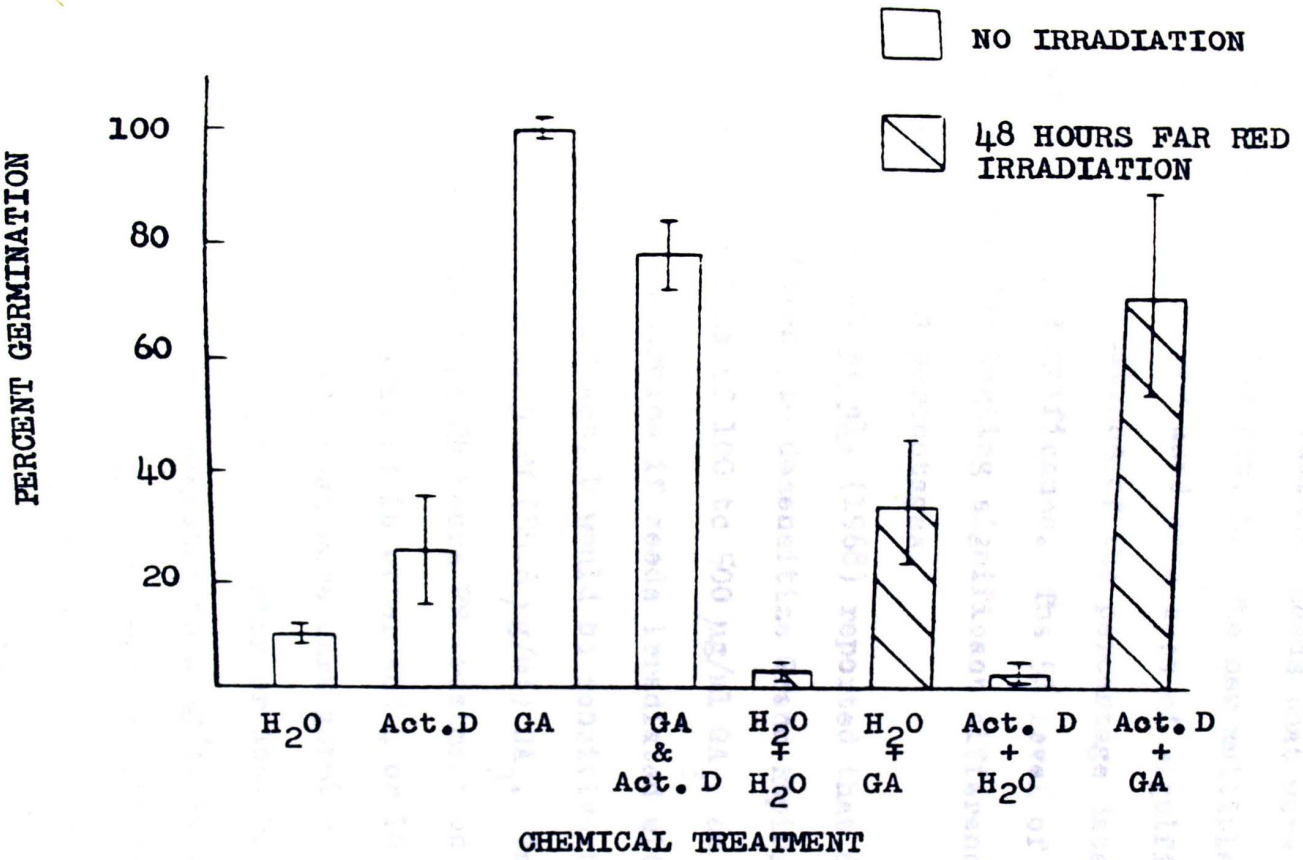


FIGURE 1. EFFECT OF CONTINUOUS FAR RED LIGHT, ACTINOMYCIN D AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS.

Unirradiated seeds were germinated in darkness for 48 hours following imbibition. Far red irradiated seeds were treated with light for 48 hours in actinomycin D (Act. D) or water, before being germinated in darkness for 48 hours at 25° in 1 mM gibberellic acid (GA_3) or water. The actinomycin D concentration was 2 ug/ml. Six replicates of approximately 100 seeds were employed for each chemical treatment.

there is no significant difference between the percentage germination for seeds irradiated with FR light before germination and those unirradiated seeds that were germinated in darkness in GA_3 plus Act. D. The new multiple range test of Duncan (1955) was used as a test of significance. Arcsine transformations of the percentage data were made before testing significance. The 5% level of significance was employed for testing significant differences among the mean germination percentages.

Since Negbi et al. (1968) reported that 6 hours far red was not sufficient to desensitize Grand Rapids lettuce seeds to concentrations of 100 to 500 µg/ml GA_3, an experiment was conducted to determine if seeds irradiated with FR light in the presence of Act. D would be sensitive to 0.5 mM (173.2 µg/ml) or 0.25 mM (86.6 µg/ml) GA_3. Table I illustrates the effect of 24 hours FR treatment on dark germination of seeds irradiated in water and 4 or 10 µg/ml Act. D. Far red treated seeds that were dark germinated in water, regardless of irradiation in water or Act. D. exhibited poor germination. Seeds irradiated in 4 µg/ml Act. D were insensitive to 0.25 mM GA_3, but were sensitive to 0.5 mM GA_3. Seeds far red irradiated in the presence of 10 µg/ml Act. D were stimulated to germinate by both 0.25 and 0.5 mM GA_3.

TABLE I

EFFECT OF CONTINUOUS FAR RED LIGHT AND VARIOUS CONCENTRATIONS OF ACTINOMYCIN D AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS

48 Hour Contimuous Far Red Light	48 Hour Dark Germination	Percent Germination
H_2O	H_2O	0.0 ± 0.0
H_2O	GA 0.25	11.8 ± 0.1
H_2O	GA 0.50	25.0 ±16.1
Act. D 4	H_2O	3.3 ± 2.1
Act. D 4	GA 0.25	33.0 ± 2.0
Act. D 4	GA 0.50	67.7 ± 4.5
Act. D 10	H_2O	19.1 ± 1.8
Act. D 10	GA 0.25	75.7 ± 4.6
Act. D 10	GA 0.50	85.9 ± 2.3

All seeds were far red irradiated for 24 hours in water or actinomycin D (Act. D) preceding dark germination in water or gibberellic acid at 25° for 48 hours. Actinomycin D concentrations are in µg/ml. Gibberellic acid concentrations are expressed in millimoles. Three replicates of approximately 100 seeds were employed for each chemical treatment.

Preliminary experiments to determine the optimal concentration of Act. D used during the irradiation period illustrated conflicting results. An experiment was conducted to determine the optimal Act. D concentration for seeds germinated in darkness in 1 mM GA_3. Figure 2 illustrates that an Act. D concentration of 4 µg/ml sensitized the Grand Rapids lettuce seeds to the greatest germination stimulation by GA_3. It must be mentioned that again these results are conflicting; as in the previous experiment, seeds were rendered more sensitive to GA_3 stimulated dark germination by 10 µg/ml Act. D.

As previously mentioned, Black and Richardson (1965) noted that chloramphenicol is capable of replacing light in stimulation of germination of Grand Rapids lettuce, as well as being able to inhibit the onset of skotodormancy. Figure 3 illustrates data from an experiment conducted to determine if the presence of chloramphenicol during FR irradiation could prevent the loss of sensitivity of Grand Rapids lettuce seeds to stimulation of dark germination. The typical germination responses as reported by Kahn (1960), Borthwick et al. (1954), and Black and Richardson (1965) were found for unirradiated seeds, with one exception. The anomaly was the germination of unirradiated seeds in chloramphenicol. Black and Richardson (1965) showed a stimulation of dark germination of over 50% by D-chloramphenicol.

