ACTION OF GIBBERELLIC ACID ON LETTUCE SEED GERMINATION [1, 2]

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Several workers have shown that gibberellic acid (GA_3) promotes the germination of seeds which ordinarily require light (6, 10, 13, 14, 16, 17, 23, 28, 30). This phenomenon raises several fundamental questions as to the mechanism of light-controlled germination: A: Does light simply act by releasing a gibberellin in the seed, B: Does the GA_3 substitute for light by bringing about chemically a reaction which is normally photochemical, or C: Does the GA_3 bring about some quite other type of growth process which independently leads to germination? The following experiments are an attempt to provide answers to these questions.

MATERIALS AND METHODS

The seeds used for germination studies were those of *Lactuca sativa* L. var. Grand Rapids (Breck's Co., Boston, Mass.). For some of the gibberellin extraction work the seeds were supplied by Pieters Wheeler Seed Co., Gilroy, Cal. Since germination in the dark was found to be gradually induced even in dry seeds, when stored at room temperature and humidity (cf. 5), special precautions were taken as to the time of purchase of the seeds and their storage. Seeds purchased at the end of January, 1959, and kept in the cold room at 4° C thereafter, showed less than 5 % germination in darkness. These were used for all quantitative experiments. They were taken out of the cold room as needed, allowing the shortest possible time of exposure to room temperature and humidity.

The ordinary method in which seeds are placed on wet blotter or filter paper gave markedly fluctuating results. For this reason the present work was carried out by shaking the seeds in solution without filter paper. Throughout the periods of imbibition and germination, the seeds were placed in 5 cm Petri dishes with the solution, and gently shaken on a rotating shaker in the dark at 25° C. Three milliliters of solution was added to each Petri dish, when 100 seeds were used, and 2 ml when 50 seeds or less were used. With this method, the seeds are treated with a precisely measured volume of water, thoroughly wetted and aerated; correspondingly the results have been much more uniform, although the overall averages, especially for the first 2 hours, are not much changed by the filter paper.

For the introduction of solutions by injection, 25 seeds whose fruit-coats had been removed were imbibed for 15 minutes on double layers of wet filter paper wrapped around two sheets of slide glass placed in a 9 cm Petri dish, and the solution was injected from the cotyledon end with a fine needle (Vita 30, Vita Needle Co., Needham, Mass.) in between the endosperm and the embryo. The endosperm layer (the innermost covering of the seed) separated from the embryo and swelled due to the liquid injected. The criterion of success in injection was that the liquid should penetrate as far as the radicle tip of the seed. According to this criterion, the reliability of the technique was more than 80 %. To complete this procedure on 25 seeds required 7 to 10 minutes. Injection was carried out at 4° C under the green safe-lamp, and the seeds were then transferred to darkness at 25° C for germination.

Gibberellic acid (GA_3) was used as a stock 100 ppm solution in distilled water, and dilutions were prepared from this just before use.

The red light source was a 100 watt incandescent lamp filtered through a Corning Signal Red glass filter (50 % transmission at 630 mμ), and the source for far-red light was a General Electric 150 watt reflector flood lamp filtered through the Corning glass filter No. 7–69 (2600) (50 % transmission at 742 mμ). Both these filters are of the steep cut-off type. An interference filter with peak transmission at 735 mμ was also employed for a few experiments, but because of its small size was found inconvenient to use. The lamps were placed 25 cm above the level of the seeds. Necessary manipulations which required light were carried out under a green safe-lamp (20 watt white fluorescent lamp wrapped by double layers each of yellow and green cellophanes). In a few cases the germination occurred in an atypical manner; these are denoted by numbers in parentheses in the tables.

RESULTS

I. NORMAL COURSE OF GERMINATION. Preliminary experiments were carried out to find the amount of moisture which must be absorbed to allow germination under the conditions employed. For this purpose the fresh weight of the seeds was determined at various periods after the beginning of imbibition at 25° C. One group of seeds was induced to germinate with 2 minutes exposure to red light at 1.5 hours after the beginning of dark imbibition, and the other groups were kept in darkness throughout. For comparison with the imbibition data, the extent of germination which had been induced by 1 minute irradiation with red light at various times after the beginning of imbibition was also determined. The results are shown in figure 1; each reading was the average of two runs.

