

STUDIES ON PHOTOBlastic GERMINATION
IN LETTUCE SEED

Thesis by
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This thesis is respectfully dedicated to my father.

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ABSTRACT

It was shown, with the aid of osmotic inhibition of germination, that the action of the far-red-absorbing form of phytochrome (Pf) in promoting germination can be completed even if the seed is held under conditions where germination is not possible. An effect of the continuing action of Pf beyond the point of complete germination promotion was demonstrated by enhancement of germination rate after removal of the osmotically active solute.

Previous reports that the rate of growth in water of seeds freed from the expansion-restricting endosperm is independent of the state of phytochrome were confirmed. However, a marked, phytochrome-mediated enhancement of the growth potential of such seeds was demonstrated through restricting water uptake by incubation in an osmoticum.

An experimental system, utilizing the appearance of a geotropic curvature in the radicle of the excised axial portion of the seed, was developed for more detailed studies of the phytochrome-enhanced growth potential. It was possible to demonstrate the light effect in water as well as in osmotica; this apparently is not possible with de-endospermed entire seeds. As in intact seeds, the effect of the continuing action of Pf is to enhance the rate of the response. Secretion of a chemical inhibitor of growth by the endosperm as a possible mechanism of induction of light sensitivity has been ruled out.

The phytochrome-dependent rate of appearance of geotropic curvature in osmotica is paralleled in time by a similar dependence of the rate of early extension growth of the embryonic axis. Only the first small increment of growth is differentially responsive to red (R) and far-red (F); the rate of later increase in length is independent of the light regime.

It was shown that the high concentrations of gibberellic acid required for germination promotion in the intact seed are due at least in part to a diffusion barrier in the endosperm, and that the occasional reports in the literature of the ineffectiveness of kinetin are probably due to the same phenomenon. It was shown that gibberellin, like red light, enhances the growth potential of the axis, but kinetin does not. The difference in rates of response obtained after R-irradiation or gibberellin treatment, together with other results reported in the literature, strongly suggests that gibberellic acid and red light promote germination by different means. The idea that kinetin promotes germination by yet another mechanism, probably operating in the cotyledons, was supported through two different experimental approaches.

The phenomenon of temperature-dependent dark germination was examined in detail, using a wide range of both temperatures and incubation times. With the aid of the half-seed system, it was demonstrated that the promotive effect of low temperature on germination could not be due to a low optimum temperature for early growth

of the radicle, since the rate of that process increased with increasing temperature, up to the highest temperature used.

It was shown that phytochrome does not function at high temperatures. This fact is of considerable importance in interpreting the phenomenon of thermodormancy, since in the literature only a small part of the effect of high temperature has been ascribed to an effect on phytochrome, and at that, only to an acceleration of dark reversion of Pf to the red-absorbing form of phytochrome (Pr). Partial denaturation of phytochrome may also make some contribution.

It was shown that the germination-promoting effect of low temperature depends on the presence of Pf, and concluded that low temperatures act by delaying or preventing transformation of Pf. Support for the assumption that Pf, not Pr, is the active form of phytochrome in lettuce seeds was drawn from the same evidence.

Attempts to stimulate germination by repeated irradiation with F over relatively prolonged incubation times resulted in failure, as have similar attempts reported in the literature. However, an enhancement of growth potential in the half-seed system by the maintenance of a small amount of Pf over long periods at ordinary temperatures by repeated irradiation with F was demonstrated.

It was observed that cold storage of the dry seed prevents or delays loss of dark dormancy during post-harvest storage. No change in the response of the half-seed in osmoticum to R and F was observed in seeds that had lost dark dormancy; that is, no internal change

took place to measurably increase the growth potential of the embryonic axis. This suggests that the endosperm is the seat of changes responsible for after-ripening of photoblastic lettuce seed.

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I. INTRODUCTION

GENERAL

The effect of light on seed germination, or, to use a term proposed by Evenari (13), photoblastism, has been very extensively investigated (see reviews 13, 17, 48, 76). Photoblastism may be positive or negative, that is, light may inhibit or promote the germination of photoblastic seeds. Negative photoblastism, however, is evidently a phenomenon dependent on a particular set of conditions for its expression (e.g., time of imbibition before irradiation, duration of light treatment) and conditions may usually be found such that light will stimulate germination of any photoblastic seed (17). Seeds in which germination is not affected by light are called aphotoblastic.

The germination of photoblastic seeds is stimulated by red light (R) (6300-6800A); this stimulation is reversed by far-red light (F) (7300-7500A). The effect of a series of brief, alternating irradiations with R and F is that of the last exposure alone (2). The two effective qualities correspond to the regions of maximum absorption in the visible spectrum of the two forms of phytochrome; the red-absorbing form (Pr), and the far-red-absorbing form (Pf) (7). It is therefore generally assumed that photoblastism is controlled by phytochrome.

Most of the information available on photoblastism has been garnered from experiments using lettuce (*Lactuca sativa* L.) seeds, which may be aphotoblastic or positively photoblastic, depending on

the variety and seed lot. Since it has been considered at least doubtful that the mechanism of the light response is identical in all photoblastic seeds (17), and since the subject of this thesis is lettuce seed germination, this survey will only consider the responses as they have been described for lettuce.

Morphology of the lettuce achene.

The layers surrounding the embryo play an important role in the expression of photoblastism (see later). The following brief description of the gross structure of the seed is therefore included for the reader's orientation.

The ontogeny of the lettuce achene (fruit) from just prior to anthesis to the fully matured structure has been described by Borthwick and Robbins (4). The layers surrounding the embryo of the mature achene, from the inside out, are as follows: (1) The endosperm consists of a distinct layer of thick-walled cells entirely investing the embryo. This layer is 2 cells thick everywhere, except at the radicle end, where it is often 3 or more cells in depth. (2) The "seed coat", derived from the integument, consists of a persistent, thick-walled outer epidermis and within that remnants of disorganized cells, and finally, adjacent to the endosperm, a suberized semi-permeable membrane which represents the cuticle of the ephemeral inner epidermis. (3) The pericarp (fruit coat) is longitudinally ribbed and consists of sclerenchymatous, strongly lignified cells.

The germination process.

Evenari (16) has distinguished 4 phases in normal germination.

(1) The imbibition phase. This phase consists in hydration of the seed. The rate of hydration is temperature-dependent; in lettuce seed, the maximum increase in fresh weight is attained after about 4 hours at 25°, but only after 12 hours at 3° (37). The full development of sensitivity to light, however, occurs well before this phase is complete, at 1.5 hours at 25°, and 4 hours at 3°. Development of photosensitivity during this phase proceeds the same under nitrogen as it does in air (37). (2) The activation phase. All the mechanisms which enable the embryo to begin growth are developed in this phase..."The trigger is set whose release will carry germination to its last phase and to the start of normal growth." (16). The length of time the seed can remain "triggered" without losing viability is relatively great. Lettuce seeds have been held in the imbibed condition at temperatures too high to permit germination for as long as 8 months without appreciable loss of viability (10). However, in photoblastic varieties, the germination response to a given dosage of R may decrease when the light treatment is given after prolonged dark incubation (see later). (3) The phase of mitosis. Cells of the radicle undergo mitosis and proceed to elongate during this phase. In normal germination these 2 processes occur simultaneously. Mitosis begins, at 26°, at the 13th hour of imbibition. After the 16th hour the frequency of mitoses declines. Haber and Luippold (24), using the aphotoblastic variety New York, and a

variety of techniques, were able to separate mitosis and elongation in time during germination. In germination at 10° , or at ordinary temperatures following gamma-irradiation, radicle protrusion preceded mitosis. The converse obtained with germination in mannitol solutions (0.3-0.5M), or at 30° . (4) The protrusion phase. Here the rootlet penetrates the coats surrounding the embryo and seedling growth commences. During this phase, germination and growth overlap. Evenari considered growth and germination to be two separate processes, and this distinction has been emphasized by Ikuma and Thimann (36, 37).

LIGHT-INDUCED GERMINATION.

The level of germination response attained after irradiation with red light may depend on factors other than the total light dose. For example, the degree of response to a brief irradiation depends on the length of the dark imbibition period preceding irradiation. Responses to long-term irradiation at low and high intensities and to irradiation with white or blue light have been observed that are not explainable in a simple way solely in terms of photoconversion of phytochrome.

The integrity of at least one of the tissue layers surrounding the embryo is essential to the light requirement of the seed.

Role of the surrounding layers.

The light requirement for germination of photoblastic lettuce seeds is lost upon removal of all surrounding layers (20, 35, 36). The naked embryo will "germinate" and grow at temperatures where

germination of the intact seed will not occur regardless of the light regime (4), and its growth rate is unaffected by irradiation with either R or F (35, 36). Removal of the fruit coat alone does not remove the light requirement (20, 33). In experiments involving depth-controlled deuteron irradiation of the intact seed, it has been shown that irradiation of the endosperm alone was necessary and sufficient to overcome dark-dormancy (46, 64). It would seem that the light requirement depends on the presence of an intact endosperm. Removal of the upper end of the endosperm (by slicing off the cotyledonary end of the seed) was not sufficient to induce dark germination, but removal of the upper half by the same operation was sufficient, as was also making a longitudinal slit through the upper half of the seed (32, 37). Removing the small portion of endosperm that invests the radicle tip removes the light requirement (40). Red light, applied to different parts of the intact, imbibed seed is effective only in the radicle-hypocotyl region (33), and light of either wavelength is just as effective in reversing the effect of a previous irradiation with the other wavelength when applied to the opposite, as when applied to the same side of the seed (59). Thus, it seems probable that the primary mechanism of response to light resides in the embryo, not in the endosperm.

Length of pre-irradiation imbibition time.

The subsequent germination behavior is unaffected by irradiation of the dry seed. Seeds placed to imbibe in water have been reported

to give a detectable response to light as early as after 10 minutes of imbibition (71). The response to a brief irradiation with white light at 26° shows a sharp optimum at the 8th hour, to red light a sharp optimum at the 3d hour (18), or at the 2d hour (37), or a broad optimum (when incubated at 20°) at the 14th hour of imbibition (3). Sensitivity to F falls sharply from the 2d to the 15th hour of imbibition at 26° (18), or, at 20° , drops less sharply over the same interval, but then increases to the 30th hour (3). Prolonged dark incubation (3-4 days) at 26° results in failure of the seeds to respond at all to brief R irradiation (19, 57, 66). Such seeds are said to be "skotodormant" (17). The decline in response to a given R dose with increasing dark preincubation is an indisputable experimental fact. The differences in the rates of decline reported by the various workers are no doubt attributable to different experimental conditions, mainly temperature and magnitude of the light dose. The latter variable may be the most important, since it has recently been shown (54) that the decline in response to a 30-second R irradiation at 26° does not occur if the irradiation time is extended to 2 minutes, at least for preincubation periods up to 24 hours.

Qualitative and quantitative effects of light.

The reciprocity relationship is valid within certain intensity and irradiation time limits for R, F, and white light (17). For brief exposures, white (incandescent) light is of the same order of effectiveness as R, even though the emission of the white source is slightly

higher at the longer than at the shorter actinic wavelength. The predominating effect of R is owing to the fact that photoconversion is several times as efficient for R as it is for F (3). For continuous irradiation with high intensity white light at 26°, germination falls off with increasing intensity, but 100% germination is still obtained for intensities of the order of 150 foot-candles (12, 14, 17). Under continuous monochromatic light of low intensity germination falls off abruptly at wavelengths greater than 7000Å, whereas at higher intensities inhibition begins at about 6600Å and is complete at 7000Å (29). The inhibition by prolonged irradiation with white or F light has been interpreted as an effect of continuous stimulation of the pigment by F (29), but a detailed mechanism was not advanced. The dormancy resulting from prolonged irradiation with F has been called photodormancy (17).

Light in the region 5000-6000Å has no effect on germination. Blue light (4000-5000Å) was found to promote germination somewhat, but only after long periods of dark imbibition. Blue light immediately following a sub-saturating dose of R somewhat lowered the germination response to R, for all periods of imbibition. Approximately 100-fold higher energies were required for a given response to blue than to either R or F. Because of the dual action and low efficiency of blue light it was concluded that both forms of phytochrome have minor overlapping absorption bands in the blue, and that higher energy (i.e., prolonged) irradiation in this spectral region serves to establish an equilibrium between the 2 forms that is dif-

ferent from that after R or F (3). In a report from another laboratory (81), blue light (4 hours) was found to reverse the promotion following a 1.5-minute R irradiation at the same intensity. Repeated reversibility, in the manner of the R-F antagonism, was demonstrated.

Escape from photocontrol.

F administered immediately after R completely reverses the effect of R (i.e., germination percentage is reduced to that obtained after F alone), but as the time of the dark interval between R and F increases, germination increases, ultimately to the level attained after R alone (e.g., 3, 34). This phenomenon is termed "escape" from photocontrol. The rate of escape from photocontrol is temperature sensitive. The interval between R and F required to effect a given percent germination decreases with increasing temperature up to 25°, then increases for higher temperatures (37).

TEMPERATURE EFFECTS

Temperature and light vs dark germination.

Photoconversion of phytochrome is temperature-independent. The degree of promotion and reversal of promotion by sub-saturating doses of R and F, respectively, was the same at 26° and 7° (3). Nevertheless, the germination behavior of photoblastic seeds is strongly affected by the temperature regime. The effect of temperature on the rate of escape from photocontrol has already been mentioned. In most seed lots, dark germination at temperatures below 20° is quite high, and consequently the difference between light and dark germination

is relatively small (12). At progressively higher constant temperatures dark germination falls off faster than light germination and the maximum difference between light and dark is found between 25 and 30°. At 35°, germination is nil, both in darkness and continuous white light (12).

The effect of high temperature during dark imbibition.

If seeds are held in the imbibed state in the temperature range 30-35° for protracted periods of time, dark germination upon transfer to 25° is practically nil, and the response to a single irradiation with R is also strongly reduced (62). Such a state is termed thermodormancy (17). The depth of thermodormancy induced increases with temperature and duration of temperature treatment (73). Pretreatment with R prevents or delays the appearance of thermodormancy. A small response to R (R-reversible) can be induced in aphotoblastic lettuce seeds by incubating them at high temperatures for several days (2, 3, 19, 23, 73).

The effect of high temperature (35°), when given only during the pre-irradiation (imbibition) period, depends on the length of the period. For imbibition times not exceeding 4 hours, such temperature treatment does not affect the germination response to a given R dose, when this is followed by incubation at 25°. It was concluded that the inhibiting effect of high temperature is due partly to acceleration of reversion of Pf to Pr, and possibly partial denaturation of phytochrome, but principally to a general inhibition of

metabolic processes occurring after irradiation (37). The reversion rate at 35° has been reported to be about twice that at 30° , on the basis of the length of post-irradiation incubation at a given temperature required to reduce subsequent germination to a given percentage. It was stressed that although dark reversion of phytochrome is temperature-dependent, inhibition of germination by high temperature is probably not principally the result of an effect on the pigment system (3).

The effect of low temperature.

As mentioned before, dark germination becomes progressively higher as the incubation temperature is lowered. The mechanism of this stimulation by low temperature is unknown, but, on the basis of failure of F to reverse the stimulation caused by a low temperature treatment, it was concluded that "the action of low temperature is to stimulate germination at some point other than that controlled by phytochrome" (37).

The effect of alternating temperatures.

Dark germination is reported to be significantly promoted if the temperature is raised from 22° to at least 27.5° for a short time and then lowered again (9). The promotion did not depend on the rate of ascent of temperature, but only on the short-term incubation at the minimum effective temperature (27.5°). It was hypothesized that the alternating temperature treatment destroyed a block to germination consisting of a "complex macromolecular arrangement." This hypothesis was proposed to counter an earlier suggestion (84) that

the effect of alternating temperatures was to raise the concentration of some metabolite above a critical level, a process which should depend on the rate of temperature ascent.

CHEMICAL INHIBITORS AND PROMOTERS.

Osmotic effects.

Dark germination of photosensitive seeds may be prevented by carrying out imbibition in a solution of any of a number of non-toxic solutes. The effect is one of osmotic curtailment of water uptake. Short-term imbibitions in such solutions do not affect germination if the seeds are subsequently transferred to water, but after long dark incubations, germination upon transfer to water is markedly reduced. Pretreatment with R or low temperature prevents or reduces this inhibition. It was suggested that osmotic inhibition in the dark is an effect of delay of germination, and that during this delay something takes place that permanently prevents germination in darkness--the establishment of so-called "osmodormancy" (39).

Inhibitors.

A number of compounds, some of them naturally occurring in fruits or seed coverings, inhibit seed germination at relatively low (non-osmotic) concentrations (15, 85). Of these inhibitors, coumarin has been most intensively investigated in its role as an inhibitor of germination of photoblastic lettuce seeds. It was found that the ratio of normalized percent germination in coumarin solutions in the dark to that in the light was not constant. This was purported to demonstrate an interaction between the "photomechanism" and

coumarin (16). The synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) evoked a similar response, but 2,4-dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, did not. Coumarin was further postulated to act on the "skotomechanism" (the author assumed that seeds germinating after R-irradiation accomplished this by some phytochrome-independent mechanism) because coumarin depressed the germination rates in light and darkness to the same value, whereas in the absence of coumarin, the rate of germination in the dark was much lower than in the light (44). Seeds of the aphotoblastic variety Progress become positively photoblastic when treated with coumarin, but not when treated with 2,4-D or DNP (12, 16, 58).

Promoters.

Gibberellic acid (GA3), at relatively low concentrations (e.g., 100 mg/l in (62)), effects complete dark germination (38, 41, 51), counteracts to some extent the establishment of thermodormancy by high temperature treatment (62, 75), and prevents or reverses inhibition by coumarin (53). Because of similarities in the time course of development of sensitivity to gibberellic acid and to R during imbibition, it was concluded that both act at the same morphological site, namely, the embryonic axis (34). It was suggested that phytochrome acts by regulating the levels of endogenous gibberellins (85). But aside from the fact that no increase in extractable gibberellins was found in R-irradiated seed (34), it was concluded that the effect of R could not be to regulate gibberellin levels since R reversed the effect of F given during a preincubation at

22° followed by high temperature treatment, whereas gibberellic acid did not (26).

Thiourea, at high concentration, substantially promotes dark germination (71, 72). It is significant that thiourea, when used at germination-promoting concentrations, strongly inhibits radicle extension in the germinated seed, and that the highest promotions are observed if the seeds are transferred to water after first being incubated in thiourea solutions.

Kinetin was reported to replace the light requirement (55), but it was later found that if care is taken to maintain absolute darkness, kinetin promotes germination only slightly, but does so significantly if a subliminal R dose, or, surprisingly, a saturation F dose is administered (56). Several other workers have reported no stimulation of dark germination by kinetin (21, 26, 35, 82). A number of kinetin analogs have been demonstrated to stimulate the germination of the variety Early Curled Simpson at 30°, where it is partially dark-dormant (67, 68, 70). Gibberellic acid and kinetin have been claimed to have a synergistic action on germination (35, 69). Most reports of stimulation of germination by kinetin do not comment on the mode of germination, but Ikuma and Thimann (35) noted that there was an increase in "atypical" germination (germination by extrusion of the cotyledons through the surrounding layers) in the presence of kinetin, both in darkness and after R irradiation. It was also observed that kinetin promoted the expansion of isolated lettuce seed cotyledons, but inhibited extension growth of the

embryonic axis of de-endospermed seeds. It was suggested that kinetin promoted germination of the intact seed by stimulating cotyledon expansion.