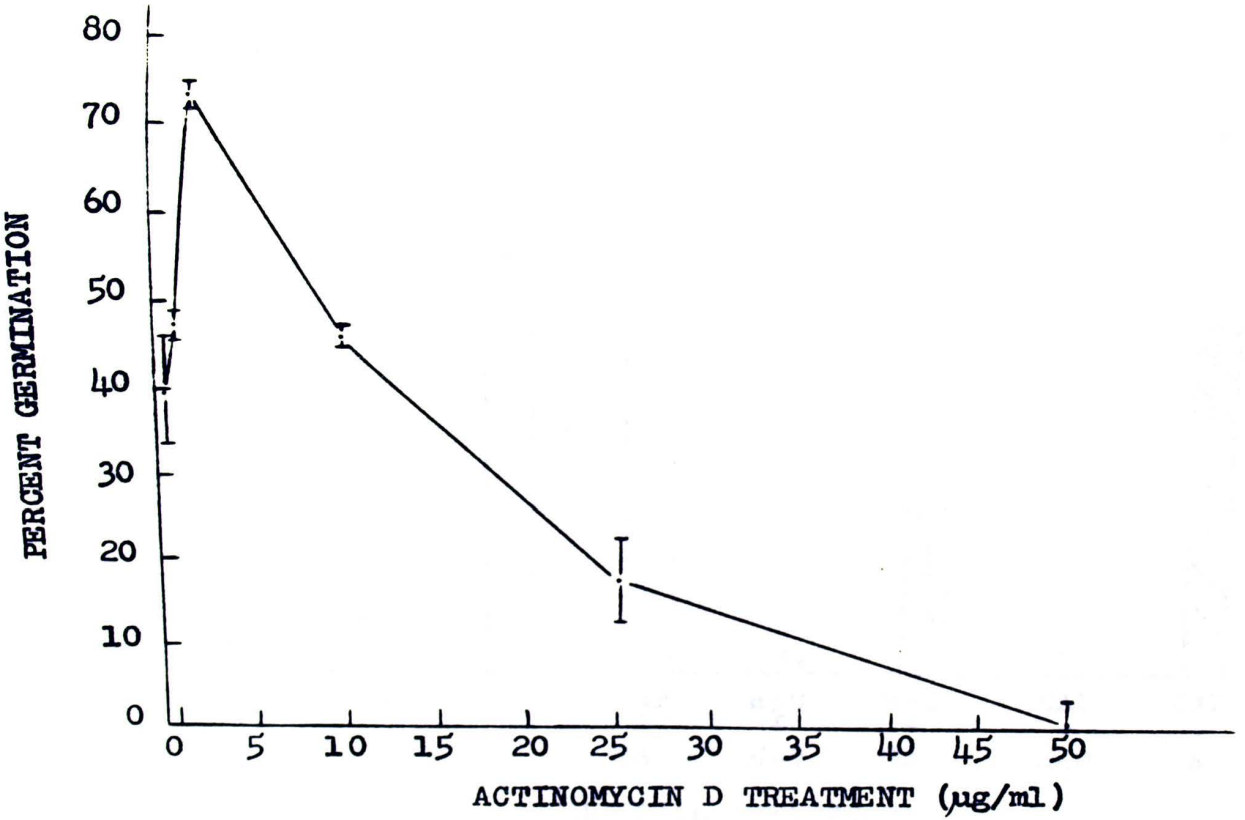


FIGURE 2. EFFECT OF CONTINUOUS FAR RED LIGHT, VARIOUS CONCENTRATIONS OF ACTINOMYCIN D AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS.

All seeds were far red irradiated for 24 hours in water or actinomycin D preceding dark germination in water or gibberellic acid at 25° for 48 hours. Gibberellic acid concentration was 1 mM. Two replicates of approximately 100 seeds were employed for each treatment.

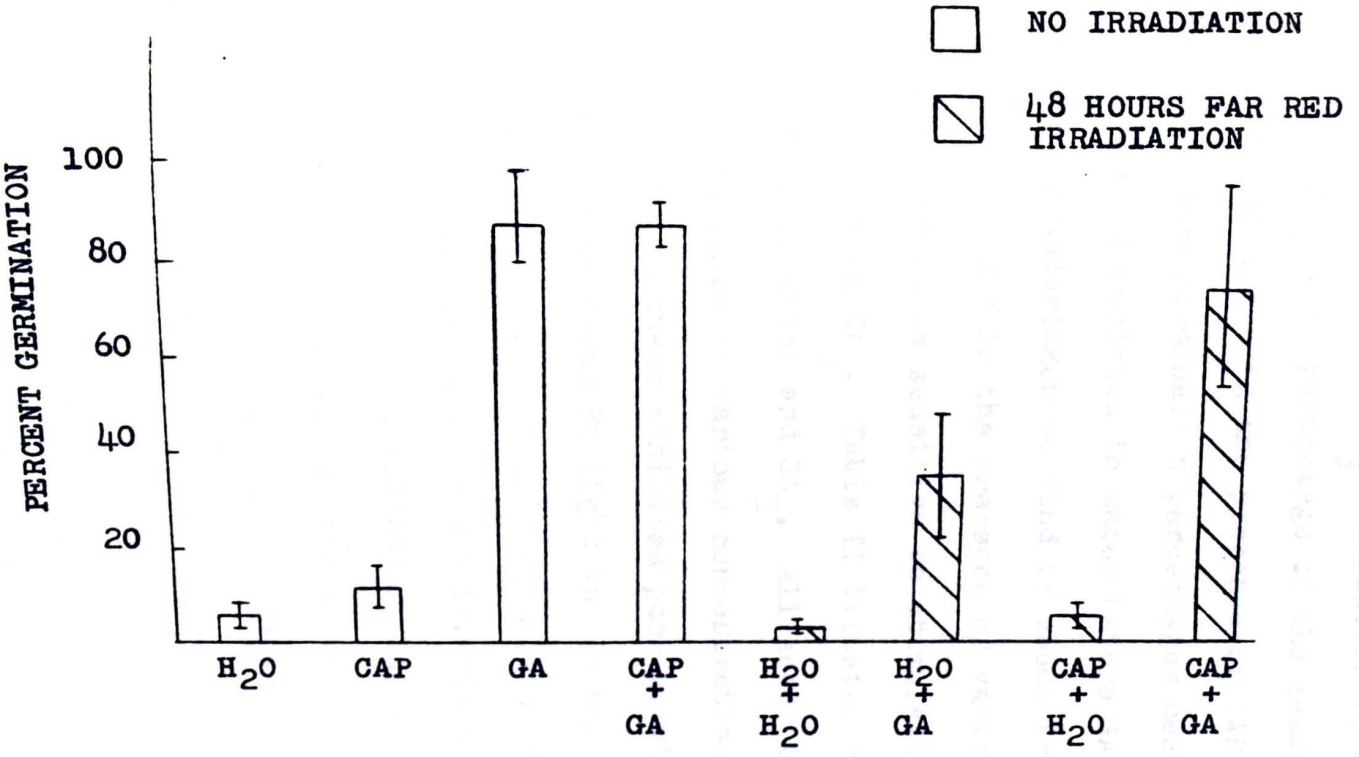


FIGURE 3. EFFECT OF CONTINUOUS FAR RED LIGHT, CHLORAMPHENICOL, AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS.

Unirradiated seeds were germinated in darkness for 48 hours following imbibition. Far red irradiated seeds were treated with light for 48 hours in chloramphenicol or water, before being germinated in darkness for 48 hours at 25° in 1 mM gibberellic acid or water. The chloramphenicol concentration was 3000 µg/ml. Six replicates of approximately 100 seeds were employed for each chemical treatment.

This marked stimulation was not observed in this study. Seeds irradiated for 48 hours with FR light in water were effectively desensitized to GA_3 stimulation of dark germination. The germination percentage of the seeds treated with continuous FR light in the presence of CAP exhibited GA_3 stimulated dark germination percentages nearly twice that of the seeds irradiated in water before GA_3 treatment.