Curves A and B show that light does not stimulate the uptake of water by the seeds, but shortly after visible germination begins a marked increase in

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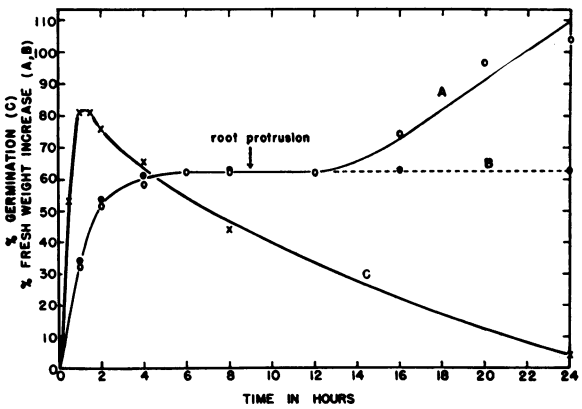


FIG. 1. Percent increase of fresh weight (A and B) during the course of imbibition and germination. A: Seeds induced to germinate with 2 minutes red light at 1.5 hours after the beginning of imbibition. B: Seeds kept in darkness. C: Effect of 1 minute irradiation with red light, given at indicated times, on germination examined 48 hours after the light treatment.

weight occurs; this parallels the elongation of the seedling (cf. fig 4). Although 1 minute of exposure to the red light is not sufficient to induce full germination at any period of imbibition, it is clear that the seeds are most sensitive, showing a single peak, at about 1¼ hours from the beginning of imbibition, when water uptake is still taking place actively and the water content of the seeds has reached about 40 % of their original weight (figure 1C). This observation indicates that seeds need not be in the fully turgid state for maximum germination to be induced.

As is well known, exposure of the seeds to far-red light inhibits germination (2, 4, 8, 9). However, Borthwick et al (2) showed that seeds which had been induced to germinate by red light escaped the inhibitory effect of far-red irradiation, if the far-red was given a sufficiently long time after red irradiation. Figure 2 shows a detailed study of this effect. The seeds were exposed for 2 minutes to red light after 1.5 hours (Curve A) or 8 hours (Curve B) from the beginning of dark imbibition, then given 2 minutes far-red light at various intervals after the red treatment. An average of two readings is plotted in this figure.

The curves of figures 2A and 2B agree fairly well in general shape, except that figure 2B tends to show a longer lag period, slower rate of escape, and less germination at the maximum level. After the first sign of root penetration, the curves reach the plateau, i.e., complete escape from far-red inhibition (see arrows). In comparison with figure 2, Borthwick et al found that the initial lag period continued up to 3 hours and their seeds became gradually less sensitive up to about 15 hours (fig 9 of ref 2). The markedly slower timing is probably due to differences in the experimental method as discussed below. However, their curve essentially coincides in its shape with those reported here.

As shown in figure 1C, at 1.5 hours after dark imbibition the seeds are in a state of maximum sensitivity and by 8 hours they have become much less responsive to red light. Some fluctuations in the data of figure 2B may reflect the gradual decrease in sensitivity due to the long imbibition period, which by the end of the experiment totals 24 hours.

II. EFFECT OF GIBBERELLIC ACID. That lettuce seeds are as responsive to GA_3 in the dark[3] as has been reported in the literature (6, 10, 13, 14, 17) is shown in figure 3, which includes data of two experiments. In one case the concentration of GA_3 needed for 50 % germination in the dark is approximately 28 ppm and maximum germination is obtained at 60 ppm; in the second experiment 50 % germination was produced by 20 ppm and 100 % at between 50 and 100 ppm. In both cases controls in water gave less than 1 % germination.

When the seeds are shaken with the solution, only a small amount of GA_3 may be taken up, so that the actual sensitivity is doubtless higher than that shown above. For this reason the action of GA_3 was also tested by direct injection into the seeds. The method of injection has been described above. In table I the results of three series of experiments are summarized. Unfortunately with this method many control seeds (injected with water) germinate, but the results are still fairly clear; maximum germination can be obtained with a concentration as low as 1 ppm. This point will be referred to in section V below.

It is well known that GA_3 has a marked effect on elongation of shoots (see 3, 26). However, Evenari et al (6), who made measurements of the hypocotyls and roots of lettuce seedlings 72 hours from the start of soaking, concluded that GA_3 had no effect on the

[3] Minimum green light was used briefly for the manipulations.