METABOLIC CONSEQUENCES OF IRRADIATION.

Information on any biochemical change developing following R-irradiation can be of great interest because of its potential value in elucidating the mode of action of phytochrome. Unfortunately, most reports on various biochemical processes in dark-held vs irradiated lettuce seeds suffer from a serious drawback; the light-induced changes were associated with the beginning of germination and hence cannot be distinguished from changes associated with the beginning of growth. Such reports will not be considered.

Respiration.

The presence of oxygen is required for a period of time following R, but not during the imbibition period (Evenari's phase I), or for photoconversion (37). Thus, lettuce seed phytochrome undergoes reversible photoconversion but will not function in the absence of oxygen.

Interesting if unelucidated reports of changes in respiratory activity following irradiation occur in the literature. Irradiated seeds have a significantly higher respiratory rate (both oxygen uptake and carbon dioxide evolution). This stimulation is evident before radicle protrusion; it was claimed to be evident as early as one hour after irradiation. In older seeds which had lost dark-

dormancy during storage, R increased carbon dioxide evolution relative to dark, but did not affect oxygen uptake; F lowered oxygen uptake (22). Similarly, in an independent study of respiratory rates at 32°, it was reported that a F-reversible, 6-fold increase in oxygen uptake occurred within 2 hours after R irradiation. In tissues where R is inhibitory to growth, for example, etiolated maize mesocotyls and etiolated pea epicotyls, R caused a depression, instead of a stimulation, of oxygen uptake (52).

Removal of the endosperm resulted in an increased respiratory rate (oxygen uptake) in both Grand Rapids and the aphotoblastic variety Progress. The rates of oxygen uptake for the 2 varieties were determined after different treatments. The following systems are listed in order of decreasing respiratory rates: (1) intact Progress, dark; (2) de-endospermed Grand Rapids, dark; (3) intact Grand Rapids, R-irradiated; (4) intact Grand Rapids, dark (14). It was suggested that a respiratory block existed in dark-dormant seed, but not in nonphotosensitive varieties, and it was postulated that this assumed block can be overcome by R, prolonged storage, or decoating (17).

Assimilation and enzyme activity.

Coats surrounding the embryo are impermeable to phosphate and sulfate, but when the labelled ions were supplied to punctured seeds, it was found that phosphate was esterified and sulfate was reduced to the level of sulfhydryl as early as 3 hours from the beginning of imbibition. C¹⁴-bicarbonate could penetrate intact seeds and the

label was detectable in certain soluble compounds as early as one hour after the beginning of imbibition, suggesting that carboxylation, the Krebs cycle, and transamination mechanisms function during the earliest phases of germination. There was no detectable effect of white or F light on these processes (27).

Catalase activity drops after the 5th hour, but rises rapidly again after the 10th hour of imbibition in dark-imbibed seeds. Light treatment accelerated the initial fall until a lower level was reached than that in the dark. The subsequent rapid rise was also more pronounced than in dark-held seeds. The initial fall was also accelerated by the germination-promoter thiourea (59).

The dry seeds contain high ascorbic acid oxidase activity. This activity increases during imbibition, but at the same rate in darkness as after light treatment. The activity was high enough to account for all of the oxygen taken up by the seed (60).

Growth substances.

In a study of the growth substances of the acid fraction of lettuce seed extracts, carried out with the *Avena* coleoptile section assay, it was found that the dry seed contained no growth promoters, but two chromatographically separable growth inhibitors. These disappeared after 12 hours' imbibition of R-treated seeds. This disappearance coincided with the appearance of two growth-promoting substances and the protrusion of the radicle (61). Quantitative determinations of the levels of the inhibitory substances during the time prior to radicle protrusion were not accomplished, but it

is possible that such a procedure might have revealed a detectable decline prior to germination.

REVIEW, AND OBJECTIVES OF THIS THESIS.

The data on germination of photoblastic lettuce seeds are very extensive and pose many interesting problems for further investigation. However, they permit relatively few reasonably certain conclusions to be drawn. It has been established beyond doubt that promotion of germination by light is in every case mediated by the phytochrome system. The action of phytochrome must be sustained for relatively long periods of time to potentiate germination of the dark-dormant seed, and this sustained action is sensitive to temperature and dependent on the presence of oxygen. Photoconversion of the pigment is on the contrary independent of temperature and aerobic conditions.

Aphotoblastic lettuce varieties are probably not qualitatively distinct from photoblastic ones, since a (small) sensitivity to light can be induced in the former by treatment with coumarin or prolonged high temperature treatment.

Through several different lines of experimental approach, it has been reasonably well established that gibberellic acid, kinetin, and red light all promote germination by different mechanisms. These mechanisms, however, are completely obscure. The morphological site of GA action seems to be the same as that of light, but evidence from experiments with excised cotyledons points to this organ as the effective locus for the action of kinetin. It was concluded

that kinetin promotes germination by stimulating cotyledon expansion, although it is difficult to see why this should result in increased atypical germination. In an endosperm subjected to increasing tension by cotyledons expanding within, the obvious place for rupture is at the pointed, radicle end of the embryo. As an alternative explanation, kinetin may bring about (atypical) germination by stimulating the secretion of cytolytic enzymes by the cotyledons, the effect being a weakening of the mechanical resistance of the endosperm at the cotyledonary end. The validity of this hypothesis still awaits experimental confirmation.

On the other hand, a great number of responses, while factually well established, cannot be assimilated into a unified picture of photoblastism, or germination control in general. The locus of light perception and the effects of the action of phytochrome are known either only from circumstantial evidence, or are not known at all. A light requirement for germination is present only as long as the endosperm of the seed is intact. Upon removal of this tissue the naked embryos will proceed to elongate and develop without receiving any light treatment, and the growth rate of such embryos is unmodified by irradiation with either R or F, at least within the limits of precision of measurements which have been reported. This might lead to the assumption that the light effect is consummated in the endosperm. However, other evidence, particularly the experiments with R and F shone on opposite sides of the seed, strongly implicate the embryo, and in particular its hypocotyl-radicle region, as the

effective locus of light perception. Because of this, the necessity for the presence of an intact endosperm is currently thought to be the result of its passive role in mechanically restricting embryo expansion, although restriction of the rate of exchange of respiratory gases, primarily oxygen, or secretion of a growth inhibitor (80) have not been eliminated as possible contributions of this tissue to the expression of photoblastism. Assuming mechanical restriction is the only effective property, the effect of red light must be either to increase the ability of the embryo to expand against the endosperm, or to decrease the resistance of the endosperm to embryo expansion (or both). There is to date no experimental evidence supporting either of the two mechanisms; however the second has been favored because of the failure to demonstrate any effect of R or F on the growth rate of de-endospermed embryos.

The chemical basis of the action of phytochrome in promoting seed germination is foremost among the obscure aspects of the physiology of photoblastism. It is not even quite certain which of the 2 forms of the pigment is the active one in controlling the behavior of the seed. It is commonly (and reasonably) assumed that Pf is the effective form of the pigment, and that Pr is inactive, but the arguments advanced for this assumption are not as convincing for lettuce seed germination as they are for other photomorphogenic systems. The unattractive possibility remains that in lettuce seed Pr is the active form, and acts by somehow reducing germinability.

The strong germination-inhibiting effect of continuous radiation with high intensity blue, far-red, or white light still awaits a simple explanation in terms of effects on phytochrome.

The decay in photosensitivity upon prolonged dark incubation, and the establishment of thermo- and osmodormancy, while they may all be related phenomena, have not been explained.

The reason for the promotion of dark germination by low temperatures remains unelucidated, as does the reason for the gradual loss of dark dormancy during post-harvest storage of the dry seed.

The objective of the present studies was to obtain information on some of the obscure aspects of lettuce seed germination, particularly on the role of phytochrome in different responses. The two questions studied most extensively were: (a) Can an effect of R and F light on the embryo be demonstrated directly: (b) Can the effects of high and low temperature be related to phytochrome? These studies also permitted some conclusions as to which form of the pigment is active in the control of lettuce seed germination.

In addition, some experiments were performed with chemical germination promoters and also on the effect of repeated F irradiation and on the loss of dark dormancy in storage. The objective of the former was to compare the response to these chemicals with that to red light in the system chosen; that of the latter to determine whether and how these phenomena relate to phytochrome.

II. METHODS AND MATERIALS

Lettuce (*Lactuca sativa*, L.) seed of the variety Grand Rapids, Lot 1132, 1962 crop, Lot 163R18, 1964 crop, and Lot 164R20, 1964 crop, was obtained from the Pieters-Wheeler Seed Company, Gilroy, California, and stored in the laboratory under conditions of ambient temperature, humidity, and occasional illumination. Some experiments were performed with seed of the variety Great Lakes, R-200, Lot 257A11, 1963 crop, obtained from the same source.

The R source was two Sylvania 40-Watt warm white fluorescent tubes wrapped with three layers of heavy red cellulose acetate and placed at a distance of 20 cm from the illumination plane. The light from two 150-Watt internal reflector floodlamps at a distance of 33 cm from the illumination plane, filtered through 3 cm of water and two layers each of blue (Rosco #138) and red (Rosco #17) gelatine filters (obtained from the Bates Lighting Company, Hollywood, California), served as the F source. Unless otherwise stated, exposure times to R and F were 10 minutes and 5 minutes, respectively, representing greater than saturation doses for all reported effects. In the standard irradiation procedure used, red saturation of the imbibed seed occurs at between 3 and 4 minutes' irradiation time; maximal F reversal of R-irradiated seed occurs at between 2 and 3 minutes' F irradiation.

Irradiations, and all dark incubations at 20°, were carried out in a darkroom maintained at 19-21°. Incubations at other temperatures

were carried out in temperature-controlled darkrooms of the Earhart Plant Research Laboratory, where the temperature was maintained constant within a range of one degree. Unless otherwise stated, "dark" manipulations of imbibed seeds were performed under dim green safelight. The source consisted of two 15-Watt green fluorescent tubes. The light from the source was filtered through several layers of dark green gelatine filters, prepared according to Withrow and Price (83). Direct exposure of imbibed seeds to the safelight for periods at least as long as $\frac{1}{2}$ -hour did not affect dark germination.

Germination tests with intact seeds were carried out as follows. One hundred seeds were placed in a 9-cm petri dish containing one disc of Whatman #1 filter paper and 2 ml of water and removed immediately to a darkroom at either 20 or 25°. Light treatments were given 2 hours later. The sample size was 300-600 seeds, and the incubation time was 3 days.

A large part of the experiments was performed with "half-seeds", containing the embryonic axis (radicle plus hypocotyl) and a small portion of the cotyledons. Half-seeds were obtained by slicing the dry seed across its longitudinal axis with a Gillette "thin" razor blade at a point approximately 0.4 seed-lengths from the radicle end. The growth of the axes of these half-seeds is in no way mechanically restricted by the endosperm.

The imbibing medium for seeds operated in any way always con-

tained streptomycin sulfate at a concentration of 100 mg/l, to minimize bacterial contamination. The germination of intact and punctured seeds is unaffected by the antibiotic at this concentration. Half-seeds, in lots of 50, were placed to imbibe on two layers of 5.5-cm Whatman #1 filter paper pressed into a 5-cm petri dish and wetted with 2 ml of liquid. Imbibition of half-seeds in an osmoticum was carried out in 0.46M d-mannitol. Where mannitol solutions were used, the dishes were immediately sealed in transparent plastic bags containing wet paper towelling, and removed to the darkroom. Light treatments were given 2 hours after the start of imbibition, except where stated otherwise. The appearance of a positive geotropic curvature in the radicle was used as a criterion that growth had taken place. The geotropic response was always accompanied by extrusion of the half-embryo from the surrounding layers.

III. PRELIMINARY EXPERIMENTS

This section includes a characterization of the seed lots used in later experiments. This characterization consists in analyses of the light- and temperature-dependent germination behavior and illustrations of two well-established phenomena; the escape from control by far-red and the loss of photosensitivity attending prolonged dark incubation. The phenomena are described in the introduction. These studies were necessary as all these responses may exhibit considerable variation, depending on the seed lots used, and on experimental conditions. By the technique of osmotic inhibition of germination, using NaCl and d-mannitol solutions, it was shown that the effect of the sustained action of Pf is detectable for periods longer than those ordinarily required for complete promotion of germination.

CHARACTERIZATION OF THE SEEDS.Germination behavior.

The relative sensitivity to light of lettuce seed of the variety Grand Rapids is known to vary greatly among different seed lots, even immediately after harvest. The germination behavior at two different temperatures, and with different light treatments, of different lots of this variety and also a lot of the aphotoblastic variety Great Lakes, is illustrated in Table 1. At the two temperatures employed, germination is maximal at about 48 hours, and does not increase with further incubation. The difference in the light requirement (i.e.,

Table 1

Germination response of 2 varieties of lettuce seed at 2 different temperatures. Germination was counted after 3 days dark incubation following the light treatment, which was 5F or 10R given after 1.5 hours imbibition. Sample size, 300-600 seeds.

Variety	Temperature	Light treatment	% Germination
Grand Rapids, lot 1132	20°	R	99
		F	2
		none	17
"	25°	R	96
		F	1
		none	1
Grand Rapids, lot 163R18	20°	R	99
		F	21
		none	43
"	25°	R	93
		F	4
		none	14
Grand Rapids, lot 164R20	20°	R	98
		F	65
		none	90
"	25°	R	86
		F	15
		none	22
Great Lakes, R-200, lot 257A11	20°	R	100
		F	100
		none	100
"	25°	R	99
		F	100
		none	100

in the depth of dark-dormancy) among the 3 lots of Grand Rapids is evident. The germination behavior of Grand Rapids, lot 1132, at 25° characterizes this lot as highly dark-dormant. For the lower temperature, the reduction in germination of F-irradiated seeds is highly significant. Such F depression of dark germination is usually observed in seed samples possessing any amount of dark-dormancy at this temperature. It is an expression of the fact that phytochrome exists as 2 species, only one of which is physiologically active. Although the red-absorbing form (Pr) is the more stable species, conditions during maturation of the seed in situ may be expected to maintain a considerable proportion of the pigment in the far-red-absorbing form (Pf) up to some stage of maturity (dessication), where the Pf/Pr ratio presumably becomes fixed, and is no longer subject to change by actinic radiation.

Rate of escape from control by F.

This process is illustrated in Figure 1, for lot 1132, at 25°. Red light was administered after 1.5 hours of imbibition, then F was administered at increasingly longer time intervals after the initial R dose. Germination was counted after 52 hours. Dark controls germinated 1.5%, and those given no F germinated 97.5%. The curve is quite representative of those found in the literature and indicates that for a given dark-dormant seed to germinate under a defined set of conditions, a given quantity of Pf must operate for a definite time interval, this interval differing greatly among different seeds. In other words, a given seed lot is strongly

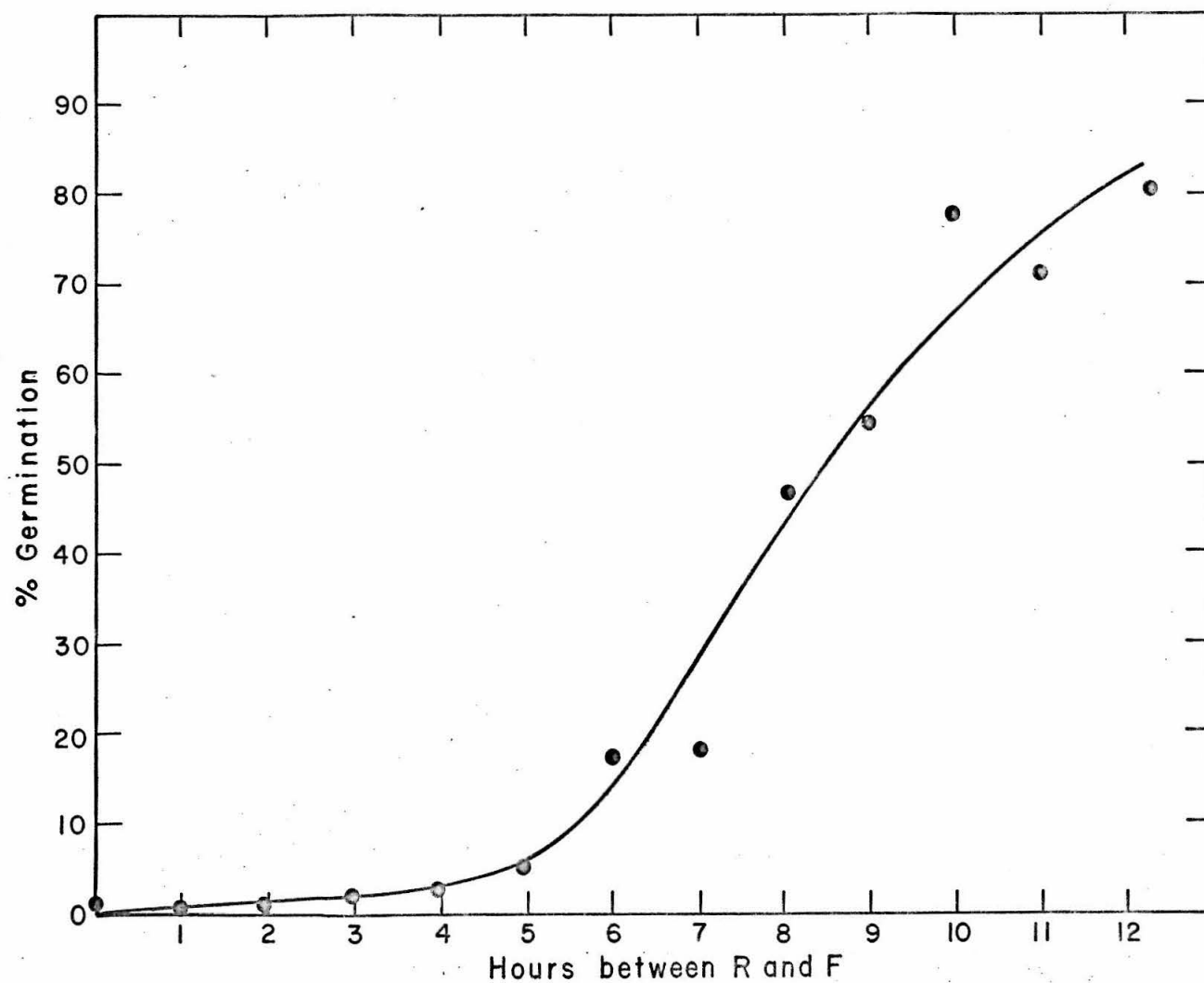


Figure 1. Effect of the interval between R and F on ultimate germination response at 25°. Sample size, 200. Lot 163R18.

heterogeneous with respect to depth of dark-dormancy.

Effect of time of imbibition before irradiation.