A study was undertaken to find if seeds irradiated with 48 hours FR light in the presence of various concentrations of CAP would be sensitive to stimulation of dark germination by 0.5 mM GA_3. Table II illustrates the typical dark germination in water and GA_3. All seeds irradiated with FR light in water or various concentrations of CAP and germinated in darkness exhibited poor germination. Seeds irradiated with continuous FR light in 750 µg/ml CAP showed greater sensitivity to gibberellin stimulated germination than the water control. Those seeds irradiated in 1500 µg/ml CAP germinated to a significantly higher percentage that the seeds light treated in water. Duncan's new multiple range test with arcsine transformations of percentage data was employed. Again, the 5% level of significance was adopted. Germination of FR irradiated seeds in the presence of 3000 µg/ml CAP before supplying 0.5 mM GA_3 was significantly higher than the seeds irradiated in water. Although the GA_3 stimulation of germination of seeds

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF CHLORAMPHENICOL AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS

Chemical Treatment		% Germination
48 Hours Far red Light	15 Hours Dark Germination	
H_2O	H_2O	1.48 ± 2.55
H_2O	GA_3	11.13 ± 3.65
CAP 750	H_2O	1.32 ± 1.51
CAP 750	GA_3	15.42 ± 6.52
CAP 1500	H_2O	1.24 ± 0.93
CAP 1500	GA_3	28.60 ± 9.49
CAP 3000	H_2O	4.95 ± 2.84
CAP 3000	GA_3	44.63 ± 11.75

Seeds were germinated in darkness at 25°. Three replicate dishes of approcimately 100 seeds per dish were employed for each chemical treatment. Chloramphenicol (CAP) concentrations are expressed in micrograms per milliliter. The gibberellic acid (GA_3) concentration was 0.5 millimolar.

FR treated in CAP was higher than those irradiated in water, the high germination percentages (70.8) of FR treated seeds stimulated by 1 mM GA_3 were not observed.

A study was undertaken to determine the concentration of CAP that optimally prevents the loss of seed sensitivity to GA_3. Figure 4 illustrates high stimulation of germination for seeds FR treated in CAP at 500 µg/ml to 3000 µg/ml. Again results have been somewhat inconsistent. In other experiments, 3000 µg/ml appeared to be the optimal concentration.

Effect of 3 Hour Imbibition of Gibberellic Acid and Continuous Far Red Irradiation in Actinomycin D on Dark Germination of Grand Rapids Lettuce Seeds

Burdett (1972) reported that the inhibitory effect of FR light on GA_3 stimulated dark germination could be overcome by puncturing the endosperm before adding GA_3, by injecting the hormone, or by supplying GA_3 buffered at a low pH. From these experiments and others, he hypothesized that FR light inhibited GA_3 action by rendering the endosperm impermeable to the hormone. As an indirect method of measuring this, seeds were pre-soaked for 3 hours in 1 mM GA_3 preceding germination in darkness or far red treatment in Act. D or water and subsequent dark germination in water. Figure 5 illustrates that the 3 hour imbibition period in 1 mM GA_3 was sufficient to stimulate the classical high

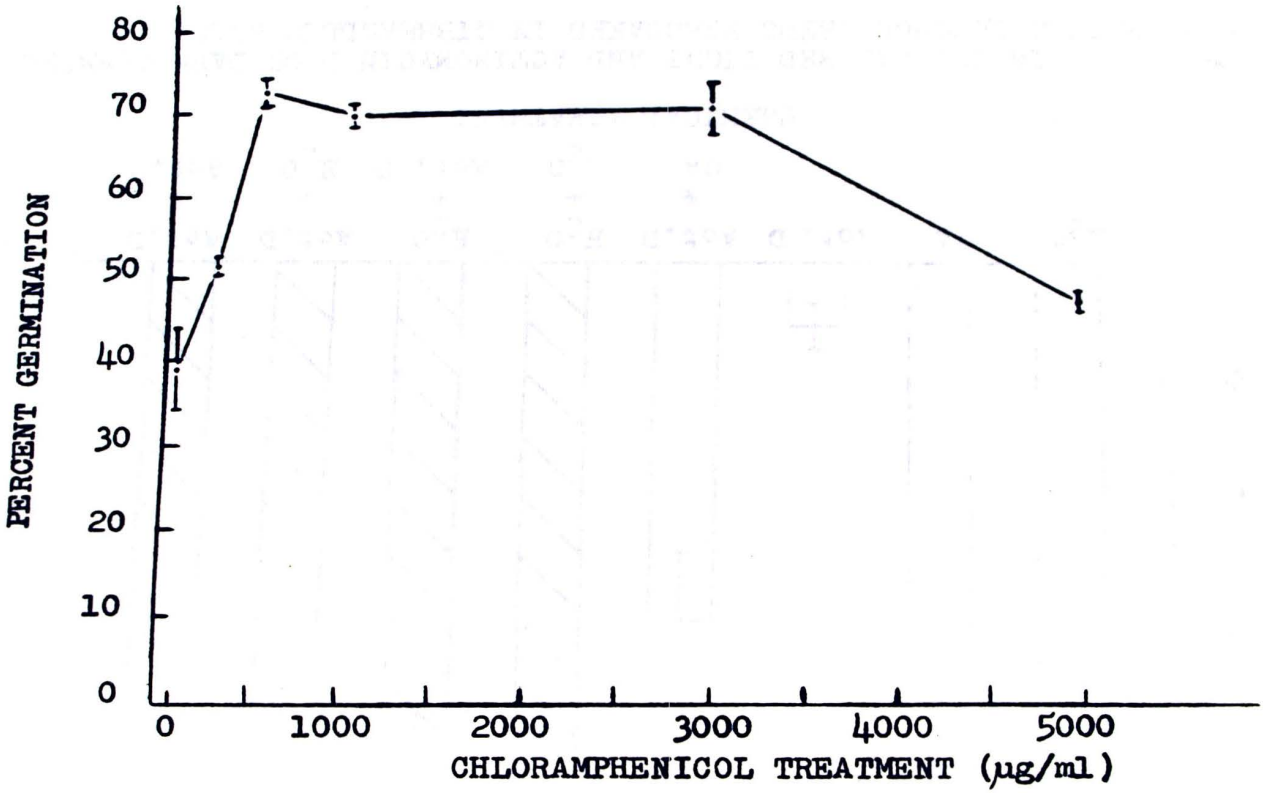


FIGURE 4. EFFECT OF CONTINUOUS FAR RED LIGHT, VARIOUS CONCENTRATIONS OF CHLORAMPHENICOL, AND GIBBERELLIC ACID ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS.

All seeds were far red irradiated for 24 hours in water of chloramphenicol preceding dark germination in water or gibberellic acid at 25° for 48 hours. Gibberellic acid concentration was 1 mM. Two replicates of approximately 100 seeds were employed for each treatment.