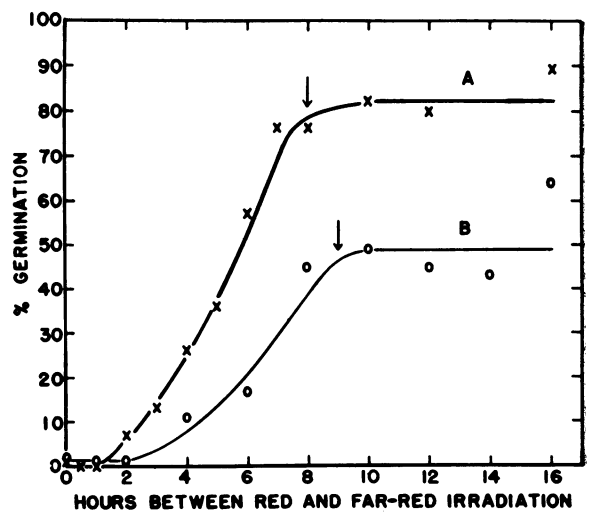
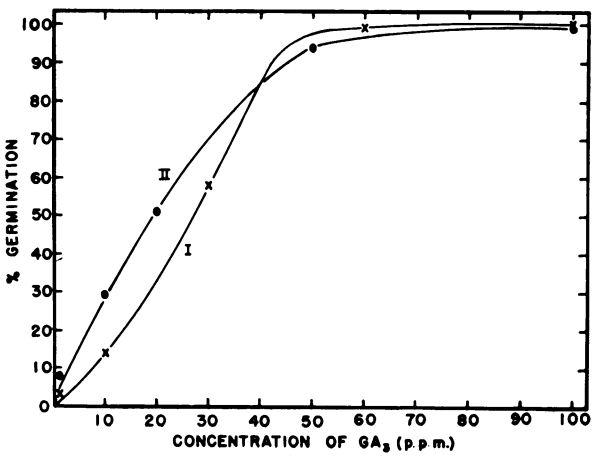
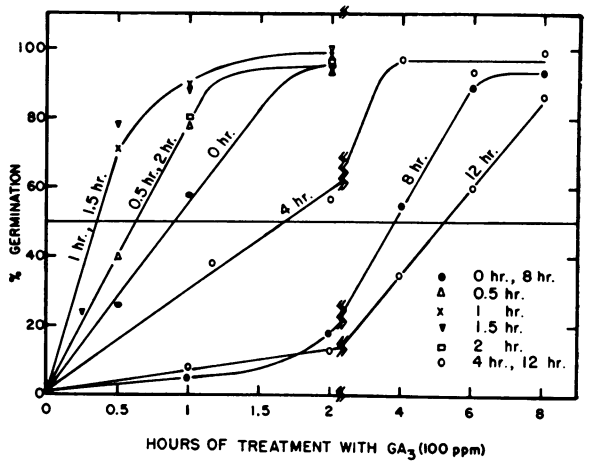


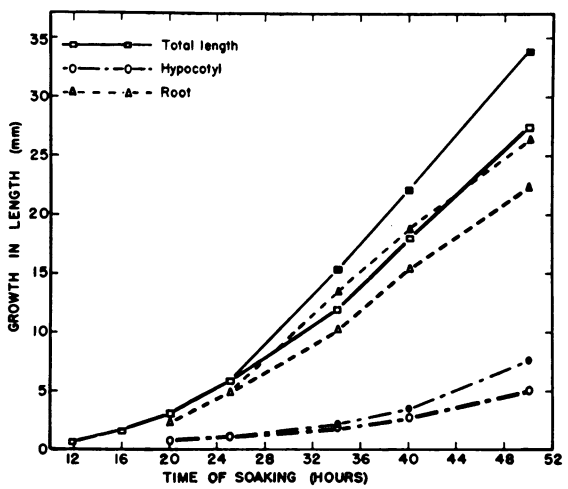
FIG. 2. Effect of far-red irradiation at various periods after the seeds have been induced to germinate with red. Red light given after 1.5 hours (A) or 8 hours (B) from the beginning of imbibition. Exposures to red and far-red 2 minutes in each case. The arrows indicate the time of the first sign of root protrusion.