Considerable differences in the effect of administration of a fixed dosage of R at different times after the beginning of imbibition are reported in the literature, but the course of these changes was quite different in different reports. In some cases, rather sharp (18, 37), in others, rather broad (3) optima for pre-irradiation imbibition time have been reported. Because of this, it was considered necessary to establish the time course of this decay in photosensitivity obtaining under the present experimental conditions. Sub-saturating (3), apparently saturating (37), and uncharacterized (18) R doses have been used in published reports of decay. In view of the uncertainty in precision of administration of suboptimal R doses, detection of decay was attempted by giving a saturating R dose after increasing times of dark imbibition, followed (an arbitrary) 13 hours by a saturating F dose. Thus, assuming that the concentration of total phytochrome in the seeds remains constant throughout dark imbibition, and that the rate of dark reversion of Pf to Pr is independent of any changes taking place in this time period, what was being tested was the effectiveness of a constant $(Pf) \cdot \text{time}$ as a function of the antecedent dark imbibition time. Figure 2, from data from an experiment performed at 25^o, demonstrates the appearance of decay after a relatively long period, but reveals no effect of duration of pre-irradiation imbibition for the interval from 1.5 to

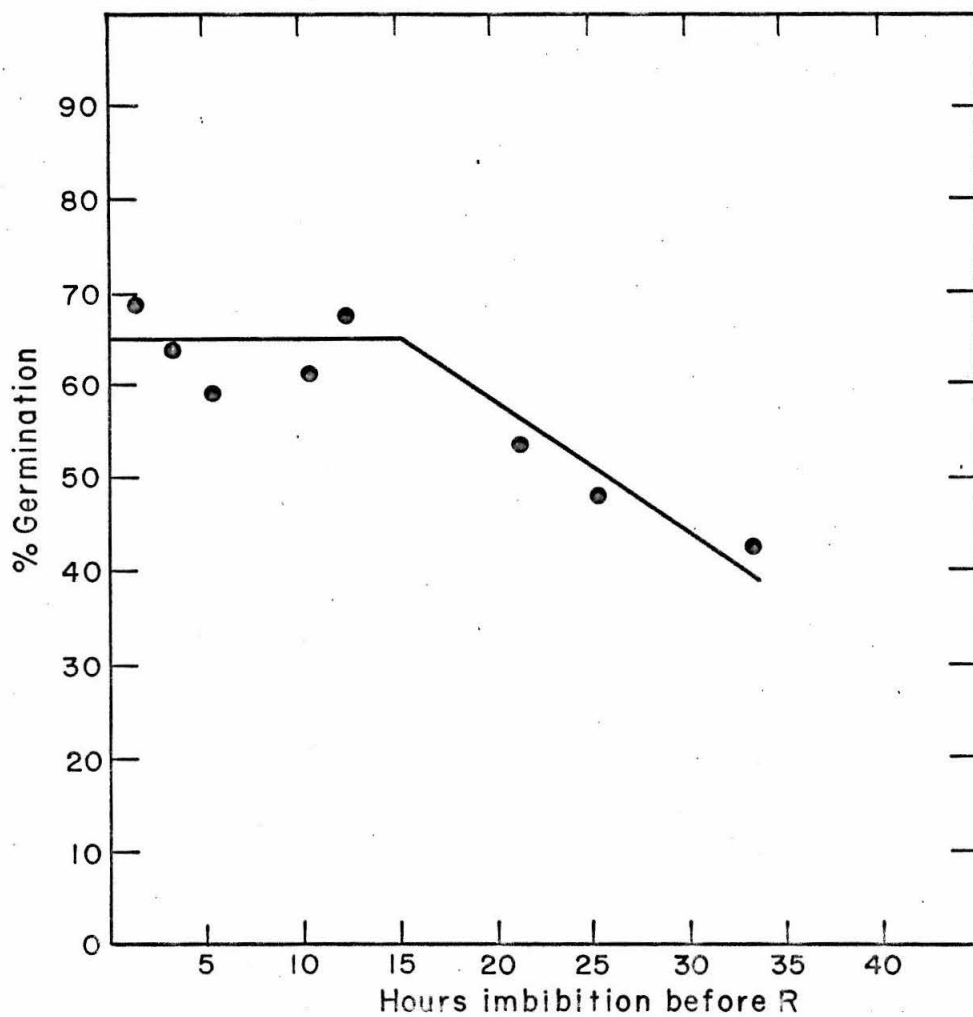


Figure 2. Effect of pre-irradiation imbibition time on the germination response at 25° to an interval of 13 hours between R and F. Sample size, 600. Lot 163R18.

about 15 hours.

TEMPORAL SEPARATION OF THE ACTION OF Pf FROM GERMINATION.

Dark germination may be brought to nil by imbibing seeds in a solution, at the appropriate concentration, of a number of different non-toxic solutes, including mannitol and sodium chloride (39). After prolonged dark incubation in an osmoticum, the seeds do not germinate upon transfer to water unless given R.

Seeds were placed to imbibe in 3 ml of either water or 0.2M NaCl in petri dishes inside plastic bags and left on the laboratory bench in continuous, white fluorescent, illumination. The ambient temperature was 22-24°. Within less than 24 hours from the beginning of imbibition, virtually all seeds in water had germinated. Six days later, when the experiment was terminated, none of the 400 seeds in the osmoticum had germinated. Thus, light cannot effect germination in a NaCl solution of this osmotic potential.

In a subsequent experiment, seeds were imbibed in 0.2M NaCl in the dark 3.5 hours, then irradiated 5 minutes with either R or F, and returned to the dark for 24 hours. They were then washed 3 times with water in safelight and both sets given 5 minutes F, then returned to darkness for 48 hours. The ambient temperature was 22-24°. Germination for R-treated seeds was 94.5%, and for those receiving only F, 0%. This result shows that the action of Pf can be completed under conditions where germination is impossible.

In a similar experiment, conducted at 20°, two sets of seeds were

imbibed in 0.2M NaCl for 3 hours in the dark, then given R, then returned to darkness. One set was washed in water after 15 hours and the other after 60 hours. No F was given. The rate of germination (after the water wash) in darkness was determined by withdrawing and observing samples from either set under a dissecting microscope and is illustrated in Figure 3. Seeds that were transferred earlier to water germinated very quickly (50% germination 4.5 hours after removal of the osmoticum), whereas the germination of those transferred later, although Pf had presumably been acting for a longer time, was substantially slower (50% germination after 10.5 hours). Without attempting to explain the germination delay, it may be called a "staling" effect of holding seeds in the imbibed state for long periods under conditions where germination is impossible, and may be related to the decay in photosensitivity after prolonged dark imbibition (Figure 2) and development of osmodormancy (39).

The ultimate germination percent of these seeds at a given temperature increased with the length of time Pf was allowed to act (Figure 1). Since the subsequent rate of germination in water is depressed by very prolonged incubations in osmotica after R has been administered (Figure 3), it was of interest to determine the effect of increasing time of Pf action on this process. Seeds were imbibed 3 hours in 0.25M d-mannitol (at this concentration, germination after R is nil for at least 5 days at 20^o), then R-irradiated, then held in

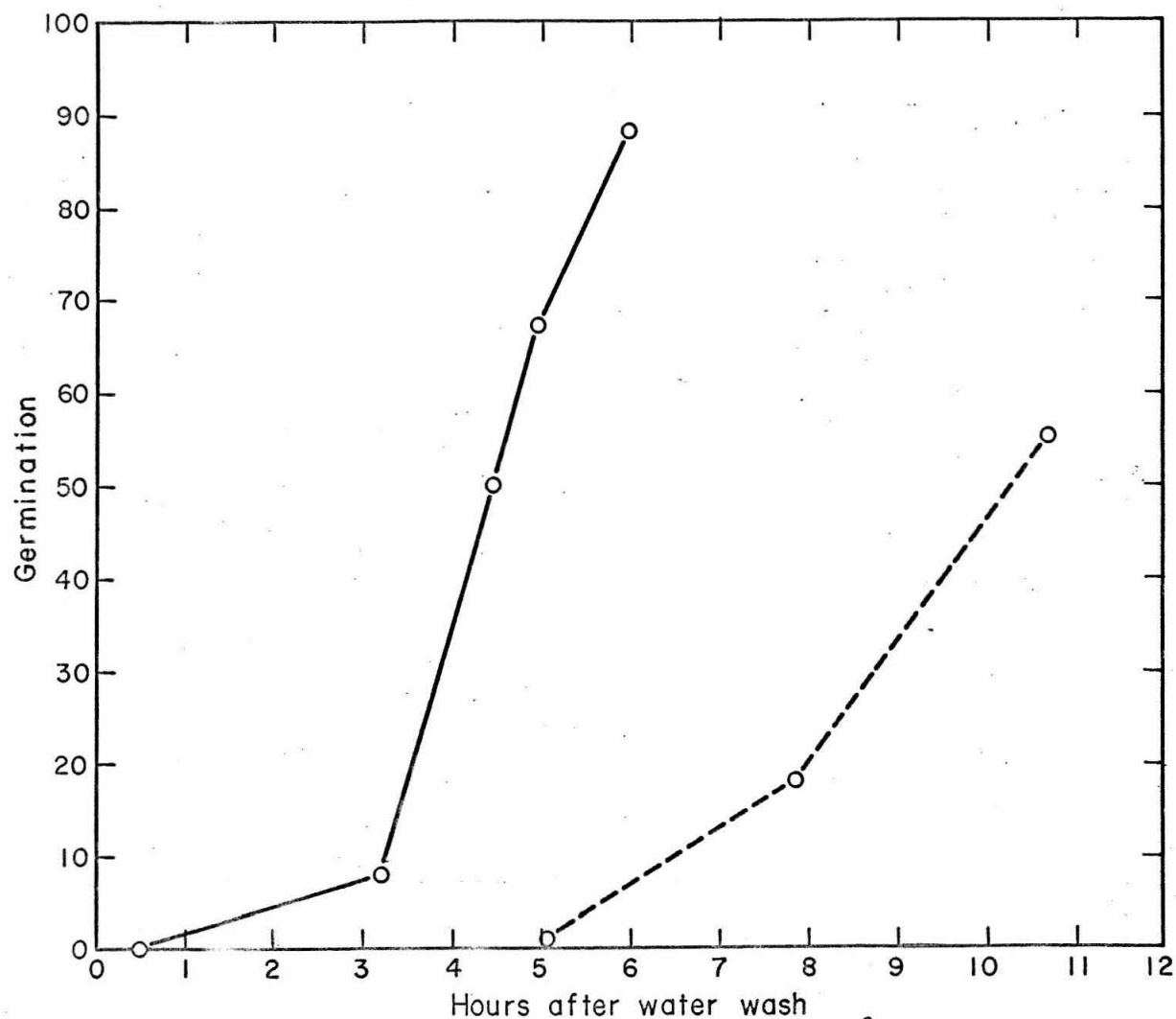


Figure 3. Time course of germination in water at 20° at two different incubation times in 0.20M NaCl after R. Sample size, 100. Solid line, 15 hours after R; broken line, 61 hours after R. Lot 1132.

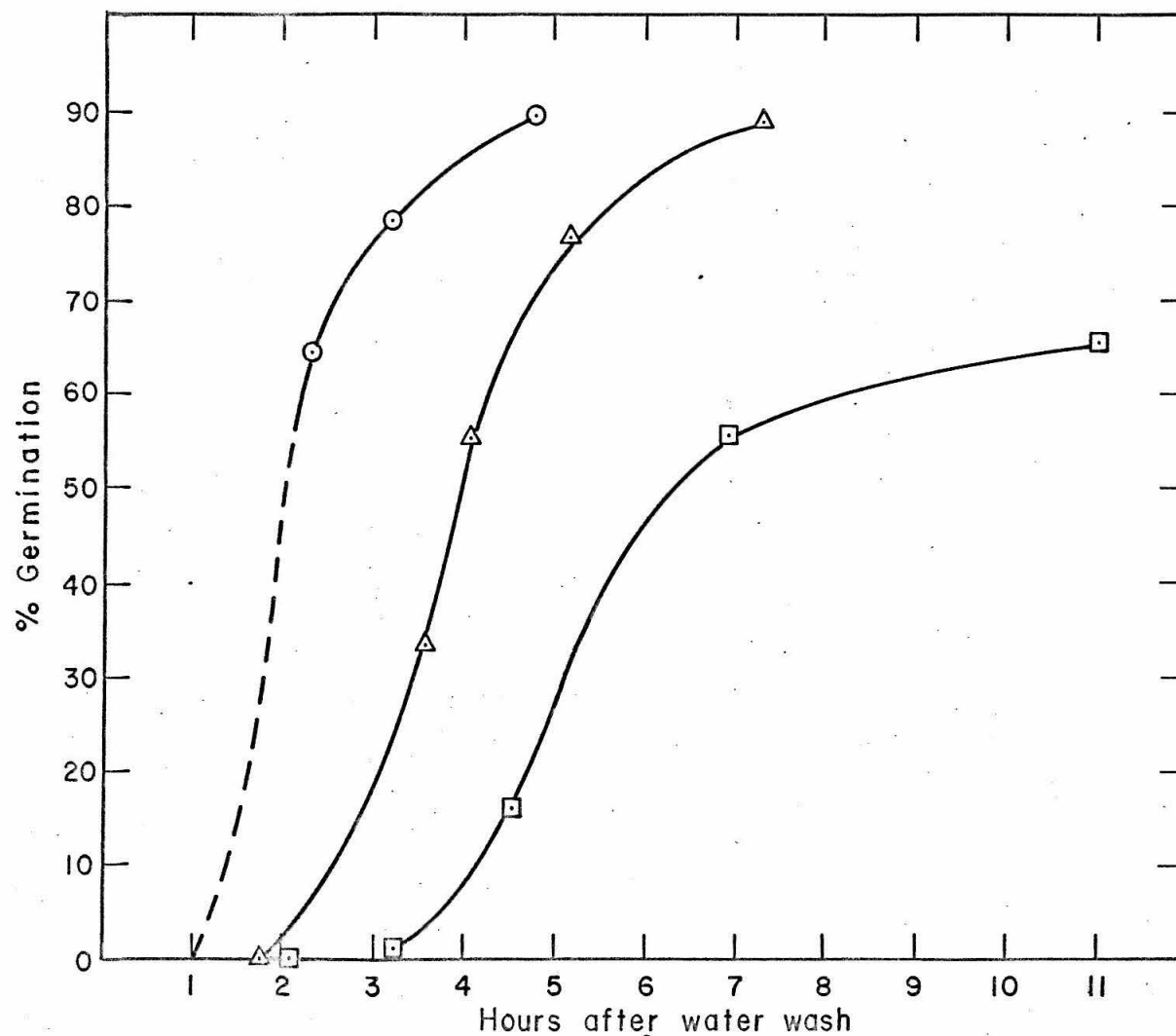


Figure 4. Rate of germination at 20° in water after increasing intervals between R and F in 0.25M mannitol. Sample size, 300. Circles, 21 hours; triangles, 16 hours; squares, 12 hours Pf. Lot 1132.

the dark at 20° for an additional 12, 16, or 21 hours, at which times 5 minutes F was given. Immediately after this, the seeds were washed and the rate of germination in darkness observed. From Figure 4 it may be seen that the longer the interval between R and F, the faster the subsequent germination, in spite of the fact that seeds undergoing the more prolonged action of Pf are also being held in the osmoticum longer and might conceivably be beginning to be subject to the "staling" effect. A 12-hour interval between irradiations is evidently insufficient to permit maximal germination. A 16-hour interval is probably as efficacious as a 21-hour interval as far as ultimate germination percent goes, but whether or not this is strictly true, the 90% that germinated after having spent a 21-hour interval between R and F must be equivalent to the same 90% that germinated after having spent a 16-hour interval, yet they germinated faster. This feature of the action of Pf cannot be demonstrated by ultimate germination response, and shows that the effect of the continuing action of Pf can be detected beyond the point necessary to overcome the mechanical resistance of the endosperm.

IV. DEMONSTRATION OF A LIGHT-INDUCED GROWTH POTENTIAL CHANGE IN THE EMBRYO

It may be assumed as a working hypothesis that the only contribution of the endosperm to the expression of dark dormancy is a mechanical restriction of embryo expansion. In this interpretation, it is obvious that red light acts by effecting either a weakening of this mechanical restriction or an increase in the ability of the embryo to expand against the restriction (or both). Removal of the endosperm abolishes the light requirement; that is, the de-endospermed seed can grow in the dark, and the growth rate is unaffected by irradiation with R or F (31, 36). This fact has led to the natural but unsubstantiated notion that light-induced germination is effected by a weakening of the mechanical resistance of the endosperm to embryo expansion, via secretion of cytolytic enzymes by the embryo.

Experiments with de-endospermed embryos.

Since it had already been twice reported in the literature (31, 36), the time course of radicle elongation in the de-endospermed embryo was not studied. However, the reported, apparent phytochrome-independence of radicle extension was confirmed in the following experiment. Forty seeds were imbibed in the dark at 25°. The endosperm was slit lengthwise with a fine needle, and removed from the embryo, this operation being carried out in white fluorescent light. The naked embryos were aligned on wet filter paper in petri dishes, then given 5 minutes of either F or R and incubated in the dark, 25°.

in a vertical orientation for 23 hours, after which the total length of the embryo was measured to the nearest 0.1 mm. The average length of R-irradiated embryos was 7.46, S.E. 0.31 mm, that of F-irradiated, 7.40, S.E. 0.35 mm. The lengths of the 3 main parts of the embryo (radicle, hypocotyl, and cotyledons) were not individually determined because of the absence of a sharp demarcation between radicle and hypocotyl in expanding embryos at this stage of development. The increase in length is most probably entirely due to radicle growth, according to the results of a similar experiment reported in the literature (36). The important point is that no difference between F- and R-irradiated embryos was detectable.

If, however, instead of water, the naked embryos are incubated in an osmoticum, a large difference in growth rates of R and F-treated embryos can be demonstrated. The foregoing experiment was repeated, using 0.40M d-mannitol as the incubation medium. Larger petri dishes containing 3 layers of filter paper were moistened with 10 ml of the mannitol solution. This relatively large volume was used to minimize any small differences in evaporative water loss during the times the dishes were open. The water-imbibed, de-endospermed embryos (total of 80) were aligned and irradiated as before, then a 55-hour dark incubation was carried out at 20° in a water-saturated atmosphere. The final lengths were: R, 6.78, S.E. 0.1 mm; F, 3.76, S.E. 0.1 mm. No initial measurements were made, but the F-treated embryos appeared not to have grown at all.

Experiments with half-seeds.

The process of de-endosperming imbibed seeds is very time-consuming. Because the seeds are relatively small, de-endosperming cannot be accomplished without the use of a dissecting microscope, which necessitates exposure of the imbibed seed to high intensity light for prolonged times. In addition, since the endosperm closely invests the embryo, some injury (gouging) of the latter is unavoidable during manipulation. Therefore, in addition to restriction of sample size, possible uncontrollable variables introduced by irradiation and trauma impose limitations on the utility of this system in experiments more complicated than the foregoing one, which was designed only to demonstrate a differential effect of F and R.