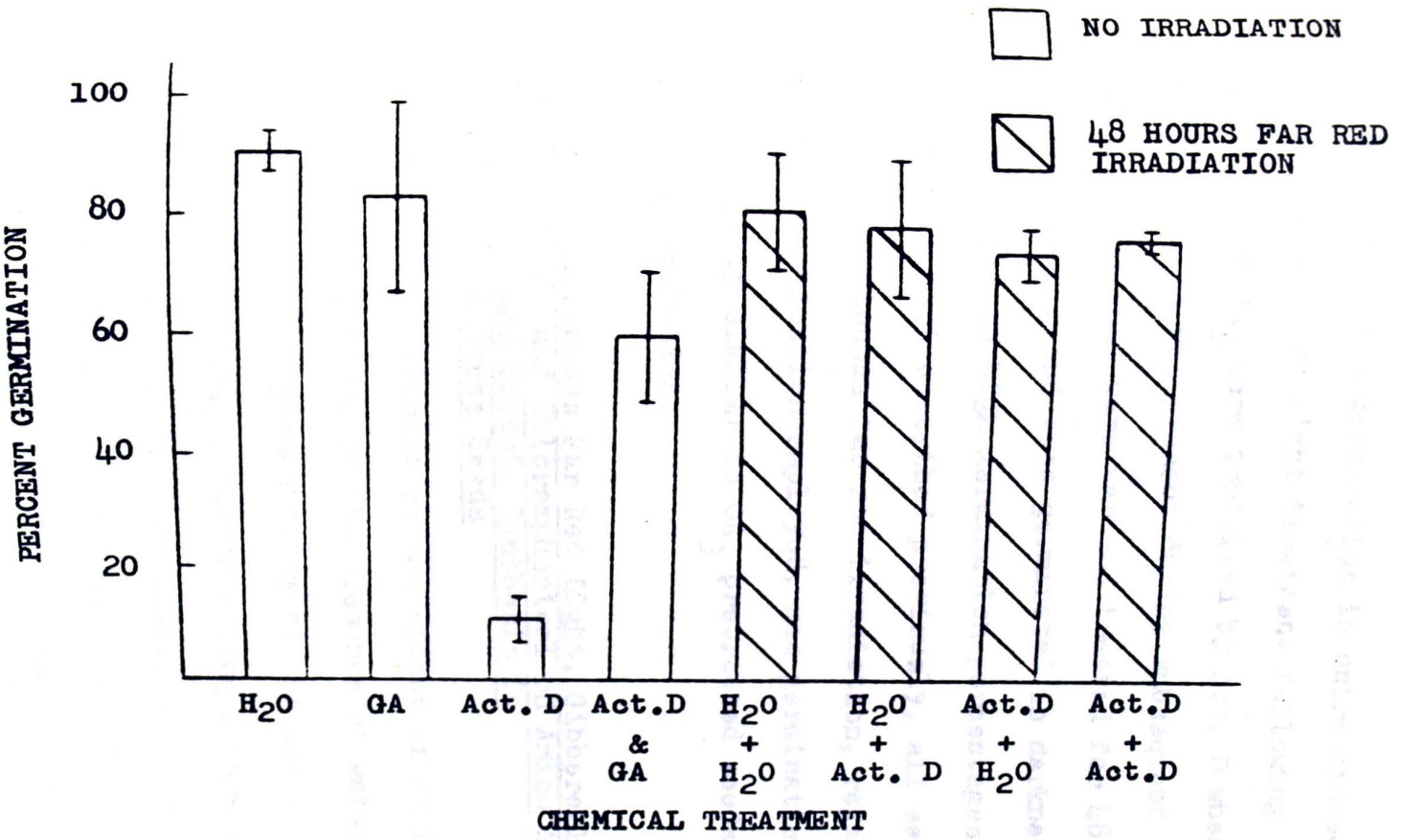


FIGURE 5. EFFECT OF CONTINUOUS FAR RED LIGHT AND ACTINOMYCIN D ON DARK GERMINATION OF GRAND RAPIDS LETTUCE SEEDS PRESOAKED IN GIBBERELLIC ACID.

Unirradiated seeds were germinated in darkness for 48 hours following imbibition in 1 mM gibberellic acid (GA_3). Far red irradiated seeds were treated with light for 48 hours at 25° in water. The actinomycin D (Act. D) concentration was 10 µg/ml. Six replicates of approximately 100 seeds were employed for each chemical treatment.

(89.85) percentage germination in unirradiated seeds. Seeds receiving no light treatment following 3 hours imbibition in 1 mM GA_3 were inhibited by Act. D when the antimetabolite was present during subsequent dark germination. Seeds that were far red treated for 48 hours in water or Act. D and then germinated in darkness in water in Act. D exhibited high germination percentages. Using the statistical test described previously, all seeds receiving 48 hours continuous far red irradiation, regardless of the chemical combination employed, had germination percentages statistically similar to GA_3 pretreated seeds showing the maximum germination.

Effect of Continuous Far Red Light, Gibberellic Acid, Actinomycin D, and Chloramphenicol on Amino Acid Permeability and Protein Synthesis in Grand Rapids Lettuce Seeds

Table III illustrates the effect of FR irradiation of Grand Rapids lettuce in the presence of water, Act. D, or CAP on subsequent permeability of the lettuce to ^{14}C-leucine in water or 1 mM GA_3 solution during a dark germination period. There appears to be little difference in the permeability of seeds irradiated with FR light in water or Act. D. to ^{14}C-leucine. There also appears to be little difference in the permeability to ^{14}C-leucine when supplied in water or GA_3 solution. Seeds irradiated with FR light in CAP exhibited greater uptake of label than the seeds

TABLE III

PERMEABILITY TO ^{14}C-LEUCINE OF GRAND RAPIDS LETTUCE SEEDS TREATED WITH FAR RED LIGHT IN VARIOUS CHEMICALS

Chemical Treatment		Soluble Leucine Pool % of Control			
24 or 48 Hrs. Far Red Light	15 Hours Dark Germination	24 Hr. Far Red* TCA	24 Hr. Far Red* EtOH	48 Hr. Far Red** TCA	48 Hr. Far Red** EtOH
H_2O	H_2O	100	100	100	100
H_2O	GA	97	92	94	104
Act. D 10 µg/ml	H_2O			80	86
Act. D 10 µg/ml	GA	71	64	111	92
CAP 3 mg/ml	H_2O			115	129
CAP 3 mg/ml	GA	108	108	133	242

TCA (Trichloroacetic acid precipitation)
EtOH (Ethanol precipitation)

* 260,000 cpm of soluble leucine-^{14}C/g of seeds in water control

** 491,000 cpm of soluble leucine-^{14}C/g of seeds in water control

One-tenth gram of seeds was employed for each chemical treatment in seeds irradiated for 48 hours, 1 gram of seeds were used when seeds were irradiated for 24 hours.

irradiated in water. It is noteworthy that seeds irradiated in CAP and germinated in GA_3 had higher soluble ^{14}C-leucine pools.

Black and Richardson (1965, 1967, 1968) found that with stimulation of dark germination in Grand Rapids lettuce by Act. D and CAP, there was a concomitant reduction in protein synthesis. In this study, seeds were irradiated in these antimetabolites but were washed thoroughly before being supplied with ^{14}C-leucine in water or GA_3 solution. Table IV illustrates the effect of irradiation in water, Act. D, or CAP on protein synthesis during subsequent germination in darkness for 15 hours in water of GA_3 solution. Seeds FR irradiated in Act. D showed lower incorporation of ^{14}C-leucine into protein during incubation in water or GA_3 than the seeds irradiated in water. Seeds treated with continuous FR light in CAP exhibited increased incorporation of ^{14}C-leucine into protein when incubated with GA_3 solution but the same incorporation as the water control when incubated with water. The decrease of protein synthesis by Act. D is consistent with finding of Black and Richardson (1967, 1968). They also reported inhibition of protein synthesis in germinating lettuce by CAP. The findings of this study, at least in the case of germination in GA_3 solution, do not show a decrease, but nearly a 100% increase in ^{14}C-leucine incorporation in CAP treated seeds.

TABLE IV

INCORPORATION OF ^{14}C-LEUCINE INTO PROTEIN IN GRAND RAPIDS LETTUCE SEEDS TREATED WITH FAR RED LIGHT IN VARIOUS CHEMICALS

Chemical Treatment		CPM/mg Protein	% of Control
48 Hours Far Red Light	15 Hours Dark Germination		
H_2O	H_2O	326	100
H_2O	GA	308	94
Act. D 10 ug/ml	H_2O	278	85
Act. D 10 ug/ml	GA	204	63
CAP 3 mg/ml	H_2O	326	100
CAP 3 mg/ml	GA	635	195

Act. D (actinomycin D)
CAP (chloramphenicol)

One-tenth gram of seeds was employed for each chemical treatment.