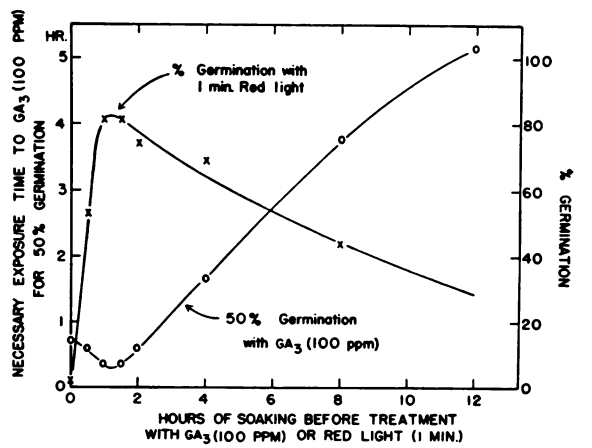
growth of hypocotyls or roots. Their data, nevertheless, do actually show an increase of 10 to 20 % in the length of the hypocotyls. The timing of their measurements, furthermore, may have been late enough so that these two parts of the seeds had already reached maximum growth. Since a possible mechanism of GA_3 action on the germination of lettuce seeds is that it promotes the initial rate of growth of these parts, this point seemed worthy of reinvestigation. For this purpose, 50 seeds were soaked in GA_3 solution (100 ppm) in darkness for various periods of time between 12 and 48 hours, then the lengths of hypocotyls and roots of all germinated seedlings were measured. As control, a group of seeds were allowed to imbibe in the dark in water, then given 2 minutes exposure to red light at 1.5 hours after dark imbibition, and measured as above. The results are shown in figure 4.

The first sign of visible germination of both light-induced and GA_3-treated seeds takes place after almost the same period of dark imbibition, or the GA_3-treated seeds may be a little later, i.e., about nine hours from the beginning of soaking. It is, however, difficult to measure the length of the elongated radicles just after their emergence. The rates of growth of hypocotyls and roots of treated and control seeds appear not to differ much at first. In both organs the gibberellin treatments show appreciable increase in growth rate only after 16 hours from the first sign of visible germination, i.e., 25 hours after the begin-









FIGS. 3-6. Action of gibberellin on germination.

FIG. 3 (*top left*). Effect of GA_3 at various concentrations on germination in darkness. I, experiment of May 30, 1959; II, experiment of July 9, 1959.

FIG. 4 (*lower left*). Growth in length of hypocotyl and root. Germination induced either by 2 minutes red light given 1.5 hours from the beginning of soaking or by 100 ppm GA_3 given throughout the period of imbibition and germination. Open symbols (thick lines) light-induced seeds; closed ones (thin lines) GA_3 treated.

FIG. 5 (*top right*). Germination of lettuce seed as influenced by duration of treatment with GA_3 (100 ppm) begun at various times after presoaking in water. Hours given on each curve (and inset) indicate the length of time presoaked in water; abscissa the duration of the GA_3 treatment.

FIG. 6 (*lower right*) Necessary exposure time to 100 ppm GA_3 to cause 50 % germination (*left ordinate*), and per cent germination with 1 minute red light (*right ordinate*), after various periods of presoaking in water.

TABLE I

EFFECT OF GIBBERELLIN IN VARIOUS CONCENTRATIONS WHEN INJECTED INTO GRAND RAPIDS LETTUCE SEED*

Conc of GA_3 (ppm)	% Germination		
	Expt 1	Expt 2	Expt 3
0	26 + (2)	36	24
0.1	40 + (4)	...	28 + (4)
0.3	...	...	32 + (8)
1.0	84 + (6)	98	66 + (6)
10	...	94 + (6)	...
30	...	98 + (2)	...

* Each reading the average of 50 seeds.
Numbers given in brackets represent atypical germination.

ning of soaking (fig 4). Thus it seems difficult to ascribe the germination caused by GA_3 to any promotion of the initial growth of emerging hypocotyls and roots.

This conclusion was confirmed by using the modified procedure described in section III, in which 50 seeds were treated only for 2 hours with GA_3 solution (100 ppm) after 1.5 hours of water presoaking in darkness, and then rinsed three times in water. Thus the external GA_3 was removed long before visible germination began. After 50 hours from the beginning of soaking, the lengths of hypocotyls were: in red light treated 5.1 mm (s.d., 1.62) and in GA_3 treated 4.7 mm (s.d., 1.50), while the lengths of the roots were 21.6 mm (s.d., 4.10) and 23.3 mm (s.d., 5.06), respectively. It is evident that the differences between the two groups are not statistically significant ($t = 1.27$ for hypocotyl and $t = 1.82$ for roots, hence $P < 0.05$ for both) and therefore that the gibberellin, when applied only before the time of visible germination, does not increase the elongation of the seedling.