In some experiments with separate parts of the seeds, it was noticed that a seed half that contains the embryonic axis may be observed to "germinate". The process consists in this case in extrusion of the half-embryo from the surrounding layers at the cut end, the extrusion being downward-directed by the development of a positive geotropic curvature in the elongating radicle. If such half-seeds, obtained as explained in the section on materials and methods, are placed to imbibe on wet filter paper, the time course of "germination" may be followed by the index of proportion of the sample that has undergone the extrusion and curvature. It was thought that this system might provide a more sensitive parameter than straight growth measurements for determining the time course

of growth response to irradiation. Half-seeds were allowed to imbibe in the dark for 1.5 hours, then given the appropriate light treatment and returned to darkness at 20°. Samples were removed after different time intervals and the proportion of each sample that had undergone the response determined by visual inspection. Immediately thereafter each sample was fixed in formalin-acetic acid-ethanol (FAA) and retained for inspection at a magnification of x10 where it was again scored, this time by the presence or absence of a curvature in the radicle tip. For the second inspection the samples were coded to eliminate prejudice in the determination, which is admittedly a subjective one. The time course of this response after R or F is illustrated, for the 2 methods of determination, in Figure 5. It may be seen that both methods give comparable results, although the second method appears to be more conservative. The small but consistent difference between R and F-treated material in water confirms that the half-seed system is a more sensitive one than the naked embryo for detecting light-induced changes in early growth characteristics. Because of its much greater simplicity, the visual inspection method was used to score most subsequent experiments.

Experiments with half-seeds in osmotica.

It was expected that incubation in an osmoticum would greatly heighten the difference between light treatments. The choice of mannitol as the osmotically active solute was somewhat arbitrary, but it was felt that use of a non-ionizable solute that is probably

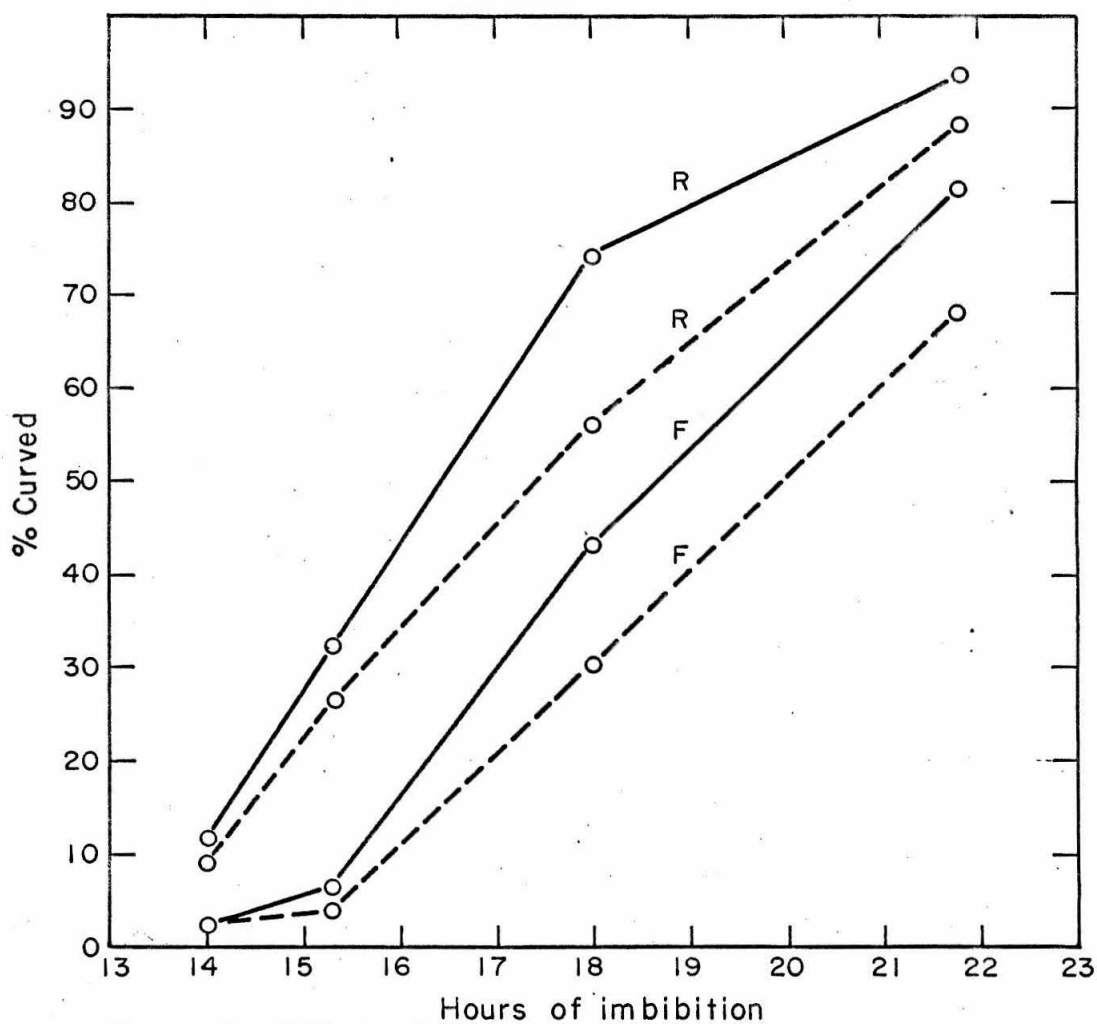


Figure 5. Effect of light quality on the time course of appearance of geotropic curvature in the half-seed at 20°. Solid lines, scored by visual inspection; broken lines, scored by observation of coded samples at x10. Sample size, 100-200. Lot 1132.

metabolically inert would tend to minimize undesirable side effects. For example, in a comparison of the relative effects of NaCl and mannitol on osmotic inhibition of alfalfa seed germination, it was found that both germination at isoosmotic concentrations and recovery upon transfer to water were always lower for NaCl than for mannitol (79).

Half-seeds were imbibed in a series of mannitol concentrations from 0.40 to 0.50M, given R or F, then killed by fixing in FAA after an incubation time of 21 hours at 20°. The results, presented in Table 2, prompted selection of 0.46M mannitol as a suitable osmoticum for further studies with the half-seed system.

R and F irradiation times were usually 10 and 5 minutes, respectively, in all experimental work, because these constitute higher than saturation doses for the germination response. Because of the importance, in some experiments, of assuming photo-equilibrated Pf/Pr ratios, it was desirable to ascertain whether the saturating doses for the half-seed response fell within these time intervals. Half-seeds were prepared and treated in the usual way, except that those to be F-irradiated were first given a 10-minute irradiation with R, so that a subsequent saturating dosage of F would be that for conversion of maximal Pf (see Table 3). R dosages were from 5 to 30 minutes, and response was recorded after 29 hours of incubation at 20°; F dosages were from 1 to 15 minutes, and the incubation time was 52 hours. The choice of different incubation times was arbitrary, but the purpose was to obtain large but submaximal response values.

Table 2

Selection of 0.46M mannitol. Half-seeds were given R or F after 1.5 hours imbibition, held in the dark at 20° for 21 hours, then fixed in FAA. Sample size, 100. Lot 1132.

<u>(mannitol). M</u>	<u>Percent curved</u>	
	<u>R</u>	<u>F</u>
0.40	58	0
0.42	71	0
0.44	64	0
0.46	71	0
0.48	43	0
0.50	19	0

Table 3

F and R dosage-response values for half-seeds in 0.46M mannitol at 20°. R-treated material scored after 29 hours, F-treated, after 52 hours incubation. Sample size, 400. A saturating dose of R was given before F. Lot 1132.

<u>R dosage (min.)</u>	<u>% curved</u>	<u>F dosage (min.)</u>	<u>% curved</u>
Dark control	9	Dark control	58
5	46	1	31
10	49	5	22
20	42	10	33
30	41	15	32

The results, presented in Table 3, show that saturation doses for R and F are below 5 minutes and 1 minute, respectively.

The experiment of Figure 5 was repeated in 0.46M mannitol instead of water, using larger samples and including dark controls. The results are shown in Figure 6. While the imposition of a water stress somewhat delays the appearance of curvature, the difference between the R- and F-treated material becomes much greater. Indeed, the latter appears to attain a maximum of only about 25% curved. The position of the dark control curve between the curves for R and F-treated material points up the similarity of the half-seed response to that of the germination response in the intact seed (Table 1). (The experiment of Figure 6 was performed using a different seed lot than the one used in the curvature-in-water experiment of Figure 5, which was lot 1132. For the behavior of lot 1132 after R and F in 0.46M mannitol, see Figure 15).

Behavior of an aphotoblastic variety.

Treatment with the germination inhibitor coumarin or preincubation at high temperatures has been shown to induce some sensitivity to R and F in seed varieties that are aphotoblastic under ordinary conditions (see Introduction). It was thought possible that the imposition of an osmotic curtailment of water uptake might also reveal potential photosensitivity in such seeds, and if so that a comparison of an induced response with that already existing in dark-dormant seeds might be of interest. The half-seed response had already been

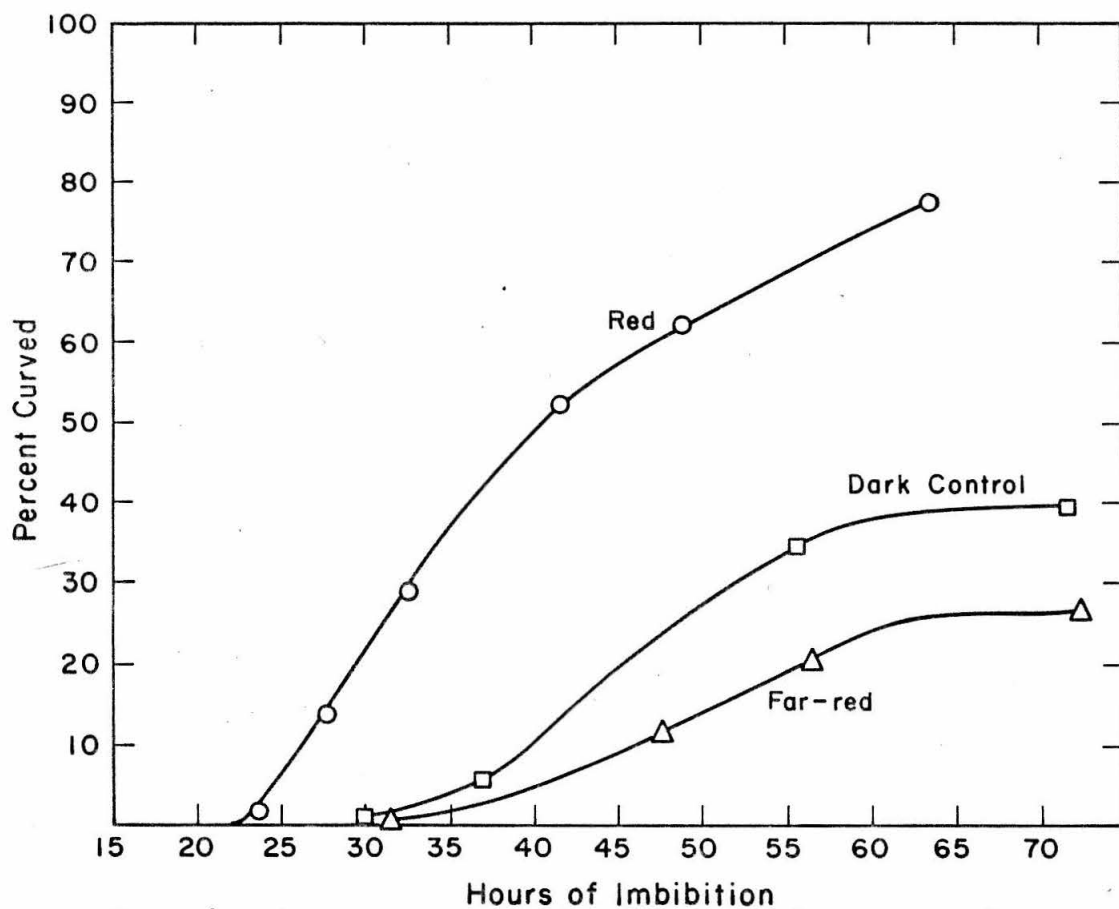


Figure 6. Effect of R and F on the time course of appearance of curvature in 0.46M mannitol at 20°. Sample size, 500. Grand Rapids, Lot 163R18.

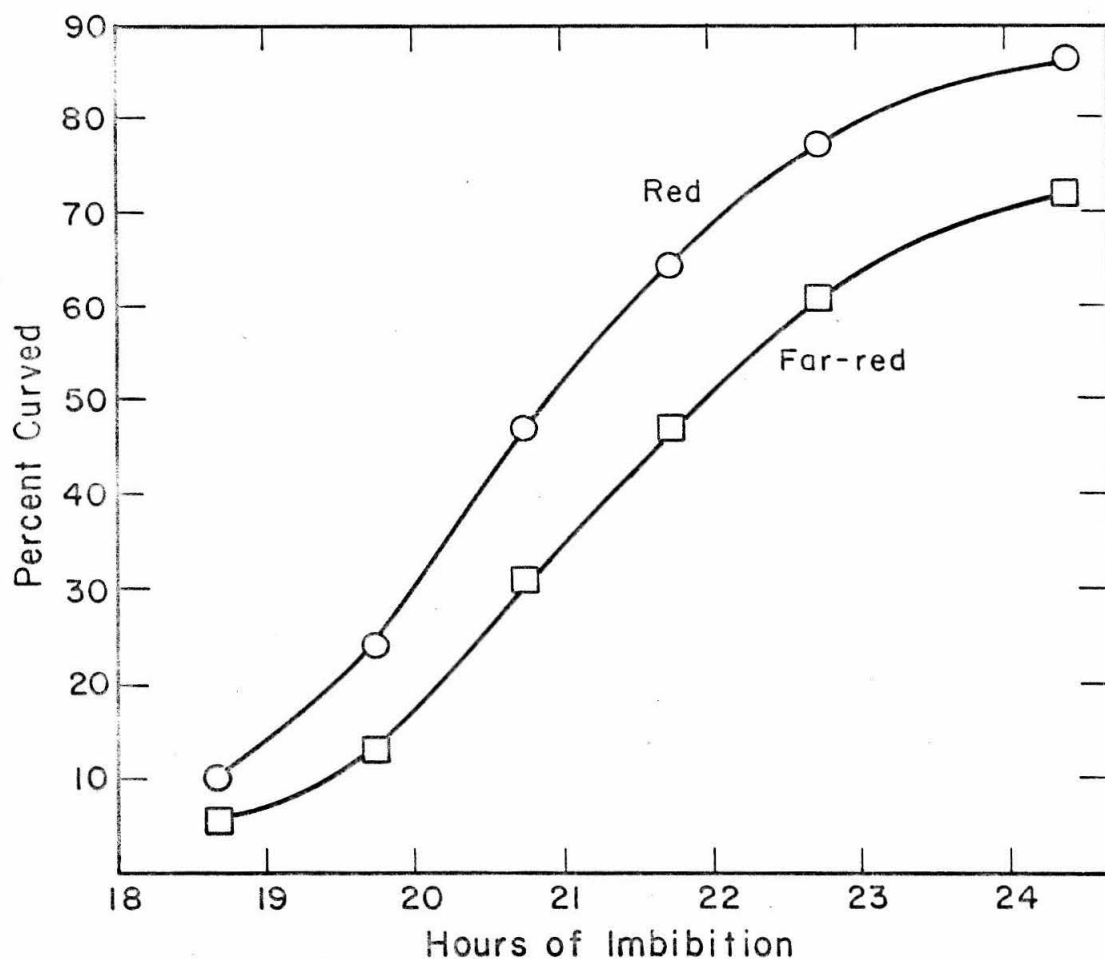


Figure 7. Effect of R and F on the time course of appearance of curvature in 0.46M mannitol in the aphotoblastic variety Great Lakes. Temperature, 20°. Lot 257A11.

used to demonstrate the growth potential increase in Grand Rapids, so this system was employed to compare that variety with an aphotoblastic one.

The results of an experiment similar to that of Figure 6, employing half-seeds of the aphotoblastic (Table 1) variety Great Lakes show a detectable response (Figure 7). However, the horizontal (time-scale) distance between the R and F curves for Grand Rapids averaged between the 10% and 20% curved levels is 28 times as great as that for the aphotoblastic Great Lakes. Thus, the response in Grand Rapids is, at least under the conditions of these experiments, about 28 times as great as that in Great Lakes.

The effect of continued Pf action.

In the germination of intact seeds, both the degree (Figure 1) and the rate (Figure 4) of the response depend on the continuing action of Pf. The following experiment was designed to test whether or not the half-seed system shows a similar dependence. Half-seeds were imbibed in mannitol and R-irradiated as usual. Irradiations with F were given at increasing time intervals after the initial R dose. Two additional 5-minute R-irradiations, spaced about 11 hours apart, were given to those half-seeds which at the time had not yet been F-irradiated. This was to insure maintenance of a high level of Pf. All samples were scored at the 29th hour of imbibition. Thus, the degree of response at the 29th hour of imbibition was examined as a function of time in Pf. The linear nature of the

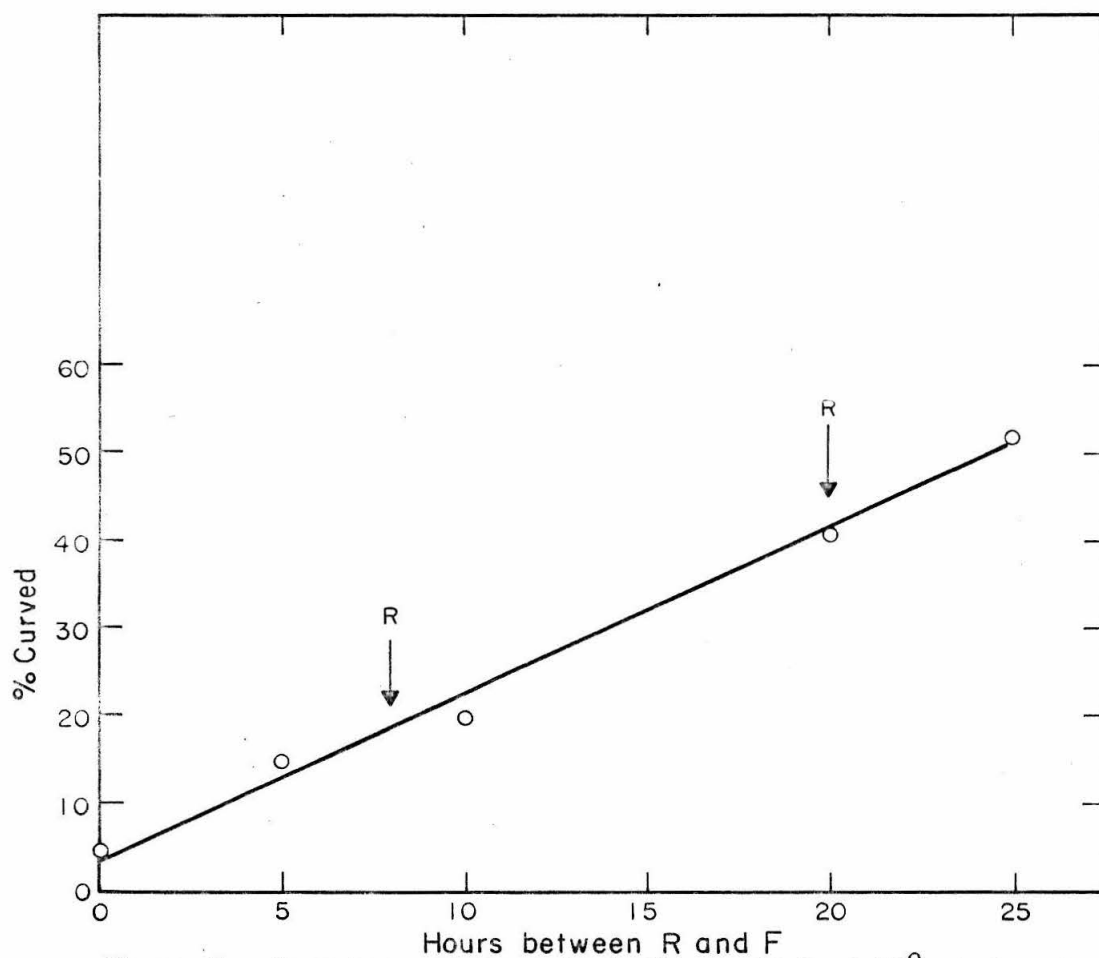


Figure 8. Curvature response in 0.46M mannitol at 20° at the 29th hour of imbibition as a function of time interval between R and F. The difference between any 2 points is significant at the 99.5% level. Additional 5-minute doses of R given at times indicated. Lot 1132.

relation (Figure 8) is probably fortuitous, but it is clear that the rate of appearance of curvature increases with the interval between R and F irradiations.