Effect of Continuous Far Red Light, Exogenous Gibberellin, Actinomycin D, and Chloramphenicol on Extractable Gibberellic Acid in Grand Rapids Lettuce Seeds

As an alternate method of investigating the effect of FR light on seed permeability, seeds were continuously irradiated in the presence of actinomycin D and CAP preceding GA_3 treatment. Gibberellins were extracted from seeds with water and separated on a Sephadex G-15 column. Gibberellic acid was estimated by the bioassay methods of Frankland and Wareing (1960). Figures 6, 7, 8, and 9 show mean hypocotyl elongation for 20 lettuce seedlings allowed to grow for 5 days in Sephadex G-15 effluent fractions. All seeds received 48 hours FR treatment preceding germination for 15 hours in 1 mM GA_3 solution. An extract from seeds irradiated with FR light in water and germinated in GA_3 (Figure 7) exhibited more extractable gibberellin-like material than did the seeds irradiated in Act. D (Figure 8) or CAP (Figure 9) that were germinated in a similar manner. Since most of the gibberellin activity was confined to two sequential Sephadex G-15 effluent fractions, total GA_3 was calculated for the 20 ml containing the highest gibberellin activity. Table V illustrates that seeds far red treated in water exhibited the highest extractable gibberellin activity, even though these seeds were inhibited in their germination response by far red light. Although lower gibberellin levels were found for seeds far red treated in

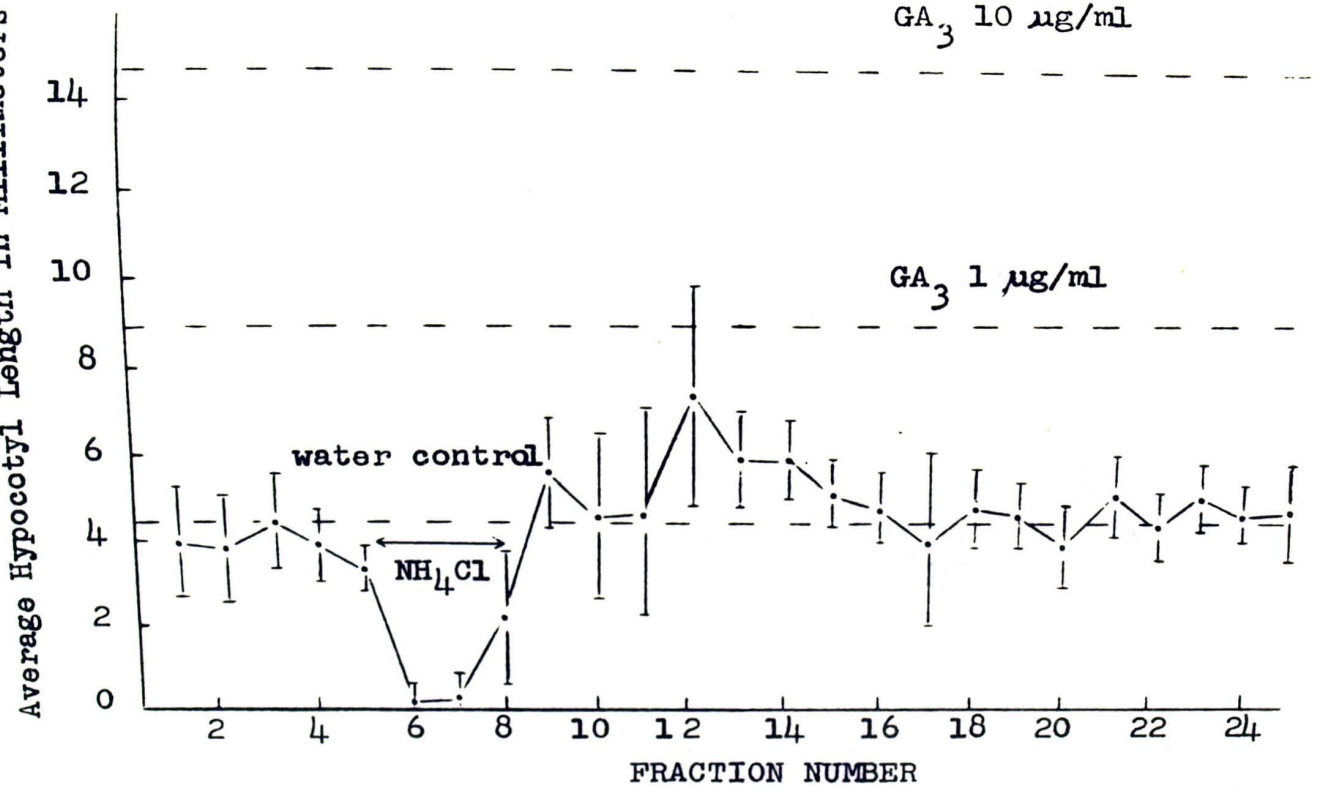


FIGURE 6. SEPARATION OF GIBBERELLIN-LIKE SUBSTANCES ON SEPHADEX G-15 FROM GRAND RAPIDS LETTUCE SEEDS IRRADIATED WITH CONTINUOUS FAR RED LIGHT IN WATER AND GERMINATED IN DARKNESS IN WATER.

All seeds were far red irradiated for 48 hours in water preceding a 15 hour dark germination period in water. The bioassay organisms were twenty lettuce seedlings grown for 5 days at 28° in 4 ml of fractionated extract. The mean is expressed for 20 seedlings. The standard deviations for the water control and 1 and 10 µg/ml gibberellic acid (GA_3) treatments were 1.00, 1.57, and 2.56 respectively. Ammonium chloride (NH_4Cl) was found in fractions 5-8.