It may be added that at no period after the beginning of water imbibition did GA_3 (100 ppm) increase the uptake of the solution by the seeds, prior to visible germination, above that by the water controls.

It has been shown that seeds whose endosperm coverings have been removed are perfectly capable of elongating their radicles in the dark (1, 11). The responsiveness of the seed to light is thus determined by the integrity of the endosperm layer; this phenomenon makes possible a clear separation between the true germination process and simple elongation of the radicle (11). The above data, therefore, suggest that the action of GA_3 on seed germination, like that of red light, is exerted on the true germination process, rather than by promoting the growth of the radicle. Furthermore, the data also offer evidence that GA_3 so applied does not persist in the seed after germination (cf. below).

III. TIME RELATIONS OF ACTION OF GA_3. According to Borthwick et al (2), when seeds are induced to germinate by red light, a previous imbibition in water of about 12 hours is needed for the light to have its maximum effect (fig 1 of ref 2). In our own experiments, however, the corresponding time, as shown in figure 1, is only about 1¼ hours. The optimum time for treatment with GA_3 was therefore investigated. Evenari et al (6) have concluded that 10 hours' soaking in gibberellin solution (2.9×10^{-5} M = 10 ppm), followed by transfer to water, gave more complete germination than soaking throughout the period in the gibberellin solution. In their experiments, however, both the quality of the seeds and the experimental method were somewhat different from those employed in our study.

A hundred seeds were first soaked in darkness in 3 ml of distilled water for various periods of time between 0 and 12 hours, then quickly dried on filter paper, and transferred to GA_3 solution, 100 ppm, for specified periods between 15 minutes and 8 hours. The drying and transfer to GA_3 solution necessitated exposure to the green safe-light for 4 to 5 minutes. After each period of soaking in GA_3, the seeds were immediately washed three times with distilled water and quickly blotted dry between filter papers.

Care was taken not to press or roll the seeds, as this might have promoted germination. The seeds were then placed in Petri dishes, each containing 3 ml of distilled water, and allowed to germinate. Forty-eight hours after transfer of the seeds to GA_3 solution, the germination was examined. Groups of seeds were prepared as controls in the same manner as indicated above, except that they were transferred for 1.5 hours to distilled water instead of GA_3 solution. The results (fig 5) show in general that the later the gibberellin is applied the less effective it is, i.e., the longer the seeds must be soaked in it to achieve the same effect. However, in the first 2 hours the trend is in the opposite direction, the gibberellin being more effective than if applied at the start. The curves for 0.5 and 2 hours, and those for 1 and 1.5 hours, overlap; evidently the most effective time to treat with GA_3 lies between 1 and 1.5 hours. At this time 50 % germination can be achieved by soaking in the solution for 20 minutes.

In order to show this more clearly, the necessary exposure time to GA_3 required to induce 50 % germination is plotted against the duration of presoaking in water before exposure to GA_3 (fig 6). The greatest sensitivity for the induction of germination by the gibberellin occurs after about 1¼ hours of presoaking in water. For comparison, curve C of figure 1, which shows the percentage germination with 1 minute of red light exposure after various periods of soaking, is plotted alongside in figure 6. As pointed out already, the seeds are most sensitive to red light after about 1¼ hours of imbibition in darkness. Thus *GA_3 and red light are both at their most effective stage after exactly the same time from the beginning of the dark imbibition of water.*

In a repeat of this experiment the results showed essentially the same pattern of curves and timing. The only difference was that the percentage of germination obtained was a little lower in every case, i.e., the slopes of the curves of figure 5 were a little less.

TABLE II

EFFECTS OF FAR-RED LIGHT ON GERMINATION OF GRAND RAPIDS LETTUCE SEEDS, AS FUNCTION OF TREATMENT WITH RED LIGHT OR WITH GA_3 (100 PPM)

TREATMENT	% GERMINATION
Red (2 min)	92
Far-red (5 min)	1
Red (2 min), then far-red (5 min)	0
Far-red (5 min), then red (2 min)	91
GA_3 (2 hrs)	93
Far-red (5 min), then GA_3 (2 hrs)	93
GA_3 (2 hrs), then far-red (5 min)	93
Water, dark control	2

IV. INTERACTION BETWEEN GIBBERELLIN AND FAR-RED LIGHT. It could be concluded from the above that the action of GA_3 is to substitute for red light. If this were true, its effect should be reversible by far-red, as is the effect of red light. However, previous reports indicate either that this is not the case (13, 17), that it is only partially reversible (6, 14), or in one case (10) that it is indeed reversible. However, in this last case, the seeds were held at 22° C for 12 hours and then transferred to 37°C; the germination was low and the effect of GA_3 was partially reversed by red light also. Thus, it is not easy to draw a firm conclusion from all these data because the extent of germination in the dark varies so greatly between the seeds and the procedures used by different workers. In order to re-examine the effect of far-red on germination caused by gibberellin, the following two experiments were carried out using the same technique as described above; i.e., the seeds were treated only for a short time with GA_3 (100 ppm) and not throughout the period of imbibition and germination.