Non-participation of the endosperm.

Most available evidence, although indirect, points to the embryonic axis as locus of the light-responsive mechanism (see Introduction). However, as explained before, the possibility remains that the endosperm in the region of the axis is directly or indirectly involved in photosensitive germination in some way other than, or in addition to, imposing a mechanical restriction on that process. For example, the possibility that the endosperm may secrete growth inhibitors has been advanced (80). While mechanical restriction by the endosperm has been eliminated in the half-seed, the possibility remains that the light requirement still depends on the presence of the endosperm. The endosperm could be involved directly in at least two ways: (a) R could induce destruction, or prevention of synthesis or release of a growth inhibitor within this tissue; (b) R could potentiate the ability of the embryonic axis to inactivate such an inhibitor.

To test this possibility, half-seeds were imbibed 1.5 hours in darkness in mannitol in the usual way. The half-embryo was then removed from all surrounding layers by gentle pressure on the radicle end, washed 3 times in mannitol, and transferred to another petri dish. F or R was then administered, and the dishes were returned to

darkness for an additional 29.5 hours. Controls were subjected to identical treatment, except for decoating and washing. The results appear in Table 4. Clearly, the inhibitor hypothesis broached above is untenable.

Geotropic curvature and straight growth.

There have been contradictory reports regarding the relative growth rates of roots held in the normal position, and roots geotropically stimulated (30, 43, 49, 65). It has also been reported that there is an interaction of the phytochrome system with geotropic reactions in the maize coleoptile (84). Because of these reports, it was thought desirable to study the effects of R and F on straight growth of the half-seed in 0.46M mannitol, and to compare it with the appearance of geotropic curvature.

In order to facilitate measurements, the osmoticum was supplied in 1.5% agar in a petri dish; the dry half-seeds were arranged in parallel rows on the agar surface and given the appropriate light treatment after 2 hours of imbibition. Thereafter, the dishes were kept in an upright position so that the radicles, not being exposed to a geotropic stimulation, would grow straight. The length of the half-seeds at various times after the beginning of imbibition was measured to the nearest 0.03 mm at a magnification of x20 (transmitted bright green light), with the aid of an ocular micrometer. Appropriate actinic radiation was re-administered after each observation. Half-seeds, treated in exactly the same manner, except that

Table 4

Effect of endosperm removal on growth of half-seeds of Grand Rapids, lot 1132. The endosperm was removed after 1.5 hours of imbibition, then light was given. The incubation time was 33.5 hours in 0.46M mannitol at 20°.

<u>Treatment</u>	<u>No. curved half-seeds per dish</u>	<u>% curved</u>
Endosperm absent:		
F	0, 1	1
R	47, 45	92
Endosperm present:		
F	1, 3	4
R	39, 38	77

Table 5

Straight growth of half-seeds on 0.46M mannitol at 20°.
Lot 164R20.

<u>Hours of imbibition</u>	<u>Light treatment</u>	<u>% half-seeds whose growth (mm) exceeded:</u>				
		<u>0.21</u>	<u>0.24</u>	<u>0.27</u>	<u>0.30</u>	<u>0.33</u>
17:20	R	0	0	0	0	0
	F	0	0	0	0	0
27:20	R	22	12	9.6	2.7	1.3
	F	14	12	9.0	1.3	0
42:30	R	59	49	43	39	29
	F	46	41	25	22	17
50:00	R	75	71	67	63	49
	F	55	52	46	32	28
64:45	R	88	85	84	80	76
	F	65	62	62	52	54

the petri dishes were stored in a horizontal position, and the observations were made in dim green light, served for a concurrent determination of the geotropic curvature response.

Grand Rapids lot 164R20, 1964, was employed in this study. This lot was considerably less dark-dormant than the others (Table 1), but possessed the desirable attribute of being coatless (doubtless a result of the cleaning process) which made precise measurements possible. Sample sizes were 150 half-seeds for determination of straight growth, and 200 for the curvature response. The incubation temperature was 20°.

The qualitative nature of the method of scoring the curvature response implies that a half-seed that has responded has undergone a minimum mean increase in length. The straight growth determinations were accordingly classified into proportions of the entire sample whose growth had exceeded certain arbitrary values at each of the (5) observation points in time, and the data appear in Table 5. A comparison with the concurrent determination of the rate of appearance of curvature revealed that the proportion of the population that had grown more than 0.27 mm was that most closely approximating the proportion that had responded in the horizontally placed half-seeds. Both parameters are compared graphically in Figure 9, where the correspondence between growth and curvature is evident.

The subsequent growth rate of half-seeds whose extension had

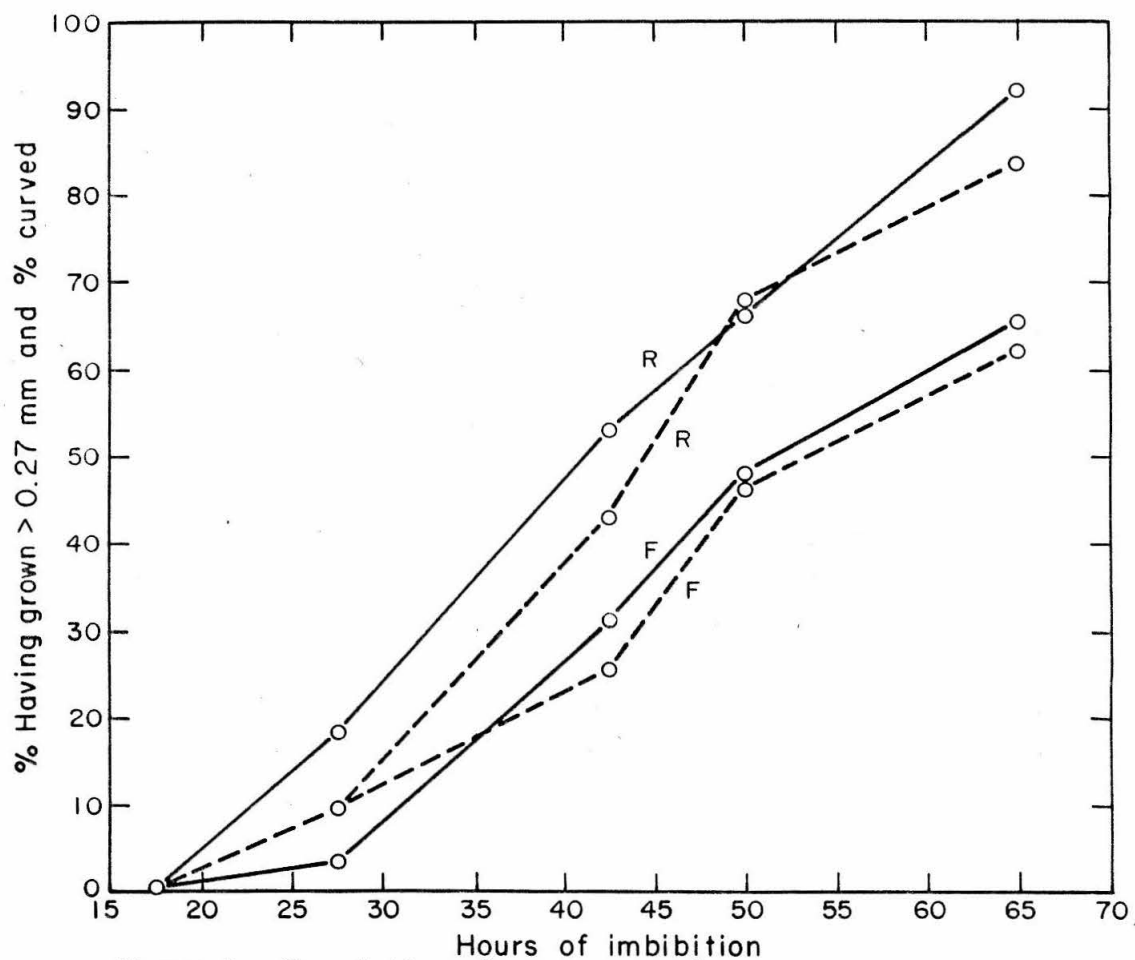


Figure 9. Correlation of appearance of curvature and straight growth on 0.46M mannitol, 20°. Solid lines, % curved; broken lines, % of sample that has grown more than 0.27 mm. Sample sizes, 150 and 200. Lot 164R20.

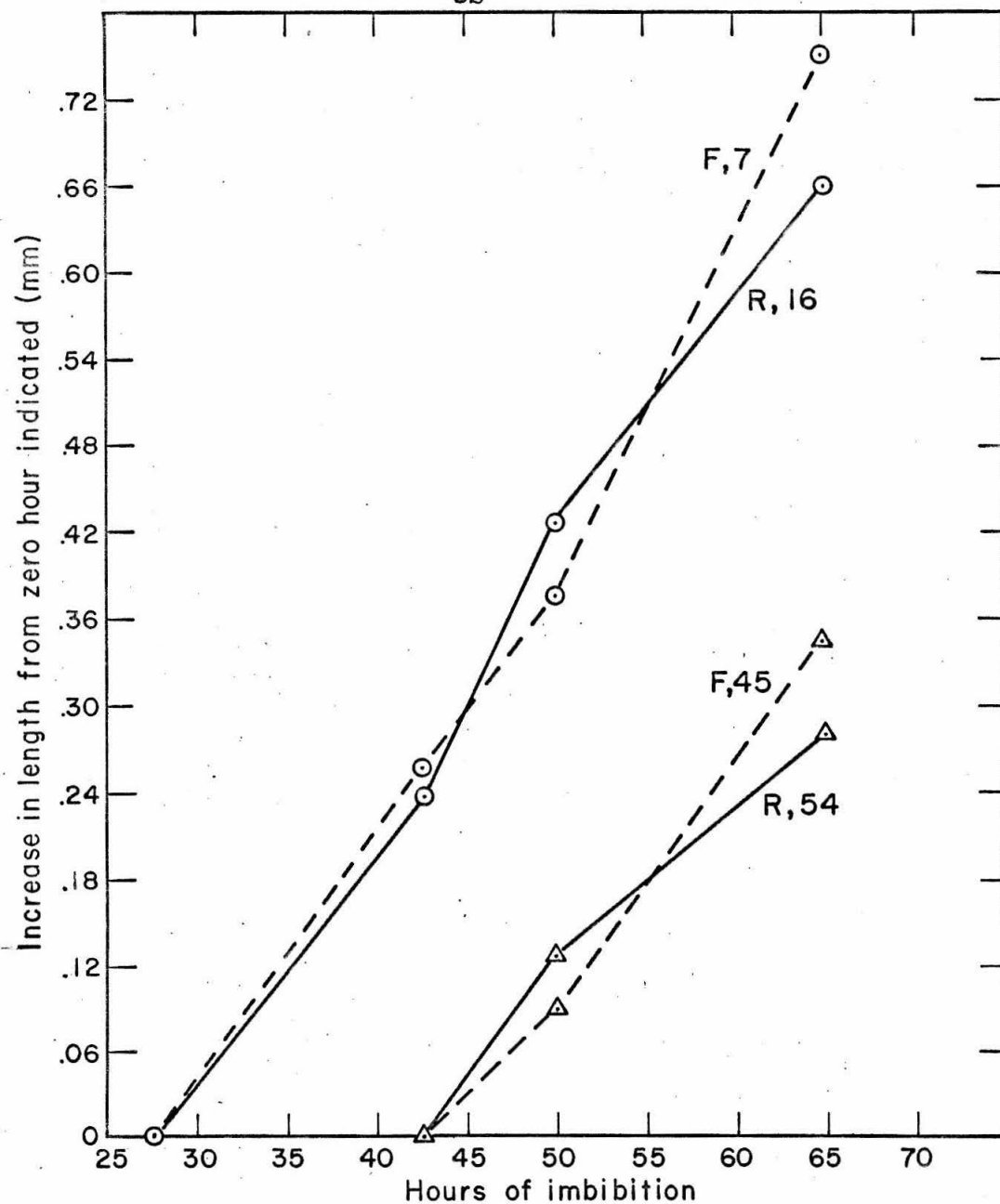


Figure 10. Time course of straight growth in 0.46M mannitol at 20° of half-seeds first exceeding 0.27 mm growth at 27.5 hours (circles) and 42.5 hours (triangles) of imbibition. Solid lines, R; broken lines, F. The number indicates the sample size. Lot 164R20.

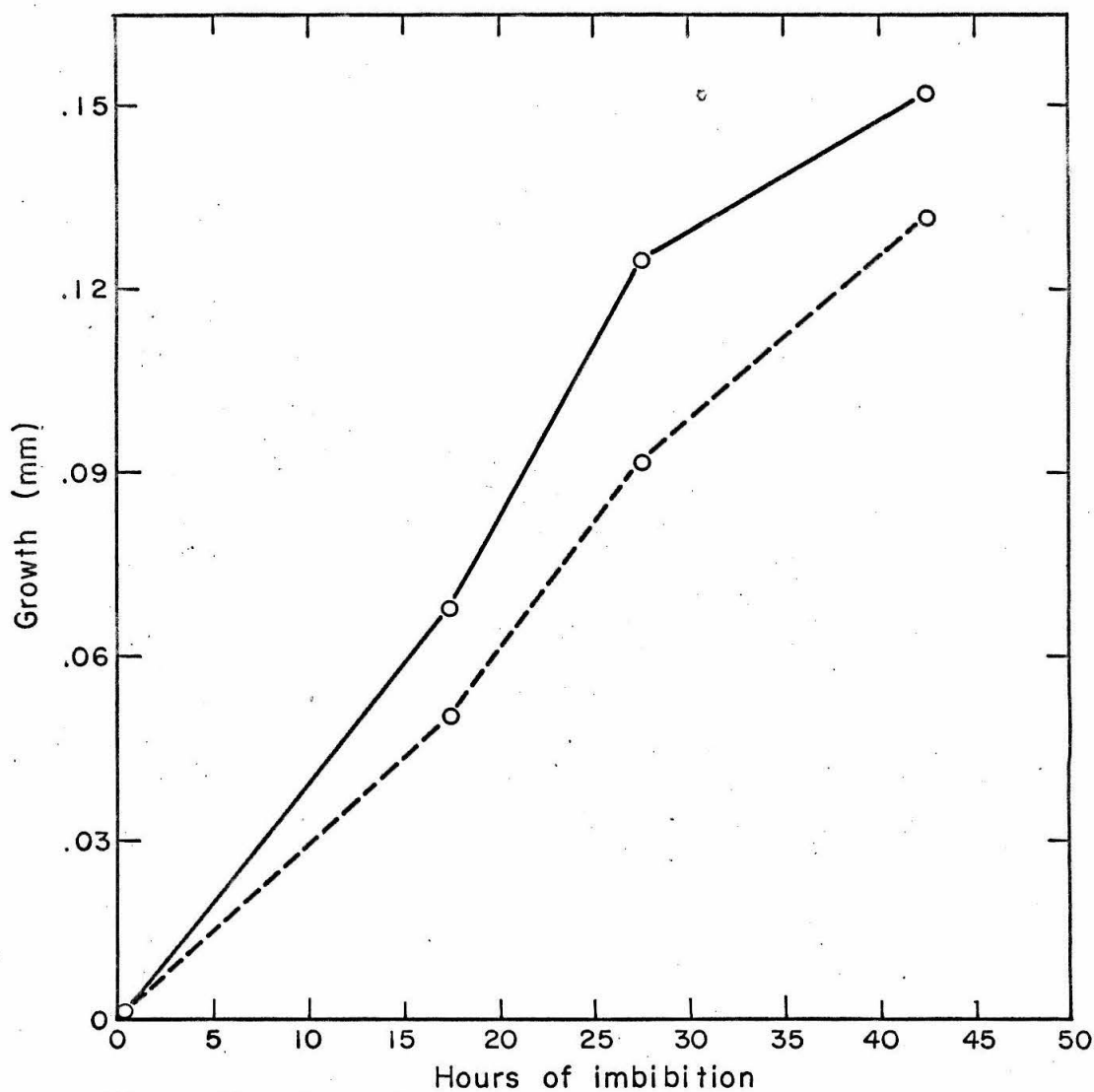


Figure 11. Comparison of straight growth in 0.46M mannitol at 20° of R-treated (solid line) and F-treated (broken line) half-seeds that have not yet achieved 0.27 mm growth. Lot 164R20.

first exceeded the arbitrary critical value (0.27 mm) at 2 different observation times (27:20 and 42:30) is depicted in Figure 10. The slight difference between the slopes of the graphs for the early and late responders (both R and F-irradiated) may be real, but the small sample size of the former precludes any conclusion on this point. The important, and rather interesting, point is that no consistent difference in growth rate between F and R-irradiated material can be distinguished once extension has exceeded the arbitrary critical value.

The time course of early growth of half-seeds that have not yet attained a growth increment of 0.27 mm is represented in Figure 11. Here, growth is plotted up to 27.5 hours of imbibition, at which time a major fraction of both populations still falls in this category. It is evident that during this early period R-irradiated half-seeds are growing faster, in contrast to the situation represented in Figure 10.