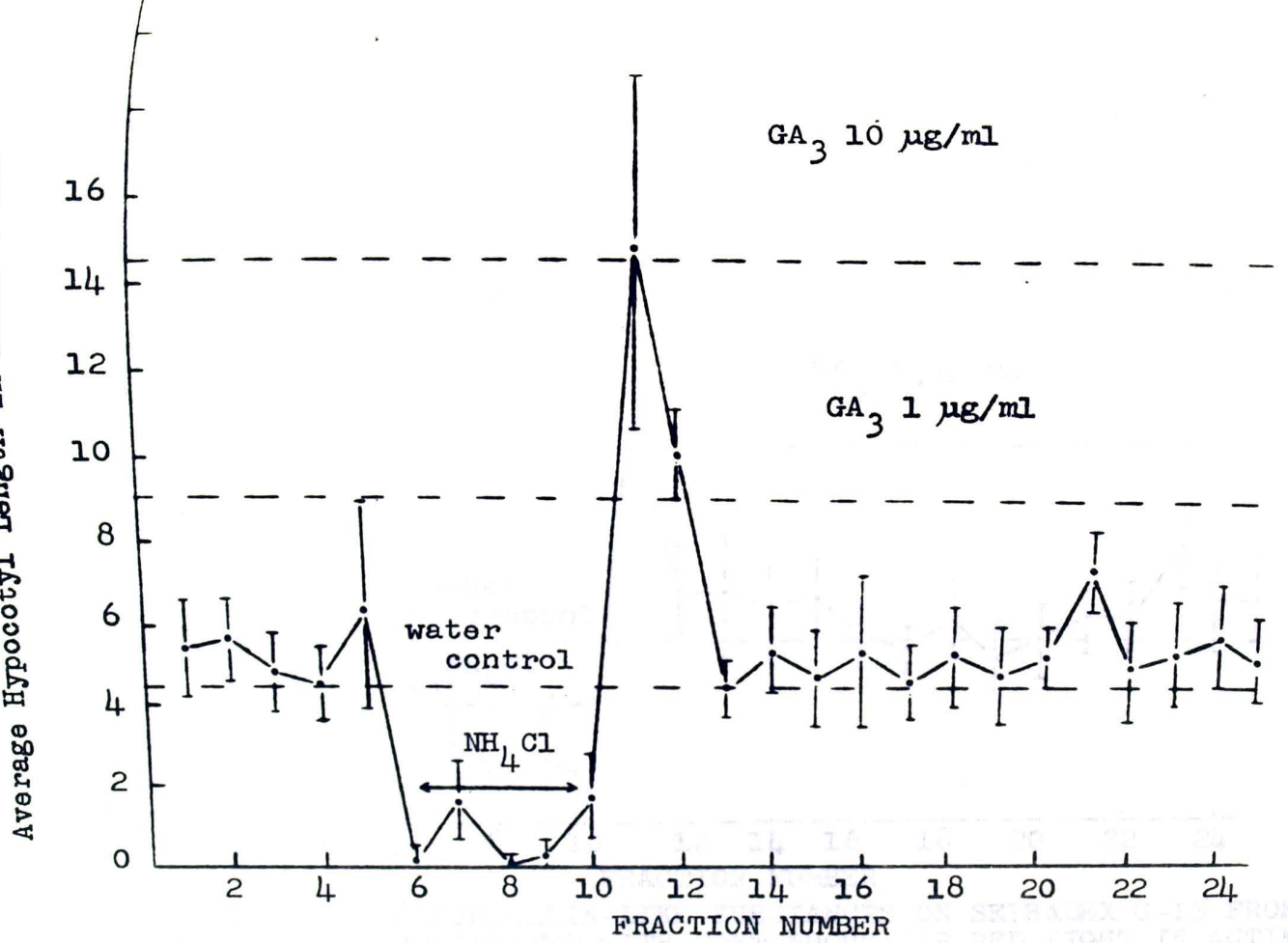


FIGURE 7. SEPARATION OF GIBBERELLIN-LIKE SUBSTANCES ON SEPHADEX G-15 FROM GRAND RAPIDS LETTUCE SEEDS IRRADIATED WITH CONTINUOUS FAR RED LIGHT IN WATER AND GERMINATED IN DARKNESS IN GIBBERELLIC ACID.

All seeds were far red irradiated for 48 hours in water preceding a 15 hour dark germination period in gibberellic acid. The bioassay organisms were twenty lettuce seedlings grown for 5 days at 28° in 4 ml of fractionated extract. The mean is expressed for 20 seedlings. The standard deviations for the water control and 1 and 10 µg/ml gibberellic acid (GA_3) treatments were 1.00, 1.57, and 2.57 respectively. Ammonium chloride (NH_4Cl) was found in fractions 6-10.

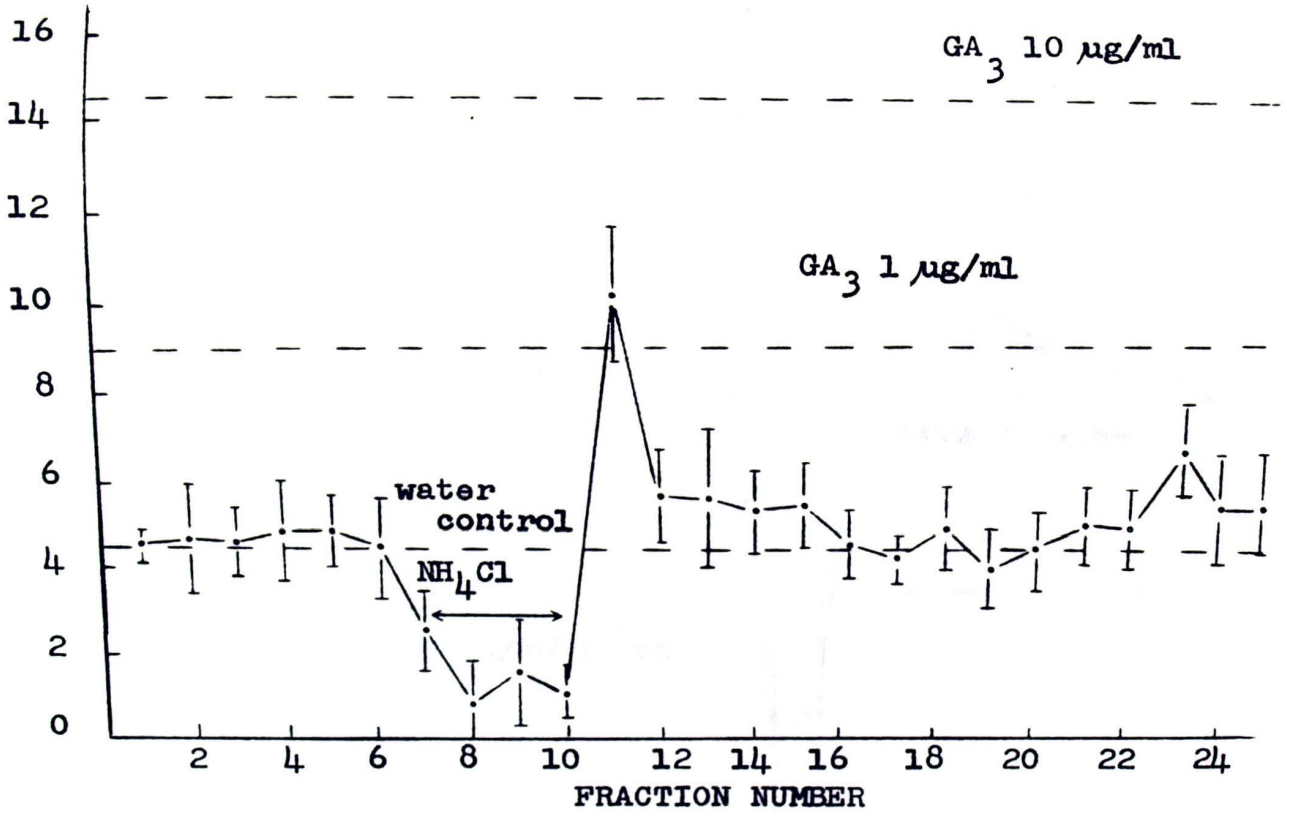


FIGURE 8. SEPARATION OF GIBBERELLIN-LIKE SUBSTANCES ON SEPHADEX G-15 FROM GRAND RAPIDS LETTUCE SEEDS IRRADIATED WITH CONTINUOUS FAR RED LIGHT IN ACTINOMYCIN D AND GERMINATED IN DARKNESS IN GIBBERELLIC ACID.

All seeds were far red irradiated for 48 hours in actinomycin D preceding a 15 hour dark germination period in gibberellic acid. The bioassay organisms were twenty lettuce seedlings grown for 5 days at 28° in 4 ml of fractionated extract. The mean is expressed for 20 seedlings. The standard deviations for the water control and 1 and 10 µg/ml gibberellic acid (GA_3) treatments were 1.00, 1.57, and 2.57 respectively. Ammonium chloride (NH_4Cl) was found in fractions 7-10.