In the first experiment, seeds were exposed to GA_3 for 2 hours after 1.5 hours of presoaking in water, (the optimum time, as shown in fig 6) then washed three times with water, and transferred to water. A generous dosage of far-red light (5 min) was given immediately before or after the exposure to GA_3. Table II shows the results, each reading being the average of 200 seeds. It is clear that, although the GA_3 treatment is capable of inducing germination to the full extent, its effect is not reversed at all by far-red light. This finding is further strengthened by the fact that in the same experiments, under the same conditions, the action of red light is fully reversed.

Haber and Tolbert (10), however, showed that the GA_3 effect was completely reversed by far-red irradiation for 20 minutes, although, as stated above, their experimental methods were different from ours. It seemed nevertheless possible that the results in table II were obtained because the exposure to far-red light was too short in comparison with the long treatment with GA_3. Therefore in a second experiment the seeds were treated with GA_3 for a sufficient period to bring about only 50 % germination (30 min or 1.5 hrs according to the length of pre-soaking time in water) and far-red light was given immediately before or after the GA_3 treatment for 10 or 50 minutes (the latter being given as five 10-min exposures evenly spread through the 1.5 hr period). The results, shown in table III, indicate that when far-red light was given *before* the GA_3 treatment, the light reduced germination by 37 to 38 %, and when given after the GA_3 by 20 %. In a third set of experiments five 10-minute exposures to far-red light were again given immediately before or after the GA_3 treatment (30 min after 1.5 hrs of presoaking in water), and the results showed essentially the same pattern as above; i.e., far-red treatment reduced germination by 63 % when given before and only by 31 % when given after GA_3. It may therefore be concluded that far-red light is capable of desensitizing the seeds to the GA_3 treatment but not of reversing completely the GA_3 action, even when given for 50 minutes. Normally 1 minute of far-red under our conditions is sufficient for complete reversal of the germination caused by red light.

These results show that far-red, especially in large dosages, reduces the sensitivity of seeds to the subsequent action of gibberellin. It is indeed actually more effective in reducing the germination if it is

TABLE III

EFFECTS OF FAR-RED LIGHT ON GERMINATION OF GRAND RAPIDS LETTUCE SEEDS TREATED WITH GA_3 (100 PPM). GA_3 TREATMENT ADJUSTED IN TIMING TO BRING ABOUT 50 % GERMINATION

TREATMENT	% GERMINATION	INHIBITION OF GERMINATION %
Far-red (10 min)	1	...
GA_3 (30 min)	45	...
Far-red (10 min), then GA_3 (30 min)	28	38
GA_3 (30 min), then far-red (10 min)	36	20
Far-red (50 min)[1]	1	...
GA_3 (1.5 hrs)[2]	46	...
Far-red (50 min)[1], then GA_3 (1.5 hrs)[2]	29 + (1)	37
Dark, water control	0 + (1)	...

Seeds were given GA_3 or far-red treatment at 1.5 hours after the beginning of soaking unless otherwise mentioned.

[1] Far-red light was given as five 10-minutes exposures during 1.5 hour period after 1.5 hours of dark presoaking in water.

[2] GA_3 was given after 3 hours from the beginning of dark imbibition for 1.5 hours.

( ) indicates atypical germination.

given *before* the GA_3 treatment than when given after. It is clear, however, that far-red does not in any real sense *reverse* the action of the gibberellin. The answer to the problem posed at the outset of this section is therefore that gibberellin does not simply substitute for red light.

V. Attempts to Identify a Gibberellin-like Substance as Product of Photoreaction. An alternative approach to the problem of the interrelationship between the effects of gibberellin and of red light is to determine if gibberellin is produced by the photo-reaction as the end-product of the process. In this case the external application of GA_3 would simply promote the germination irreversibly. It is now well established that gibberellin-like substances occur in higher plants (18, 19, 20, 21, 22, 24, 25, 27, 29) and both GA_1 and GA_5 have actually been isolated from higher plant material (18, 27, 29). Although these findings are not necessarily connected with the action of light, they obviously increase the probability of a photoproduction of gibberellin.