This investigation was repeated at 25⁰ with seeds of Lot 163R18, more dark-dormant than lot 164R20 (Table 1), but not having been decoated. Since it was impossible to precisely measure the elongating half-seeds while they were still enclosed in the fruit coat, the naked half-embryos were removed from the imbibed half-seeds by gentle pressure on the radicle end, and then placed on the agar surface. The half-seeds to be used for determination of the curvature response were treated exactly as before. Thus, the straight growth material

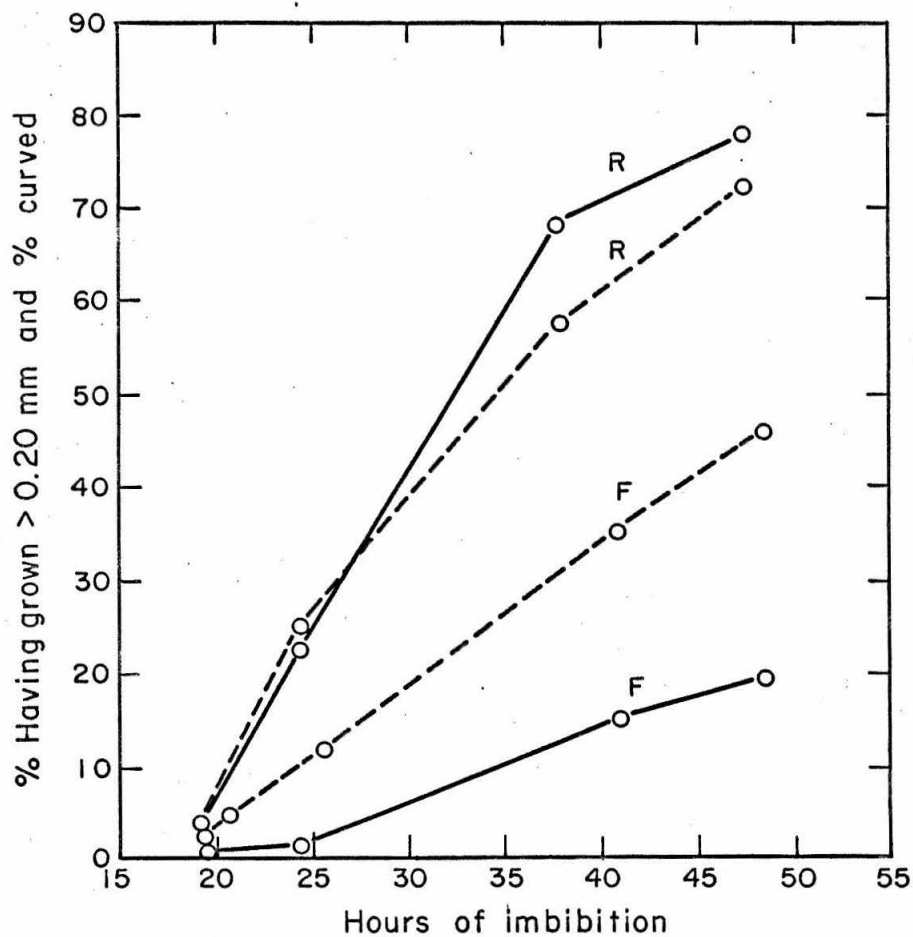


Figure 12. Correlation of appearance of curvature and straight growth in 0.46M mannitol at 25°. Solid lines, % curved; broken lines, % of sample that has grown more than 0.20 mm. Sample size, 200. Lot 163R18.

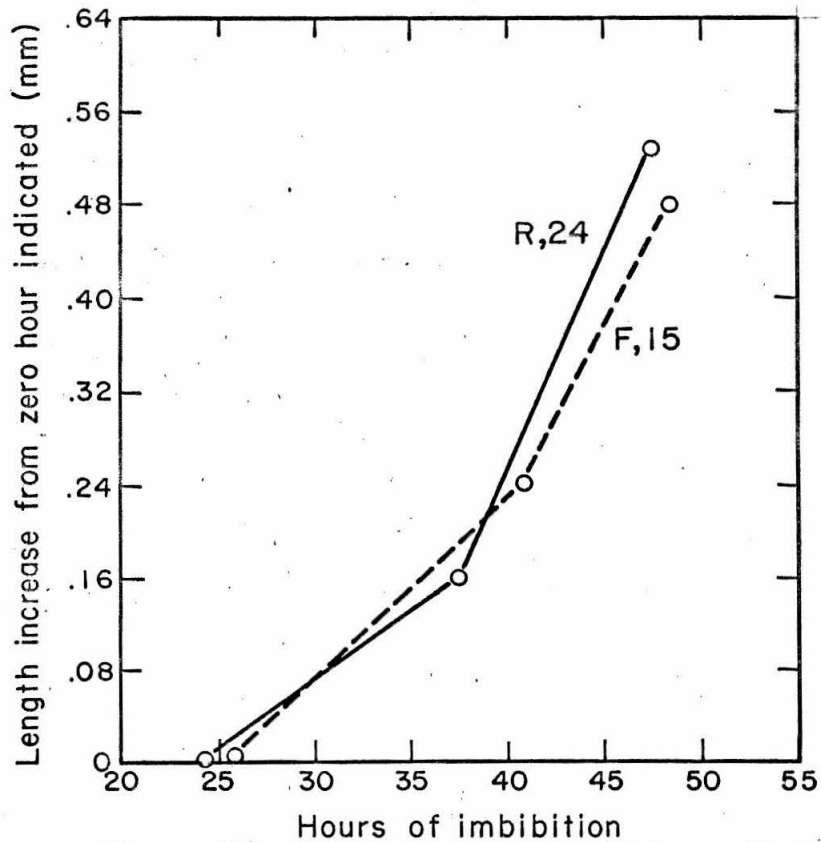


Figure 13. Time course of straight growth in 0.46M mannitol at 25° of half-embryos first exceeding 0.20 mm growth at 25 hours of imbibition. Solid line, R; broken line, F. The numeral indicates the sample size. Lot 163R18.

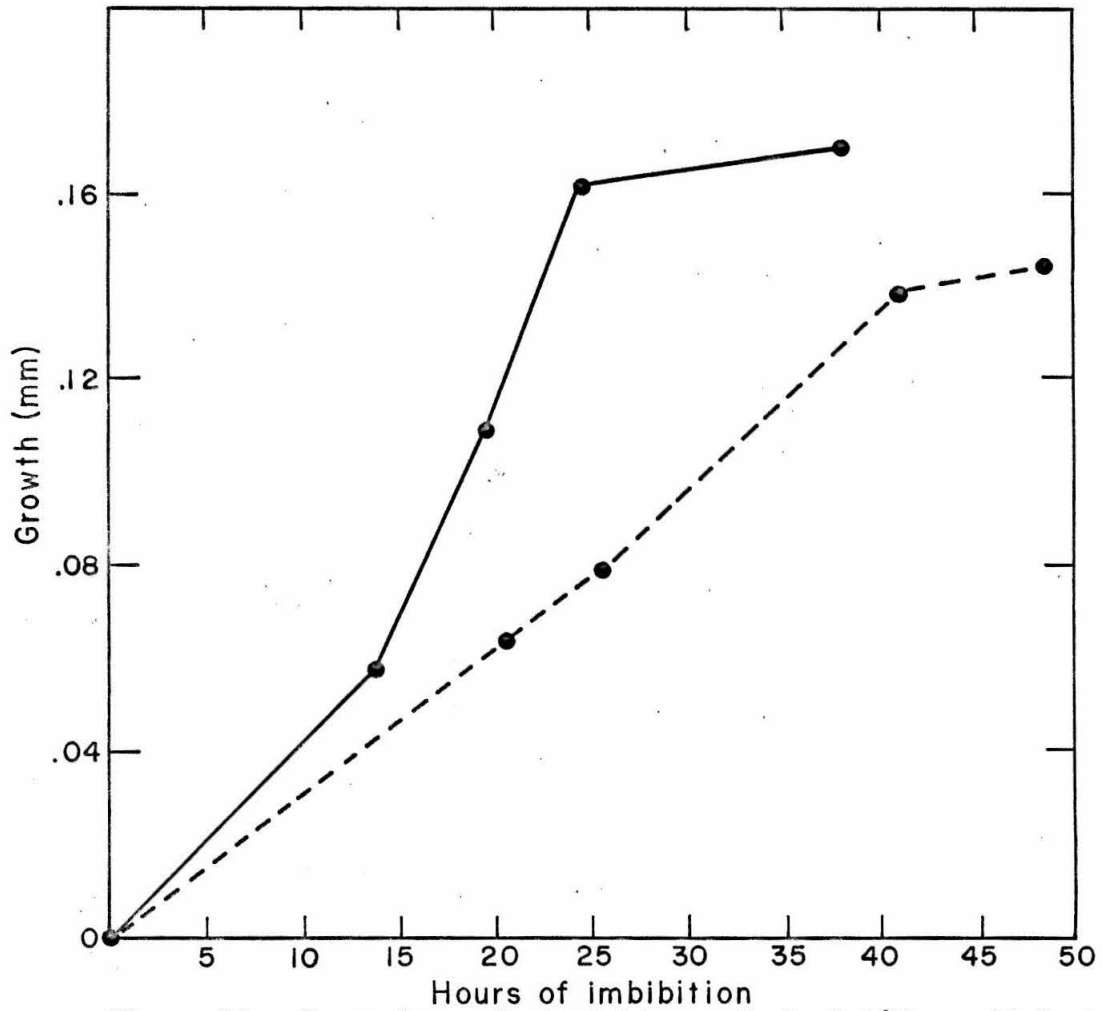


Figure 14. Comparison of straight growth in 0.46M mannitol at 25° of R-treated (solid line) and F-treated (broken line) half-embryos that have not yet achieved 0.20 mm growth. Lot 163R18.

differed from the curvature material as before, by the light source used for measurements and in the angle of orientation with respect to the gravity vector, but also by this additional manipulation. The data were classified as before. In this experiment, it turned out that the attainment of a growth increment of greater than 0.20 mm (instead of 0.27 mm) most closely followed the curvature response. Figure 12 illustrates the relation between attainment of the arbitrary critical growth increment and appearance of curvature, Figure 13 shows that the growth rate of half-embryos that have already grown more than the critical value is independent of the light regime, and Figure 14 shows that R increases the growth rate of seeds that have not yet attained such a growth increment.

To summarize, an arbitrary growth increment may be chosen so that the straight growth response of the half-seed may be scored plus or minus, allowing a comparison with the rate of appearance of curvature, which is scored in the same manner. When this is done, it may be seen (a) that the early longitudinal growth rate of the radicle parallels the rate of appearance of curvature in sensitivity to F and R, and (b) that once the half-seed has grown more than the arbitrary increment, the rate of subsequent growth is independent of the previous light regime. This suggests that the promotive action of Pf is exerted on the radicle per se, because of some condition peculiar to it that distinguishes its growth behavior from that of the elongating seedling root.

V. EFFECTS OF CHEMICAL GERMINATION PROMOTERS

Dark germination of photosensitive lettuce seeds is promoted by gibberellic acid (GA3) at relatively low, and by thiourea at relatively high concentration. The concentration of gibberellin required for complete germination promotion of intact seeds is of the order of 100 mg/l, which is quite high, compared to the sensitivity of other gibberellin-responding systems. It may be reduced 10 to 100-fold by injection of the GA solution into the space between the endosperm and embryo (34), although injecting water alone by this technique raises germination from 2% to about 30%. This result suggests that high concentrations of gibberellic acid are required because of low rates of penetration of this compound through the endosperm.

Experiments with whole seeds.

It was found that the concentration of GA3 required to promote germination to 100% could be reduced about 4 orders of magnitude through the simple expedient of puncturing the seed. Dry seeds were pierced through the middle with a thin pin at a point about 0.4 seed-lengths from the radicle end. (If the puncture was made much closer to the cotyledonary end than this, control (water) germination was considerably raised, the embryo extruding itself through the cotyledonary end of the endosperm, i.e., exhibiting so-called "atypical germination" (36)). Twenty punctured seeds were placed in 0.07 ml of liquid contained in a shell vial of 14 mm inside

diameter, whose height had been reduced to about 8 mm for handling convenience. F was administered after 2 hours of imbibition to reduce as much as possible the contribution of Pf to subsequent germination. The incubation time was extended to 3 days, since the response to gibberellin seemed slower than that to red light (see also Figure 15). The effect of a concentration series of GA3 on germination of punctured seeds, compared with its effect on intact seeds treated in the same manner, is presented in Table 6. The response in the punctured seed is saturated at about 0.03 mg/l, that in the intact seed at about 200 mg/l. The enormous increase in sensitivity obtained by puncturing the seed is no doubt due in part to circumventing a diffusion barrier in the endosperm, but the creation of a large absorbing area (damaged tissue surrounding the puncture) in the vicinity of the embryonic axis probably facilitates the access to that region of constituents of the imbibing medium. Puncturing may also create structural weaknesses in the endosperm in regions other than in the immediate vicinity of the puncture. This is suggested by an increase in "atypical germination" of the controls if the puncture is made too near the cotyledonary end. Further, if oxygen is limiting because of a diffusion barrier in the endosperm (although this does not seem as likely), then puncturing should eliminate this condition. Thus, although control germination is the same in both intact and punctured seeds (Table 6), it should be emphasized that possible multiple effects of puncturing other than

Table 6

Effect of puncturing the seed on response to GA3. 180-seed samples of Grand Rapids, lot 1132, incubated 66 hours at 20°. Five minutes F given at second hour of imbibition.

<u>(GA3). mg/l</u>	<u>Percent germination</u>	
	<u>Punctured</u>	<u>Intact</u>
0	5	5
0.001	10	--
0.003	20	--
0.01	67	--
0.03	96	--
0.10	96	--
1	--	19
10	--	19
25	--	29
50	--	39
100	--	77
200	--	95

circumventing a diffusion barrier to gibberellin in the endosperm, while being in themselves insufficient to overcome the barrier to germination, might be additive to the action of gibberellin.

Since the source of conflicting reports in the literature regarding the ability of kinetin to stimulate dark germination might also be due to difficulty of passage of this compound through the endosperm, a germination test was carried out, using punctured seeds and kinetin at $2 \cdot 10^{-5}$ and $5 \cdot 10^{-5}$ M. The seeds were imbibed in the kinetin solutions in the dark at 20° for 2 hours, then given a 5-minute dose of F, to reduce the contribution from phytochrome. The plates were then returned to the dark for 58 hours. The results, presented in Table 7, indicate a very substantial promotion of germination. It will be noted that most of the germination effected by kinetin was atypical, in contrast to GA-induced germination, where the endosperm was ruptured at the radicle end of the seed.

Effect of germination promoters on the half-seed.

Since dark germination of photosensitive lettuce seeds has been shown to be promoted by gibberellic acid and kinetin, it was thought to be of interest to examine the effectiveness of these compounds in the half-seed system. Half-seeds were imbibed in 0.46M mannitol containing 10 mg/l GA3, then given F at the usual time. The clear-cut promotion by GA3 is similar in magnitude to that induced by R, but is delayed by at least six hours (Figure 15). This delay is probably not attributable to a suboptimal concentration of gibberellin,

Table 7

Effect of kinetin on 60-hour germination of punctured seeds following F at 20°. 300 seeds per sample. Lot 1132.

<u>Imbibition medium</u>	<u>Typical germination %</u>	<u>Atypical germination %</u>	<u>Total germination %</u>
Water	21	0	21
Kinetin, $2 \cdot 10^{-5}M$	34	43	77
" $5 \cdot 10^{-5}M$	29	55	84

Table 8

Rate of appearance of curvature in half-seeds of Grand Rapids, lot 1132, in 0.46M mannitol, with or without $3 \cdot 10^{-5}M$ kinetin, at 20°. Five minutes F was given at the second hour of imbibition. Sample size, two lots of 50 half-seeds.

<u>Hours from beginning of imbibition</u>	<u>Percent half-seeds showing curvature</u>	
	<u>Mannitol alone</u>	<u>Mannitol plus kinetin</u>
29	0	0
36.5	25	24
42.5	52	35

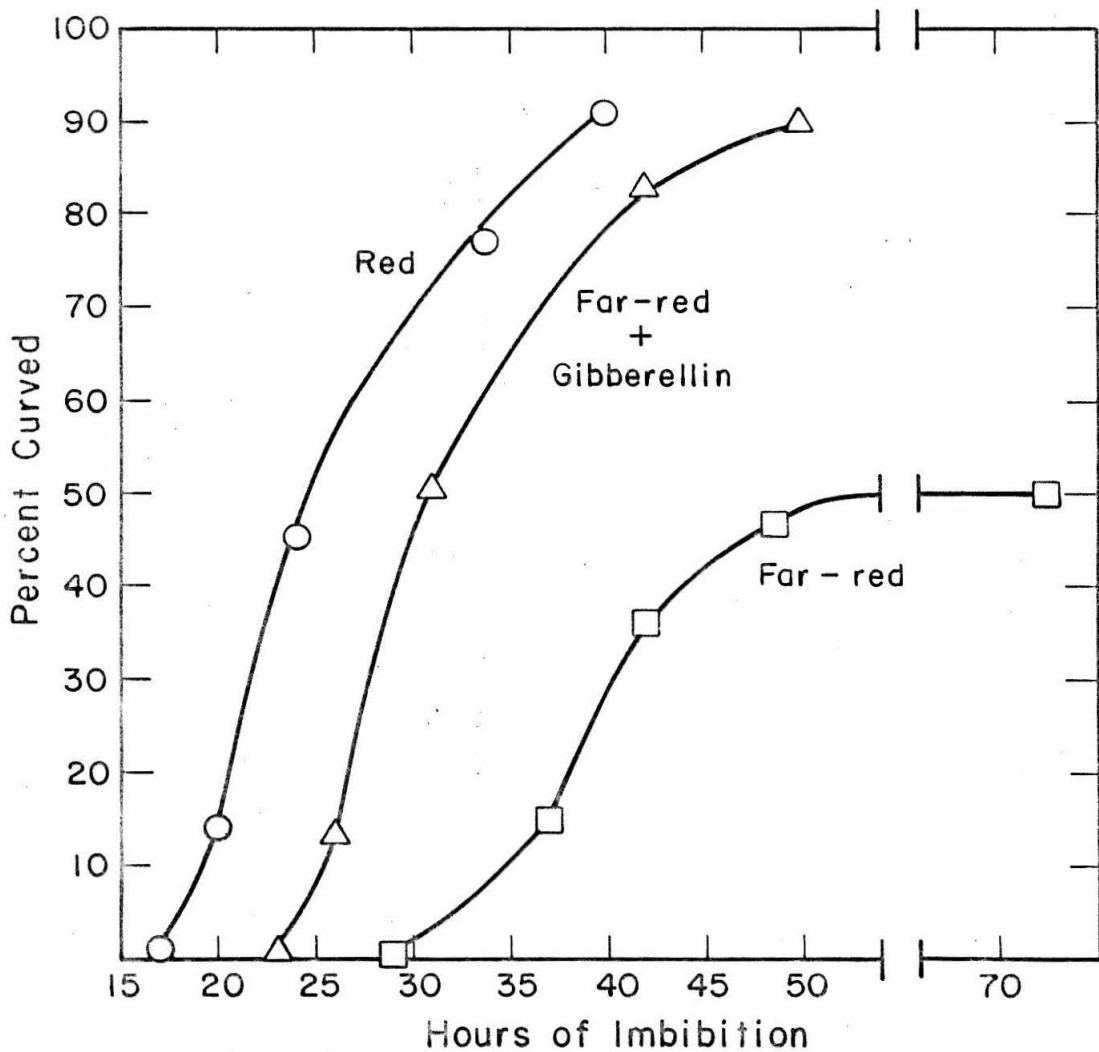


Figure 15. Effect of gibberellic acid on the time course of appearance of curvature in 0.46M mannitol at 20°. Sample size, 600. Grand Rapids, lot 1132

since the response of punctured seeds to the hormone is saturated at about 0.03 mg/l (Table 6). The delay constitutes additional evidence suggesting that the gibberellin effect is mediated by a different mechanism that is that of phytochrome.

The results of an experiment designed to detect a stimulatory action of kinetin in the half-seed system are presented in Table 8, and show that the only effect of kinetin here is an inhibitory one. This is in contrast to promotion of germination of punctured seeds via "atypical germination", where the endosperm is ruptured at the cotyledonary end of the seed. The conclusion that the germination-promoting action of kinetin is effected through the cotyledons and not the embryonic axis (35) is thus supported.