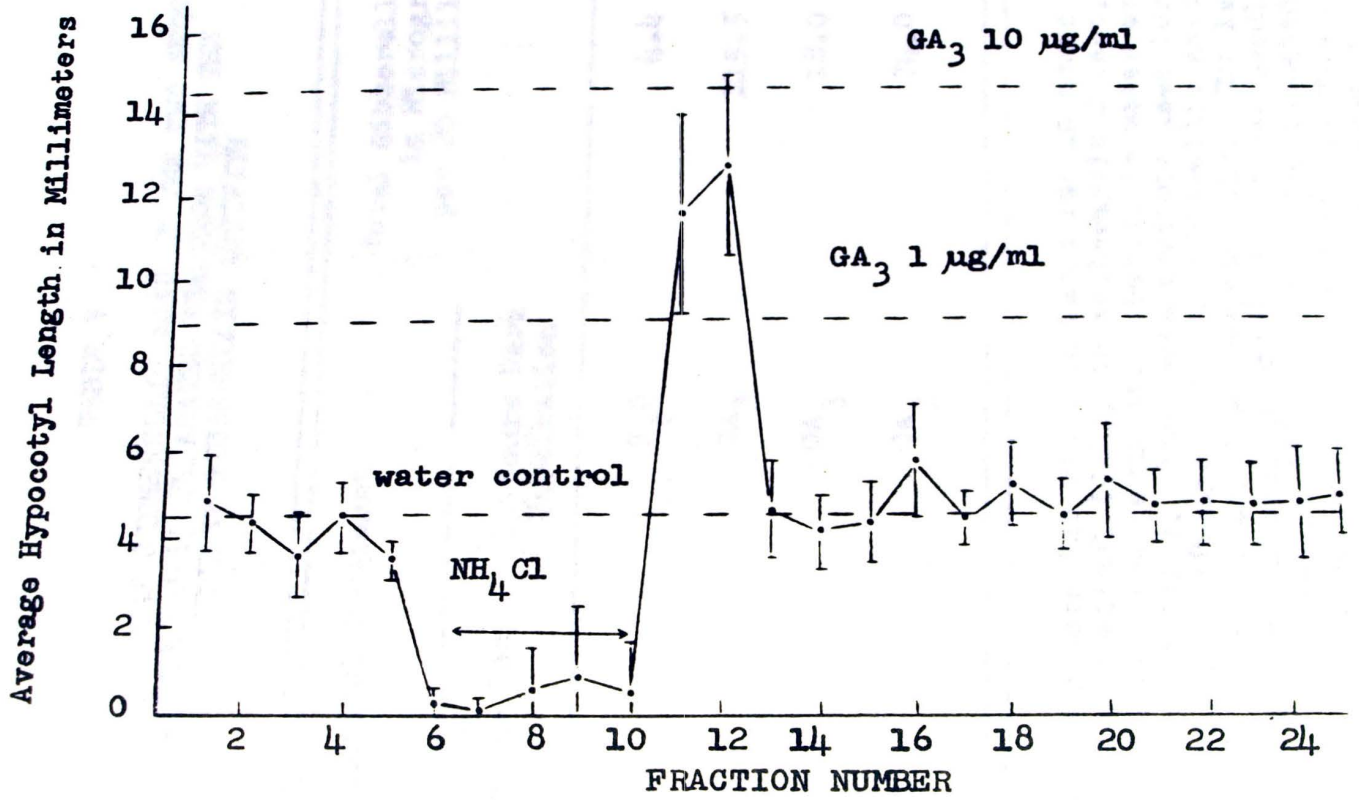


FIGURE 9. SEPARATION OF GIBBERELLIN-LIKE SUBSTANCES ON SEPHADEX G-15 FROM GRAND RAPIDS LETTUCE SEEDS IRRADIATED WITH CONTINUOUS FAR RED LIGHT IN CHLORAMPHENICOL AND GERMINATED IN DARKNESS IN GIBBERELLIC ACID.

All seeds were far red irradiated for 48 hours in chloramphenicol preceding a 15 hour dark germination period in gibberellic acid. The bioassay organisms were twenty lettuce seedlings grown for 5 days at 28° in 4 ml of fractionated extract. The mean is expressed for 20 seedlings. The standard deviations for the water control and 1 and 10 ug/ml gibberellic acid (GA_3) treatments were 1.00, 1.57, and 2.57 respectively. Ammonium chloride (NH_4Cl) was found in fractions 6-10.

TABLE V

ESTIMATE OF TOTAL GIBBERELLIC ACID IN THE TWO SEQUENTIAL SEPHADEX G-15 SEPARATED FRACTION WITH THE HIGHEST GIBBERELLIN ACTIVITY

Chemical Treatment		Total Gibberellic Acid in Micrograms per 20 Milliliters
48 Hours Far Red Light	15 Hours Dark Germination	
H_2O	H_2O	4.4
H_2O	GA_3	115.5
Act. D	GA_3	18.0
CAP	GA_3	74.0

All seeds were far red irradiated for 48 hours in water, chloramphenicol (CAP), or actinomycin D (Act. D) preceding a 15 hour dark germination period in water or gibberellic acid (GA_3). The concentrations used for chloramphenicol, actinomycin D, and gibberellic acid were 3000 µg/ml, 10 µg/ml, and 1 mM respectively. The lettuce seed bioassay was employed in which 20 lettuce seedlings were grown for 5 days at 28° in 4 ml of fractionated extract. Values were estimated from total stimulation of hypocotyl growth in the two adjacent tubes from the Sephadex separation containing the greatest gibberellin activity. Stimulation of hypocotyl elongation in experimental solution was compared to stimulation in known concentrations of gibberellic acid to obtain estimates.

chloramphenicol and actinomycin D before treatment with GA_3, these seeds exhibited high germination in darkness following the far red treatment. These results indicate that the seeds are permeable to exogenously supplied gibberellic acid, since those seeds irradiated in water and germinated in water had very low levels of gibberellin activity (Table V).

CHAPTER V

DISCUSSION OF RESULTS

In agreement with the findings of Borthwick et al. (1954) and Black and Richardson (1965, 1967, 1968), unirradiated Grand Rapids variety lettuce seeds do not germinate well in darkness, are stimulated somewhat in their germination response by the antimetabolites Act. D and CAP, and germinate maximally in GA_3 (Figures 1, 2, 3, 4, Table I, II). These figures also illustrate that Grand Rapids lettuce seeds irradiated with FR light in Act. D or CAP are not desensitized to GA_3 stimulated germination. Seeds that are FR treated for 24 or 48 hours in water are not sensitive to GA_3. This is in agreement with data reported by Kahn (1960), Negbi et al. (1968), and Burdett (1972). The method of irradiating the seeds in Act. D or CAP may be added to the methods of puncturing, injecting, and buffering GA_3 at a low pH used by Burdett (1972) as ways of preventing far red desensitization of Grand Rapids lettuce to GA_3 germination stimulation.

The obvious question is how does the presence of Act. D or CAP during FR treatment prevent the loss of germination sensitivity to GA_3. As previously mentioned, Black and

Richardson (1967, 1968) noted that dark germination in light sensitive Grand Rapids lettuce may be stimulated by Act. D or CAP. They reported that these substances inhibit protein synthesis in lettuce. They suggested that if there is a causal relationship between the suppression of protein synthesis and the stimulation of germination, a plausible explanation is that the protein synthesis which occurs in imbibed seeds in darkness is associated with some inhibitory mechanism. They further noted that although the nature of the inhibition must remain a matter of conjecture, the synthesis of an inhibitor which itself requires the prior synthesis of an enzyme might occur. Whatever mechanism, this view ascribes a role to Act. D or CAP as an "inhibitor of an inhibition," i.e. by preventing the far red inhibition.

Evenari (1965b) noted that the endosperm was the tissue responsible for the prevention of growth in the embryo. Black and Richardson (1968) illustrated that the endosperm was the most active tissue as far as protein synthesis is concerned. Burdett (1972) reported that insensitivity to GA_3 of Grand Rapids lettuce, induced by far red, could be lessened by several factors. One of these was to supply exogenous GA_3 to far red treated seeds buffered at a low pH. Since only the endosperm is in contact with the buffered medium, he suggested that this implies that pH influences endosperm permeability. Both Hendricks and Borthwick (1967)

and Tanada (1968) have suggested that phytochrome exerts its effect by regulating membrane permeability.

Since Hendricks and Borthwick (1967) and Black (1969) have suggested that P_{fr} exerts an effect on membrane permeability and because Burdett (1972) suggested a relationship between far red inhibition of GA_3 stimulation and permeability, an experiment was conducted to indirectly determine if FR light inhibition of GA_3 action was due to reduced permeability to the hormone. Figure 5 illustrates data from this experiment. All seeds presoaked in GA_3 before 48 hours far red treatment exhibited high germination percentages. Burdett (1972) also noted that seeds held in FR light in the presence of GA_3 did not germinate. The seeds, however, exhibited a high percentage germination when germination was scored 48 hours following removal from the FR light. If GA_3 is supplied before or during the early part of the far red treatment and is absorbed into the seeds, far red does not prevent GA_3 stimulated dark germination. From this experiment and those of Burdett (1972), a logical assumption is that far red treatment does indeed prevent GA_3 enhanced germination by reducing seed permeability to the hormone. Another possibility is that the presence of GA_3 during the first 3 to 6 hours of imbibition potentiates a sequence of events involved in germination.