From the results presented in table I, calculations were made before experimentation as to the maximum yield of gibberellin-like substances which should be expected in the extracts if red light acts by releasing such material in the seed. Since about 0.002 ml of a 1 ppm solution of GA_3 causes maximal germination, then, allowing for only 20 % recovery in the extracts, 10 gm seeds should be required to yield 4 μg gibberellin. If we further consider that the gibberellin acts only at a specific locus, and only 20 % of that injected reaches this locus, then the expected yield would drop further to 0.8 μg. Even such an amount is large in comparison with the known responses of test plants. Therefore, 10 gm seeds were extracted with acetone-water (24) for 10 hours at 34° C 2 or 8 hours after the light treatment (10 min exposure to white light at 1.5 hrs after the beginning of dark imbibition), and the dried extract was extracted with 1 ml of water (including 0.05 % Tween 20), of which 0.1 and 0.5 ml were applied to dwarf-1 mutants of corn. As controls, dry seeds and dark-imbibed seeds were also extracted in the same manner. In none of the extracts, however, could the presence of gibberellin-like substances be detected. The bioassay responds clearly to 0.001 γ g. of GA_3 (24). Furthermore, paper chromatography was carried out on a strip of Whatman No. 1 filter paper, but no indication of any spot fluorescing in ultraviolet light after treatment with concentrated sulfuric acid was shown with two solvent systems employed.

In the meantime, extraction of a gibberellin-like substance from dry lettuce seeds was reported (A. Lang. Personal communication of unpublished work by S. Blumenthal-Goldschmidt and A Lang.) (20). A second series of experiments were, therefore, conducted using 500 gm of seeds. The seeds were given 10 minutes of white light at 1.5 hours after the beginning of dark imbibition (the optimum time, as shown in fig 1), and 2 hours later were extracted with acetone-water (1 : 1), and the extract fractionated with ethyl acetate (29). Dry seeds and dark-imbibed seeds were extracted in the same way, and the extracts of all three were again tested on the dwarf-1 mutant. The final dried residue of the ethyl acetate reextract was dissolved in 10 ml of distilled water (containing 0.05 % Tween 80), and 0.1 ml of this was used for assay. The lengths of the leaf-sheaths are given in table IV. The total elongation of the first leaf during the 7 days was: dwarf control 2.0 cm, dry seeds 2.8 cm, dark imbibed 2.0 cm, light treated 2.9 cm. The data show a small positive reaction in the assay, but the amount was not increased by light treatment over that present in the dry seed.

According to Neely (21) the best values for calibration of the test are given by adding together the lengths of the two leaf sheaths; these data are given in the last column of the table. It may be deduced

Table IV

Final Lengths (cm) of 1st and 2nd, Leaf Sheaths of Dwarf-1 Corn, Treated With Extract from Lettuce Seeds, and of Controls *

Solution	Amt. applied per d-1 plant	1st Leaf sheath	2nd Leaf sheath	Sum
Extract from 1,000 gm dry seeds in 10 ml	0.1 ml	3.25cm	3.10cm	6.35cm
Extract from 500 gm dark-imbibed seeds in 10 ml	0.1 ml	1.90	2.35	4.25
Extract from 500 gm light-induced seeds in 10 ml	0.1 ml	2.10	2.35	4.45
GA_3 (Crystalline)	0.0001 μg	2.10	2.53	4.63
	0.001 μg	2.60	3.30	5.90
	0.01 μg	3.83	4.87	8.70
0.05 % Tween-80 water	0.1 ml	1.90	2.43	4.33
Tall sibling plants	...	5.97	8.05	14.02

* Time of measurement, for first leaf sheath 7 days, for second leaf sheath 10 days, after treatment. Nutrient solution XL 36, white light 1450 ft-c, 25° C. Each figure the mean of two or three plants.

from this calibration that 10 gm seeds yield only about 0.003 μg. (dry seeds) or 0.0002 μg. (light-induced seeds) instead of the calculated 0.8 μg.