VI. TEMPERATURE EFFECTS

As explained in more detail in the introduction, there are two principal effects of temperature on lettuce seed germination. These are the promotion of dark germination by low temperature, and the inhibition of dark and light germination by high temperature. It is commonly thought that part of the high temperature effect is due to an increase in the rate of dark reversion of Pf to Pr, but that effects on other processes preceding radicle protrusion predominate. No mechanism explaining the promoting effect of low temperature has been advanced, but it has recently been speculated that low temperature acts at some point other than that controlled by phytochrome (37).

Temperature-dependent dark germination.

Whereas the dark germination of photosensitive lettuce seed at 26° is generally close to nil, substantial germination percentages may be achieved at lower temperatures. The relative response to low temperature varies from lot to lot, and increases with increasing age of the seed. The time course of dark germination at different temperatures was investigated to determine more fully the scope of this interesting response.

Seeds were sown at room temperature, 200 per dish, in a 9-cm petri dish containing two layers of filter paper and 3 ml of water. The dishes were sealed in plastic bags, placed in foil-covered trays, and removed immediately to a darkroom maintained at the appropriate temperature. Five-dish samples were removed periodically and the

germination percentage determined. The results are presented in Figure 16. Maximum germination at all temperatures save 11° seems to be attained by the second day. The relatively high variability of the values for 15° and 19° is not due to insufficient sample sizes, but can probably be attributed to variations in rate of attainment of temperature equilibrium amongst the individual dishes, especially considering the fact that at the next higher temperature (23°) there is practically no germination. The experiment was repeated for the 15 and 19° conditions, using sample sizes of 4000 seeds and taking the precaution of sowing in temperature-equilibrated petri dishes directly in the appropriate darkroom. Germination at the third day was approximately 24% at both temperatures, with no significant difference between the two.

Temperature dependence of the rate of appearance of curvature.

Temperature dependence of the initial rate of growth of the radicle was tested using the half-seed system. Half-seeds were placed to imbibe in water in the usual manner in temperature-equilibrated petri dishes in constant temperature darkrooms covering the range 19 - 27° . No light was given. The curvature response was determined after a 15-hour incubation period. The results (Table 9) clearly show that the rate of early growth of the radicle increases with temperature, at least up to 27° . This shows that the effect of low temperature on germination has nothing to do with an unusually low optimum temperature for radicle elongation.

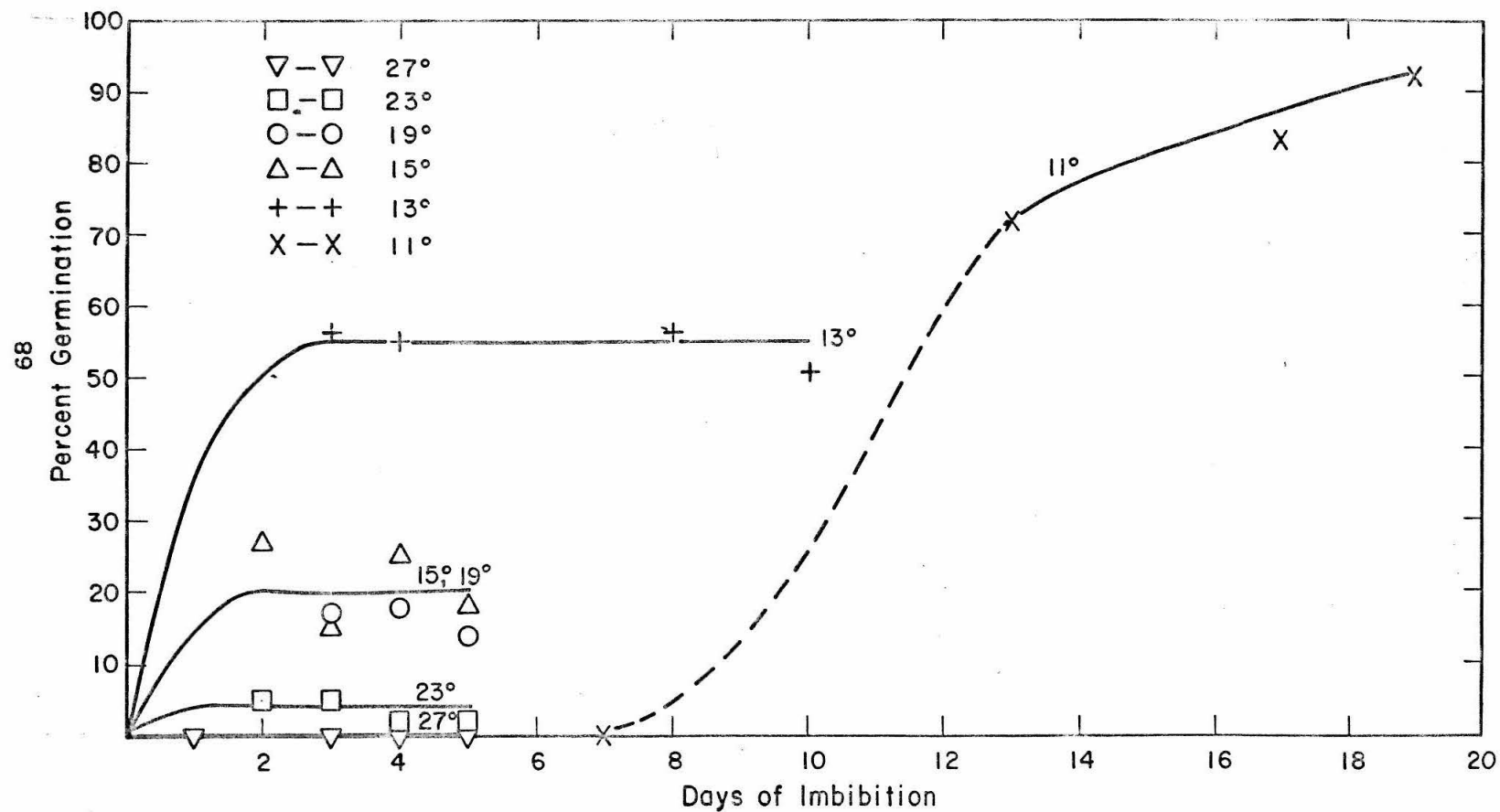


Figure 16. Time course of dark germination at different temperatures. Sample size, 1000. (rand Rapids, Lot 1132)

The effect of high temperature.

Dark germination of photosensitive lettuce seed at ordinary temperatures is very much reduced by prolonged preincubations at 30-35°, and pre-irradiation with R increases the duration of high temperature treatment required to reduce dark germination to a given level (73). After a 48-hour preincubation at 30° subsequent dark germination at 26° was reduced from 30% to 1.5%, which in turn was raised to only 12% if a 30-second dose of R was given after transfer to the lower temperature (62). Aphotoblastic varieties can be made to respond to R by holding the imbibed seed for several days at high temperatures (3, 19, 23, 73).

It was thought to be of value to obtain experimental data which would aid in the attempt to evaluate the effect of high temperature on dark reversion of phytochrome relative to the effect on "other processes". Some interesting features of the action of high temperature were revealed in the following way. Intact seeds were incubated on wet filter paper for 1.5 hours in the dark at 20°, then given R or F. Immediately after irradiation, some of the seeds were removed to a 37° room for 12.5 hours, while the rest were kept at 20°. At the end of the 12.5-hour period, the heat-treated seeds were returned to 20°; some samples from both temperature incubations were given no additional light treatment, others were re-irradiated with either R or F. Germination was counted after an additional 2 days at 20°, and the results appear in Table 10.

Table 9

Temperature dependence of the rate of appearance of curvature in water. Half-seeds were imbibed for 15 hours in the dark at the temperature indicated. Sample size, 400. Lot 1132.

<u>Temperature (°C)</u>	<u>% curved</u>	<u>S.E. (%)</u>
27	75	3.2
25	71	2.6
23	56	3.6
19	24	2.8

Table 10

Demonstration of inactivity of Pf at 37°. Intact seeds were dark-imbibed 1.5 hours at 20°, then given the appropriate light treatment. Some were then held at 37° for 12.5 hours, then returned to 20° and given a second light treatment; controls were kept at 20° throughout and given second light treatment at the same time as the heat-treated series. Treatment sequence codes: the first letter denotes the quality of the first irradiation; the numeral indicates the temperature of the 12.5-hour incubation; the second letter denotes the quality of the second irradiation given upon return to 20°. Lot 163R18.

Heat-treated

Treatment sequence:	R-37	R-37-R	R-37-F	F-37-F	F-37
Percent germination:	42.7	87.4	10.8	8.4	1.0
Standard error:	3.7	1.4	1.1	1.5	0.3

Controls

Treatment sequence:	R-20	R-20-F	F-20-F	F-20
Percent germination:	98.7	91.8	32.4	24.2
Standard error:	0.3	0.9	3.2	1.8

First of all, there is a deleterious effect of prolonged incubation at 37° which may have nothing to do with phytochrome, and which is reflected in reduced germination (relative to the 20° controls) in all heat-treated samples. Although it would be unreasonable to attempt to explain the high temperature effect solely in terms of effects on the pigment, part of the deleterious effect referred to might be due to partial denaturation of phytochrome.

Secondly, the temperature effect on dark reversion is quite pronounced, as evidenced by the doubling of the germination response evoked by the second irradiation with R immediately following the high temperature treatment (cf R-37 and R-37-R). A comparison of treatment F-37 and F-37-F reveals that the presumably small equilibrium amount of Pf remaining after F irradiation was probably completely removed during the heat treatment but was reinstated by the second F dose, which would be expected to re-establish photoequilibrium between Pf and Pr. The higher germination in 20° -held seeds given a second F-irradiation may be real, but is not statistically significant because of the high variance of that sample.

The most interesting point is brought out by the fact that there is no difference in the germination response obtained after treatments R-37-F and F-37-F. This shows that Pf cannot function at 37° , even though by the end of the heat treatment there is still enough Pf present to effect a substantial germination after return to 20° . The curtailment of Pf action by high temperature may be the result

of a direct effect on the pigment, e.g., (reversible) inactivation by changing the tertiary structure of the protein moiety, or else an indirect effect brought about by modification of some other process upon which the activity of Pf depends, but these two possibilities cannot be distinguished by these results. However, concerning the establishment of thermodormancy, it is apparent that much of the effect of high temperature, commonly relegated in the literature to debilitatory effects on processes not associated with phytochrome, actually depends on the fact that Pf does not function for the duration of the high-temperature treatment.

Evidence for phytochrome mediation of the low-temperature effect.

Dark-imbibed lettuce seeds probably always contain a certain proportion of Pf, since germination is usually reduced by exposure to F (Table 1). In addition, it has been established (42) that a small but significant fraction of the phytochrome is present as Pf, even after equilibrium dosages of F of high spectral purity. It was considered that the promotive effect of low temperature on dark germination might be in part or wholly due to retardation of dark transformation of this Pf residue.

This hypothesis was tested in the following manner. Seeds were imbibed in the usual way for 2 hours at 20°, then given a saturating dose of F to lower the ratio Pf/Pr to a constant value. The plates were set inside plastic bags in foil-covered trays and placed in a room kept at 37°. Trays were removed after 2, 4, 6, 8, 10, and 20

hours, cooled briefly in a 5° coldroom, then removed to a darkroom where half of each set was given a second saturating dosage of F, while the other half remained in the dark. Immediately after this, the dishes were placed in an 11° darkroom for 17 days, after which germination percentages were determined. Each sample comprised 5 dishes, each of which contained 200 seeds. Dark and F-treated controls received no 37° treatment but were placed at 11° directly after illumination. The results are presented in Table 11. As before (Figure 16), germination in the dark controls was very high, while that in the F-treated controls was lower, but still substantial. For the seeds not receiving a second dose of F following the heat treatment, a 2-hour heating seems sufficient to reduce subsequent germination to a minimum (the difference between the 2 and 4-hour samples is not statistically significant). Further reduction of germination by heat treatments exceeding 4 hours is probably a reflection of effects of high temperature other than that on Pf reversion.

The second dosage of F caused a reversal of the high temperature effect for all durations of heat treatment. The promotion by heat treatments up to 4 hours was not expected but may be due to acceleration of metabolic processes preceding germination. Acceleration of the action of Pf itself is ruled out by the experiment of Table 10. The main point is that F (which ordinarily reduces germination) promotes germination when administered under conditions in which

Table 11

Germination of Grand Rapids, lot 1132, after 17 days at 11° following high temperature and light treatments, as described in the text.

<u>Hours at 37°</u>	<u>Percent germination</u>		
	<u>Controls</u>	<u>Second dose F</u>	<u>No second dose F</u>
0 (Dark control)	82.7	--	--
0 (Single F control)	32.2	--	--
2		56.3	2.7
4		59.3	2.1
6		36.1	0.4
8		19.6	0.7
10		11.9	0.4
20		1.9	0.0

phytochrome may be presumed to be exclusively, or nearly so, in the red-absorbing form. It is concluded that the germination-promoting effect of low temperature depends on the presence of Pf, and that this effect is most probably achieved through delay or prevention of transformation of Pf to an inactive form.

Pf as the active form of phytochrome in lettuce seeds.

It is commonly assumed that Pf is the physiologically active form of the pigment and that Pr is inert. This assumption is based largely on the argument that conversion of a small proportion of Pr to Pf evokes a large physiological response in certain materials (28). However, apparently 50% pigment conversion is required for a half-maximal germination response in lettuce seed (28). Granting this, the possibility may be entertained that in lettuce seed Pr is the active form and functions as a suppressor of germinability. The very existence of decay in photosensitivity with time of dark imbibition (3, 34, 35, 36, 37, and Figure 2) could well be attributed to some positive inhibitory action of Pr, especially considering the fact that the decay rate increases with temperature (37).

However, the data of Table 11 can be invoked as evidence against this hypothesis. Firstly, under the experimental conditions, a large response is obtained to what must be a relatively small proportion of Pf. In the second place, the substantial increase in response obtained with relatively short periods at an elevated temperature (provided the second dosage of F is given), while probably attributable

to processes other than phytochrome activity, certainly cannot be the result of depression of a positive inhibitory activity of Pr (by irreversible thermal inactivation, for example). The reason for this is that the effect depends on the second irradiation, which would only serve to further decrease the Pr concentration, provided dark (thermal) reversion takes place in the usual direction. Further, and for the same reasons, the effect of low temperature cannot be ascribed to greater reduction of Pr inhibitory activity relative to reduction in the rates of other metabolic processes necessary for growth and germination.

VII. EFFECTS OF REPEATED FAR-RED IRRADIATION

In the preceding section, it was concluded that the promotive effect of low temperatures on dark germination may be ascribed to a retardation of the disappearance of physiologically active phytochrome. This conclusion suggests that high germination may be achieved at ordinary temperatures, provided a small amount of Pf (the amount obtaining after a saturating F dose) operates continually for a long enough time. Attempts to induce lettuce seed germination with continuous or repeated administration of F reported in the literature (38, 74) resulted either in no effect or in depression of germination. However, Downs (11) recently showed that maximum germination of light-sensitive seeds of certain bromeliads, which exhibit typical phytochrome-controlled germination, can be obtained in continuous F. This finding supports the expectation that lettuce seed, given the proper conditions, may also be induced to germinate by repeated or continuous irradiation with F.

Whole seeds.

Because of the difficulty in controlling seed temperature under prolonged F irradiation with the equipment used in the present investigation, and because of the strong inhibitory effect of such a light regime reported in the literature (see Introduction), continuous F was not used. Instead, attempts to stimulate germination by repeated doses of F were carried out for 6-day incubation periods. In one experiment, performed at 2 different temperatures, 2 sets of seeds

were imbibed for 2 hours and given an initial 5-minute F dose. One set was given additional 5-minute F-irradiations after 6, 10, 12, 21, 25, 28, 39, 48, 55, 68, 71, 77, 81, 94, 102, and 118 hours. The results appear in Table 12. At the higher temperature, there was no difference in the effect of the 2 different treatments. At the lower temperature, repeated irradiation with F resulted in a small but significant depression of germination.

In another 6-day experiment, conducted at 20° only, the initial 5-minute dose of F was preceded 4.5 hours by a 10-minute R irradiation (the 4.5 hours in Pf was expected to raise the ultimate germination capacity to the point where small positive differences induced by repeated F-irradiation might become detectable), and additional 2-minute doses of F were given at 12, 24, 29, 35, 48, 54, 60, 72, 80, 96, 101, 108, 120, 132, and 144 hours after the initial. In this experiment, there was also no significant difference in germination; 48.6%, S.E. 2.6% for the once-irradiated seeds, and 43.3%, S.E. 1.8% for those receiving repeated doses.

The half-seed in mannitol.

It had been observed that the effects of occasional brief examination in white light followed immediately by F in the half-seed in mannitol system was to raise the ultimate response (percent curved) to a level far above that of those receiving only the usual single irradiation. This observation prompted the use of the half-seed system in an attempt to detect stimulation by repeated F treatment.

Table 12

Failure of repeated irradiation with F to promote germination at 2 different temperatures. Sample size, 500. Incubation time, 6 days. Lot 1132.

<u>Temperature</u>	<u>Treatment</u>	<u>% germination</u>	<u>Standard error (%)</u>
20°	Single F	17.4	1.6
"	Repeated F	11.8	1.1
25°	Single F	5.8	1.2
"	Repeated F	5.0	0.5

Table 13

Demonstration of increase in half-seed growth potential by repeated irradiations with F. Incubation time, 5 days at 20°. Sample size, 550. Lot 1132.

<u>Treatment</u>	<u>Percent curved</u>	<u>S.E.</u>
Single F	47.3	3.0
Repeated F	77.3	1.8

Half-seeds were imbibed in mannitol and given an initial F dose in the usual manner. One set was then darkened by wrapping in aluminum foil. Thereafter, both sets were exposed to 5 minutes F at 19, 24, 45, 53, 65, 89, and 103 hours from the beginning of imbibition, and counts of the coded samples were made after a total of 5 days' incubation. The results are shown in Table 13. As is usual for this seed lot (Figure 15, Figure 18, Table 15), the final level of curvature response to a single F irradiation was about 50%. The substantial increase in response of the repeatedly irradiated material supports the hypothesis that small amounts of Pf allowed to act for extended periods of time do contribute to an increase in growth potential, and gives impetus to the expectation that, under some set of conditions, intact lettuce seeds may likewise be induced to respond to repeated F treatment with increased germination.

VIII. LOSS OF DARK DORMANCY DURING DRY STORAGE

Grand Rapids lettuce seed gradually loses dark dormancy upon prolonged dry storage under ordinary conditions. In fact, this phenomenon is the basis for the commercial practice of withholding seed of this variety from the market for quite some time after harvest. This storage effect, analogous to after-ripening, may owe its expression to one or more of the following causes:

1. An increase in the ability of the embryo to expand against external restraint. This could be realized by the gradual loss or destruction of some chemical inhibitor of growth within the embryo.

2. An increase in the ratio Pf/Pr in the dry seed. It has been reported that the dark germination of Grand Rapids seeds stored in the light is significantly higher than that of dark-stored seeds, provided the relative humidity is between 60 and 70%. At lower humidities, the difference is not manifested (18). It has also been demonstrated many times that seeds become responsive to irradiation long before imbibition is complete. Although seeds are not commonly stored in the light or at high humidity, it is obvious that if storage conditions are such that permit an increase in the amount of Pf within the seeds, then dark germination will be increased.