As a more direct method of measuring the effect of far red light on seed permeability, FR light treated seeds were supplied with ^{14}C-leucine during a subsequent dark germination period. A comparison was made between the permeability of seeds treated with FR light in the presence of water or CAP or Act. D to ^{14}C-leucine supplied during the following dark germination. If FR irradiation in Act. D or CAP increased permeability over seeds irradiated in water, one would expect a higher soluble leucine pool in the seeds treated with the inhibitors. Table III illustrates that seeds treated with CAP during FR light exposure had ^{14}C-leucine pools higher than seeds irradiated in water before being treated with ^{14}C-leucine. Actinomycin D treated seeds did not show an increase in the level of soluble labeled amino acid pool, but illustrated as much as a 35% decrease in labeled precursor pool. Obviously, the CAP data is consistent with the idea that GA_3 action is inhibited by a decrease in permeability, but the Act. D data is completely incongruous with this proposal.

It has previously been noted that Black and Richardson (1968) found Act. D and CAP inhibit protein synthesis in Grand Rapids lettuce, even though these chemicals concomitantly stimulate dark germination. Table IV illustrates data from an experiment in which seeds were FR irradiated in water, CAP, or Act. D before being supplied with ^{14}C-leucine

during dark germination. Table IV illustrates that protein synthesis during this period was inhibited in seeds irradiated with FR light in Act. D. Seeds treated with FR light in CAP exhibited marked stimulation of protein synthesis. The increase in ^{14}C-leucine incorporation in CAP treated seeds may be a reflection of higher soluble leucine pools due to increased permeability rather than a true stimulation of protein synthesis. It is interesting to note that seeds irradiated with FR light both in CAP and Act. D are not desensitized to GA_3 stimulated dark germination even though protein synthesis is enhanced in CAP and inhibited in Act. D treated seeds. Black and Richardson (1968) proposed that Act. D and CAP, both of which inhibited protein synthesis in their experiments, might stimulate dark germination in Grand Rapids lettuce seeds by "inhibiting an inhibition." Apparently another mechanism is involved in preventing desensitization to FR light by CAP, since this chemical appears not to inhibit protein synthesis during the dark germination phase.

An experiment was conducted to study further the effect of FR light, CAP, and Act. D on permeability to exogenously supplied GA_3 in Grand Rapids lettuce seeds. Seeds irradiated with continuous FR light for 24 or 48 hours were incubated in GA_3 in darkness for 15 hours. They were homogenized and the homogenate extract was separated on a Sephadex G-15 column.

Effluent fractions from the Sephadex column were assayed for gibberellin activity using the lettuce seedling hypocotyl elongation as a bioassay. Seeds exogenously supplied with GA_3 that were FR irradiated in CAP and Act. D showed lower extractable gibberellin activity than those irradiated in water. Actinomycin D treated seeds showed extremely low gibberellin activity (Figure 6, Table V). Table V illustrates data in which the concentration of extractable GA_3 is estimated in Grand Rapids lettuce seeds treated with continuous FR light, Act. D, CAP, and GA_3. It is of interest that an extractable GA_3 concentration of over 5 µg/ml in the seeds irradiated with FR light in water and germinated in GA_3 failed to stimulate dark germination. Seeds treated with continuous FR light in CAP and Act. D and germinated in darkness in GA_3 had estimated extractable GA_3 concentrations of less than 4 and 1 µg/ml respectively. Seeds that are inhibited in their germination response contain the highest levels of extractable gibberellin. This suggests that possibly a mechanism other than permeability is functioning in the far red stimulated desensitization of Grand Rapids lettuce seeds to GA_3.

It is well known that the response of Grand Rapids lettuce seeds to a given energy-level of far red light decreases if the imbibed seeds are held in darkness for some time (Borthwick, et al., 1954). This condition is

described as skotodormancy (Black and Richardson, 1965). One question concerning the effect of far red light, GA_3, CAP, and Act. D is the following: Does continuous FR light accelerate skotodormancy and does CAP and Act. D prevent the development of skotodormancy in Grand Rapids lettuce seeds? Black and Richardson (1965, 1967) reported that skotodormancy is inhibited by CAP. Skotodormancy may be the result of the synthesis of an inhibitor. Since CAP has been shown to inhibit organelle protein synthesis (Ellis, 1969) Freeman, 1969), this suggests skotodormancy may be associated with an organelle such as the mitochondrion. It would be interesting to determine the ATP level in seeds that have been exposed to continuous far red light in the presence or absence of CAP and Act. D. The phenomenon of skotodormancy may be related to the levels of metabolic energy required for germination. Although, it must be mentioned that in this study no inhibition of protein synthesis by CAP was observed during dark germination following FR irradiation.

From these experiments, it appears that FR irradiation of Grand Rapids lettuce in Act. D does not increase the permeability of the seeds to GA_3. However, experiments with CAP suggest an enhancement of permeability. In studies with ^{14}C-leucine, seeds far red treated with CAP showed somewhat higher soluble leucine pools than seeds irradiated in water.

Extractable exogenously supplied GA_3 from seeds irradiated with FR light in CAP was lower than extractable GA_3 from seeds irradiated in water, but higher than Act. D treated seeds. The possibility that FR treatment in the presence of CAP renders the Grand Rapids lettuce seeds more permeable to GA_3 cannot be completely discounted, although it seems doubtful that the mechanism of stimulation of CAP and Act. D are different.

Further experimentation is needed to elucidate the mechanism by which FR irradiation in CAP and Act. D prevents desensitization of Grand Rapids lettuce seeds to exogenous GA_3 supplied after far red light treatment. Several experiments might provide insight into the problem. Presoaking the seeds in GA_3 preceding FR irradiation in water, CAP, of Act. D followed by a gibberellin extraction and bioassay, might be of value in answering this question, i.e. what is the exogenous GA_3 level imbibed during the first 3 hours that stimulates germination even in continuous FR light?

CHAPTER VI

SUMMARY

The present study was undertaken to determine the following: (1) if far red irradiation of Grand Rapids variety lettuce seeds in CAP or Act. D prevents the loss of sensitivity to GA_3, (2) if CAP and Act. D increase the permeability of the far red treated seeds to GA_3, and (3) if the presence of CAP and Act. D during far red treatment inhibits protein synthesis in lettuce seeds during a subsequent dark germination phase.

Grand Rapids variety lettuce seeds that were treated with continuous far red light in water exhibited low germination in response to GA_3 treatment. Seeds receiving continuous FR irradiation in CAP or Act. D illustrated a much greater sensitivity to GA_3 stimulation of germination. This stimulation of germination was found to be present even when relatively low concentrations of GA_3 were employed.

Studies undertaken to determine if seeds FR treated in Act. D and CAP were more permeable to GA_3 than those irradiated in water gave conflicting results. Seeds presoaked in GA_3 were not sensitive to far red inhibition of GA_3 germination results. Seeds presoaked in GA_3 were not sensitive to

far red inhibition of GA_3 germination stimulation. Seeds that were far red treated in the presence of CAP showed an increase in permeability to ^{14}C-leucine over similarly treated seeds that were irradiated in water. Those seeds that were irradiated with far red in Act. D showed a decrease in permeability to ^{14}C-leucine. The extractable gibberellin found in the seeds irradiated in Act. D and CAP preceding GA_3 treatment was lower than those seeds FR irradiated in water.

Protein synthesis during the dark germination phase following FR irradiation in CAP, Act. D, and water were studied. Seeds treated with FR light in Act. D exhibited an inhibition of protein synthesis during the following incubation period. Those seeds irradiated in CAP during the subsequent germination phase exhibited a marked increase in incorporation of ^{14}C-leucine. However, the greater amounts of ^{14}C-leucine incorporation into protein in chloramphenicol treated seeds may be a reflection of increasing permeability and not a true index of protein synthesis.

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