3. A decrease in the ability of the endosperm to restrict embryo expansion. It is quite conceivable that the mechanical integrity of the (subsequently imbibed) endosperm could be weakened by processes probably best referred to as "weathering", but to be more

specific, weaknesses produced by, for example, autoxidation or through stresses induced by fluctuations in relative humidity and temperature. The appearance of such weaknesses in the endosperm, particularly in the intercellular cementing substance in the region of the radicle, would be expected to contribute to an increase in germinability.

An increase in dark germination of seeds (lot 1132) stored in the laboratory under ambient conditions of temperature, humidity, and occasional illumination, was first observed approximately 18 months after harvest. Figure 17 reveals the progress of dormancy loss evinced by an increase in dark germination at 20° from 8% to 78% over a 4-month period. Over the same period, germination of F-irradiated seeds increased from 2.5% to 21%. This excludes the simple explanation that an increase in the Pf/Pr ratio in the seeds is the sole factor responsible for dormancy loss, but does not rule out such photoconversion as a possible contributing factor.

Effect of storage at low temperature.

A sample of seeds of the same lot, stored in the same type of container (plastic screwcap bottles), was placed in a 5° coldroom for a period of 6 months beginning about 7 months after harvest. This was not done with an experiment in mind, but in anticipation of loss of dark dormancy, with the hope that cold storage would delay this process. The germination behavior of this sample some time after removal from the coldroom is compared with that of the

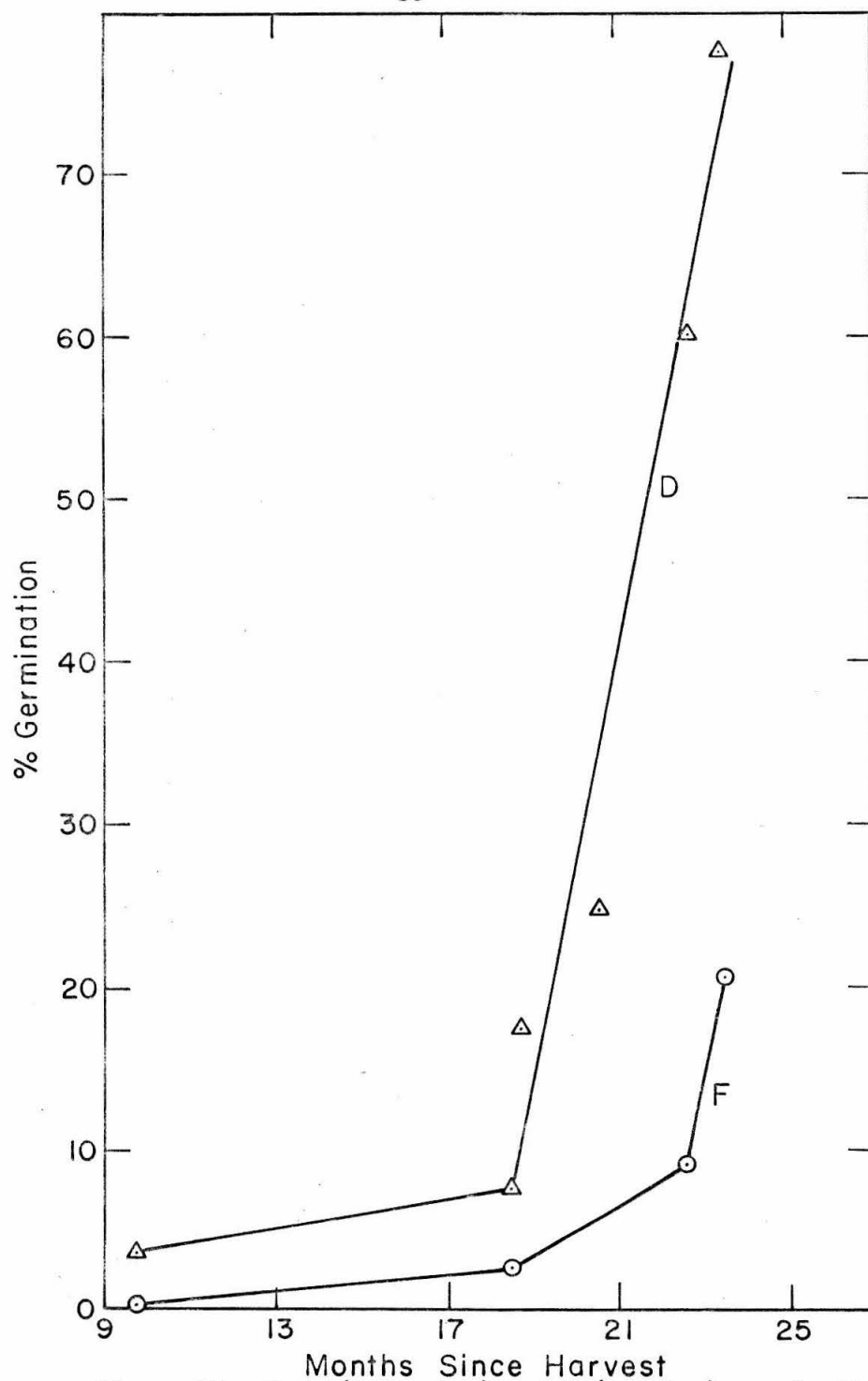


Figure 17. Dark (triangles) and F (circles) germination at 20° as a function of storage time at room temperature. Lot 1132.

Table 14

Germination of 2-year-old seeds of Grand Rapids, lot 1132, stored at room temperature. Incubation was for 3 days at 20°.

<u>Storage temperature</u>	<u>Percent germination</u>	
	<u>Dark</u>	<u>F-irradiated</u>
Ambient	77.8	20.6
Ambient, interrupted by 6 months at 5°	51.2	15.9

Table 15

Curvature response of after-ripened half-seeds of Grand Rapids, lot 1132, after 94 hours dark incubation in 0.46M mannitol at 20°. F or R was given at the second hour of imbibition.

<u>Light treatment</u>	<u>Percent curved</u>	<u>Standard error</u>
R	96.8	1.06
F	50.4	2.04

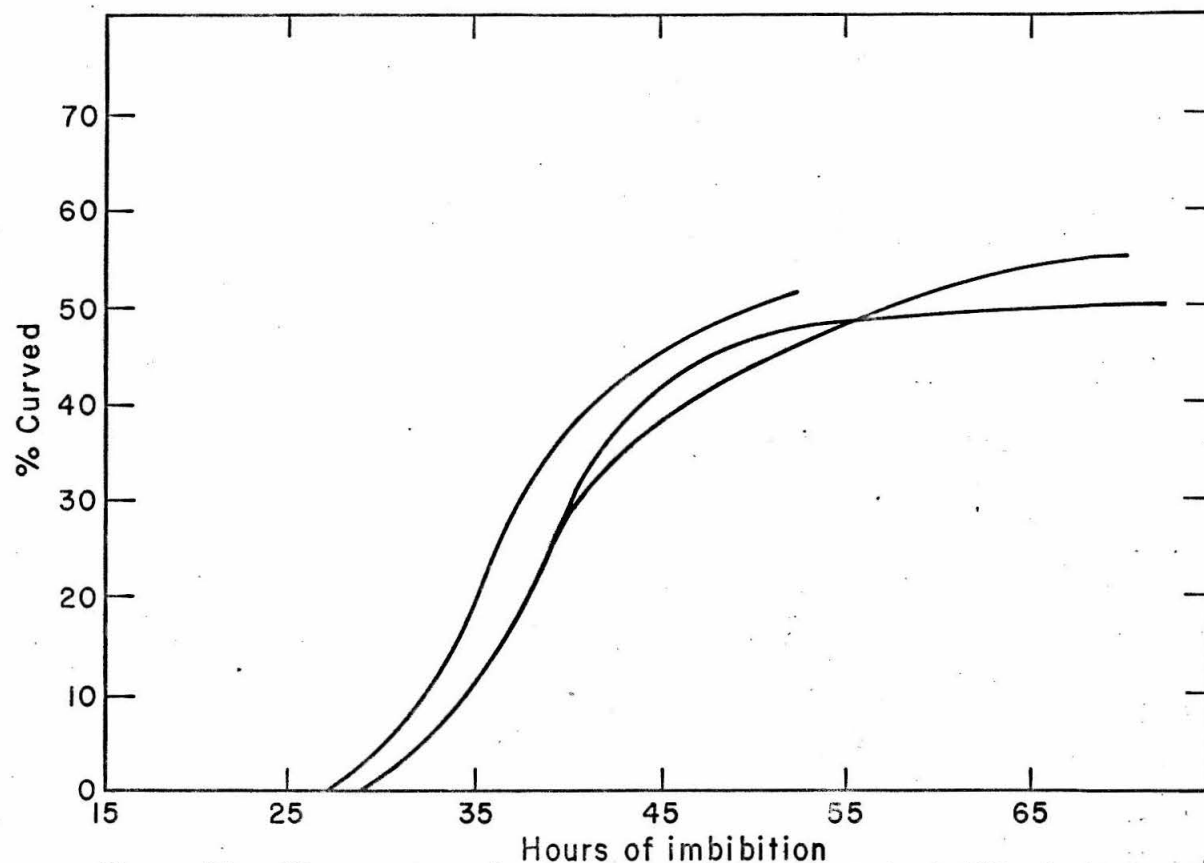


Figure 18. Time course of appearance of curvature in 0.46M mannitol, 20°. Three separate experiments utilizing seeds of lot 1132 before the beginning of loss of dark dormancy. Sample size, 400.

laboratory-stored supply in Table 14. The sample size for each treatment was 1000 seeds and the differences are very highly significant (t is 12.5 for F-irradiated seeds). While a definitive statement as to the cause for the difference in loss of dark dormancy must await a controlled experiment, it may be tentatively concluded that low temperatures prevent or delay this loss.

The growth potential of after-ripened seeds.

The behavior of these after-ripened seeds in the half-seed in mannitol curvature system was investigated with the idea that any internal changes in the embryo that increase germinability should be reflected in an increase in growth potential of the axis. Half-seeds were prepared and irradiated with R or F in the usual manner and the (presumed) ultimate percent curvature was determined after 94 hours of imbibition at 20°. The results appear in Table 15. The close agreement of the 50% maximum after F with the results of 3 independent curvature time course experiments conducted before the loss of dark dormancy (Figure 18) shows that the pronounced changes in germinability of the seed that occurred during post-harvest storage were not paralleled by any detectable increase in the growth potential of the axis. This result indicates indirectly that the endosperm is the site of changes responsible for after-ripening.

IX. SUMMARY

In order to be able to relate the results of subsequent experiments to the experimental data and conclusions reported in the literature, the behavior of the seed sample used in most of the experimental work described herein was determined under a set of carefully controlled conditions. In agreement with many reports in the literature, depression of dark germination by a brief irradiation with far-red light was observed, which indicates a certain proportion of the phytochrome of the dark-imbibed seed is present in the far-red-absorbing form. The phenomenon of "escape from photocontrol" by far-red, that is, the decreasing effectiveness of far-red in reversing the effect of an initial irradiation with red as the dark interval between the two light dosages is increased, was observed to correspond closely to previous reports. A decrease in the germination response to a standardized degree of phytochrome activation after prolonged dark pre-irradiation incubation was noted, which corresponds to earlier published reports of "decay of photosensitivity" with increasing imbibition time.

With the aid of osmotic inhibition of germination it was shown that full promotion of germination by phytochrome can take place under conditions where rupture of the endosperm by the radicle was prevented by limiting water uptake. Using a similar technique, and observing germination rates after transfer from the osmoticum to water, it was possible to demonstrate that Pf continues to exert its effect beyond

the point of complete germination promotion.

The elongation rate of embryos from which the endosperm has been removed has been reported to be the same after far-red as after red irradiation. This apparent lack of an influence of phytochrome on the growth behavior of lettuce seed radicles was confirmed by the observation that de-endospermed embryos attain the same length during a 23-hour incubation in water following irradiation with either effective wavelength. However, when de-endospermed embryos were incubated in an osmoticum (mannitol) instead of water, a very marked increase in the ability to expand was evoked by irradiation with red light. Thus, a phytochrome-dependent enhancement of the capacity of the embryo to expand against an externally imposed restraint (growth potential) in the absence of any contribution from the endosperm was demonstrated for the first time.

For subsequent, more detailed, studies of changes in growth potential, a system using the axial half of the seed was devised. In the half-seed, the appearance of a geotropic curvature in the radicle tip, accompanied by extrusion of the half-embryo from the surrounding layers, can be taken as a criterion that growth has taken place. It is inherent in this qualitative criterion that a certain amount of axis elongation precedes the appearance of curvature and extrusion. For time course studies, quantitation of this response was accomplished by recording the proportion of a given sample that had undergone

curvature as a function of time. Using this system, it was possible to demonstrate a striking acceleration of the rate of appearance of curvature in osmotica by red light, relative to the rate of the response of half-seeds irradiated with far-red. However, the phytochrome-dependent rate of appearance of curvature in half-seeds was evident in the absence of any osmotically active solute; this demonstrated the superior sensitivity of the half-seed system over the de-endospermed entire embryo. As in germination of the intact seed, the rate of response in unirradiated half-seeds was intermediate between the rates observed after irradiation with the two antagonistic wavelength regions.

Studies on the linear growth rate of half-seeds revealed that the red light-enhanced rate of appearance of curvature in osmotica was paralleled by a similar, phytochrome-dependent enhancement of the straight growth rate during the initial period of elongation. Once elongation had proceeded beyond a certain small initial value, the rate of further growth was found to be phytochrome-independent. Thus, in photoblastic lettuce seeds, the radicle of the embryo can be distinguished from the elongating root of the germinated seed by its capacity to undergo a phytochrome-dependent increase in the ability to expand against an external restraint.

Half-seeds of an aphotoblastic variety also showed a clear differential response to red and far-red, although of much smaller

magnitude than that in photoblastic seeds. This slight but significant response suggests that the photoblastic seed is not qualitatively distinct from the aphotoblastic seed, but differs in some quantitative way.

The rate of appearance of curvature was shown to depend on the continued action of Pf, in agreement with the analogous phytochrome-dependent enhancement of germination rate of the intact seed after transfer from osmoticum to water.

It is commonly assumed that the requirement of an intact endosperm for demonstration of light sensitivity in photoblastic lettuce seed is based solely on a mechanical restriction of embryo expansion by this tissue. Another equally valid explanation for the endosperm requirement is the possibility that this tissue in addition controls embryo expansion through the secretion of a growth inhibitor. This possibility has not been eliminated in any of the experimental results reported in the literature. Using the half-seed system, where mechanical restriction by the endosperm has been eliminated, it was shown that the naked half-embryo is as fully responsive to red and far-red as is the half-seed, in which the embryo is still in close contact with the surrounding layers. This ruled out secretion of inhibitory materials as a possible attribute of the endosperm.

It was found that gibberellic acid induces a response in the half-seed system equal in magnitude to that induced by red light. However, the response to gibberellin was considerably delayed relative

to that to red light. This was interpreted as additional evidence for the hypothesis advanced in the literature that gibberellic acid and red light promote germination in the intact seed by different mechanisms.

It was found that the germination-promoter kinetin does not stimulate the half-seed response. This constitutes direct evidence that kinetin promotes germination by a different mechanism than does gibberellin (or red light), and indirect, independent evidence supporting the proposal advanced in the literature that the locus of action of kinetin is in the cotyledons, not in the axial portion of the embryo.

The concentrations of gibberellic acid required for complete promotion of germination as reported in the literature are unusually high considering the remarkable potency of this compound in most other gibberellin-responding systems. This reported lack of sensitivity of whole seeds in response to gibberellin was confirmed in the present studies. However, it was found that puncturing the seed reduced the optimal gibberellin concentration 4 orders of magnitude, indicating the existence of a diffusion barrier to this compound in the endosperm. Similarly, the use of punctured seed resolved conflicting reports in the literature regarding the efficacy of kinetin as a germination-promoter, and indicated the endosperm as a barrier to the accessibility of this compound to the embryo.

The well-established fact that dark germination is promoted by

low temperature treatment was confirmed, using a wide range of temperatures and incubation times. Ultimate germination percentages increased with decreasing incubation temperature, throughout the entire range; the rate of attainment of maximal germination was lowest at the extreme low-temperature end of the range. Using the half-seed system, by the criterion of rate of appearance of curvature in water in the dark at different temperatures, it was found that this rate increased with the temperature of incubation throughout the range employed. This shows that promotion of dark germination by low temperatures cannot be attributed to an unusually low optimum temperature for early growth of the radicle.

It was shown that low temperature lacks any promotive effect on lettuce seed germination provided precautions are taken to maximally reduce the Pf/Pr ratio in the seed. Under these conditions, irradiation with F increases germination by establishing a certain, increased ratio of Pf/Pr. From these results it can be concluded that promotion of germination by low temperature depends on the presence of at least a small fraction of the phytochrome in the far-red-absorbing form, and that the action of low temperature consists in delaying or preventing dark transformation of physiologically active phytochrome to an inactive form.

The principal argument advanced in the literature in favor of Pf as the active form of phytochrome, the evocation of a large photomorphogenic response to photoconversion of small fractions of the

pigment to this form, does not hold well for photoblastic lettuce seed germination, since it has been estimated that roughly 50% conversion to Pf is required for a 50% germination response. Thus, it is possible that in lettuce seed Pr is the active form and functions as a suppressor of germinability. But it was concluded that the sole or at least the principal effect of low temperature is a direct one on delay of dark transformation of Pf. The demonstration that the equilibrium concentration of Pf after far-red irradiation, which is almost certainly only a small fraction of the total phytochrome, can indeed evoke a large germination response, provided dark transformation is delayed, constitutes good evidence for the assumption that Pf is the active form in lettuce seed.

The inhibitory effects of high temperature were studied, with the purpose of determining what part, if any, phytochrome plays in prevention of germination by high temperature and establishment of thermodormancy. Using a combination of temperature and light treatments, it was shown that Pf cannot function at 37°. It was not determined whether or not this effect of high temperature is a consequence of some temperature sensitivity of phytochrome itself, but knowledge of the fact is important to the interpretation of thermodormancy, and might prove to be of potential value in the elucidation of the mechanism of action of the pigment.

The observation that a relatively small proportion of total phytochrome present in the far-red-absorbing form (the proportion

obtained after photoequilibration with F) can effect a substantial germination response at low ambient temperatures, prompted re-investigation of the effect on germination of repeated irradiations with F administered over long-term dark incubation periods at ordinary temperatures. In agreement with earlier reports in the literature, no stimulation of germination by such repeated irradiations was observed. However, a very striking enhancement of the response in the half-seed system was obtained under such a regime. This demonstrated that small amounts of Pf acting over a relatively long time interval do substantially contribute to an increase in the growth potential of the embryonic axis, and lends weight to the expectation that a similar response in the intact seed may be realized, given the proper conditions.

The loss of dark dormancy during prolonged post-harvest storage of the dry seed, a phenomenon well documented in the literature, was observed to occur in one seed lot. The possibility that a light-induced increase in the Pf/Pr ratio in the dry seed was solely responsible for dormancy loss was ruled out. There was no detectable increase in the growth potential of the embryonic axis of such seeds, as demonstrated by the curvature response of the half-seed in osmoticum. This indirectly infers that the endosperm is the site of changes responsible for loss of dark dormancy during storage. It was observed that storage of the dry seed at a reduced temperature delayed or prevented dormancy loss.

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