Since the test solution might have contained injurious materials or growth inhibitors, it was thought that the growth promotion of the test plants might have been depressed. An attempt, therefore, was made to remove any inhibitor present by paper chromatography in a solvent (*n*-butanol: glacial acetic acid: water = 95: 5: 30) which separated the GA_3 spot (Rf = 0.80–0.81) from the rest. A section of paper chromatogram between Rf = 0.7 and Rf = 1.0 was reextracted with acetone-water (1: 1), the dried residue dissolved in Tween 80 as above, and again bioassayed. The very small yield of the extracted gibberellin-like substance from the control seeds was again not increased, but even somewhat decreased, by the light treatment.

The method employed by West and Phinney (29) leads to the isolation of GA_5 (Bean Factor II), to which dwarf-3 or -5 corn is more sensitive than dwarf-1. Furthermore, A. Lang (Personal communication of unpublished work by S. Blumenthal-Goldschmidt and A. Lang.) has informed us that the material extracted from dry lettuce seeds behaves rather like GA_5. Therefore a third series of experiments was carried out using dwarf-5 corn seedlings as assay plants. Lettuce seeds (30 gm) were extracted four times with acetone-water (1: 1) as above. After condensing each extract to about 2.5 ml, about 20 % of it was paper-chromatographed with *n*-butanol: glacial acetic acid: water = 95: 5: 30 and the chromatograms cut into five equal sections according to the Rf value. Each section was eluted with 1: 1 acetone-water four times, the eluate dried completely, taken up into 1 ml water, and 0.1 ml of this bioassayed by applying 0.04 ml between the coleoptile and the first leaf, and 0.06 ml inside the unfolding first leaf, within a 12 hour period. Figure 7 shows the growth of the first and second leaves, and table V gives the final lengths of the leaf sheaths. These data show a definite growth promotion; the highest value at Rf = 0.8 — 1.0, slight activity at Rf = 0.6 — 0.8, and none at lower Rfs. However, again the light treatment clearly produced no increase in the amount of extracted gibberellin.

The calibration with a sample of pure GA_5 (obtained from Dr. J. MacMillan of Imperial Chemical Indus.) indicates that the maximum amount extracted (i.e., from the dark-imbibed seeds) corresponds to 0.002 μg of GA_5; there are, therefore, 0.03 μg per 10 gm seeds. This compares well with Murakami's figure (21) of 0.025 μg per 10 gm. It is about ten times as much as was shown in table IV using dwarf-1 corn. The amount is clearly not increased, but even decreased, by the light treatment.

From these results it is confirmed that Grand Rapids lettuce seeds contain a small amount of gibberellin-like substance whose biological activity is more like that of GA_5 than GA_3, but that *the amount of the substance in the extracts is not increased by light treatment.* This repeated failure to demonstrate any increase in the amount of the gibberellin-like substance in the seeds after exposure to light makes it improbable that light can act by releasing a gibberellin within the seed.

TABLE V

FINAL LENGTHS (CM) OF 1ST AND 2ND LEAF SHEATHS OF DWARF-5 CORN, TREATED WITH EXTRACT FROM LETTUCE SEEDS, AND OF CONTROL*

SOLUTION	RF VALUE	AMT APPLIED/ D-5 PLANT	1ST LEAF SHEATH	2ND LEAF SHEATH	SUM
Extract from 30 gm dark-imbibed seeds in 1 ml	0.0–0.2	0.1 ml	1.85cm	2.15cm	4.00cm
	0.2–0.4	0.1 ml	1.75	2.00	3.75
	0.4–0.6	0.1 ml	1.80	1.90	3.70
	0.6–0.8	0.1 ml	2.00	2.55	4.55
	0.8–1.0	0.1 ml	3.15	3.25	6.40
Extract from 30 gm light-induced seeds in 1 ml	0.0–0.2	0.1 ml	1.75	2.15	3.90
	0.2–0.4	0.1 ml	1.85	2.15	4.00
	0.4–0.6	0.1 ml	1.85	1.95	3.80
	0.6–0.8	0.1 ml	1.95	2.60	4.55
	0.8–1.0	0.1 ml	2.50	2.55	5.05
GA_5 (crystalline)	...	0.00001 μg	2.05	1.85	3.90
	...	0.0001 μg	2.55	2.40	4.95
	...	0.001 μg	2.70	2.95	5.65
	...	0.01 μg	3.40	4.20	7.60
0.05 % Tween-80 water	...	0.1 ml	1.93	2.13	4.06
Tall sibling plants		...	4.57	6.20	10.77

*Nutrient solution XL 36, white light 1450 ft-c, 25° C. Each figure the mean of two or three plants.

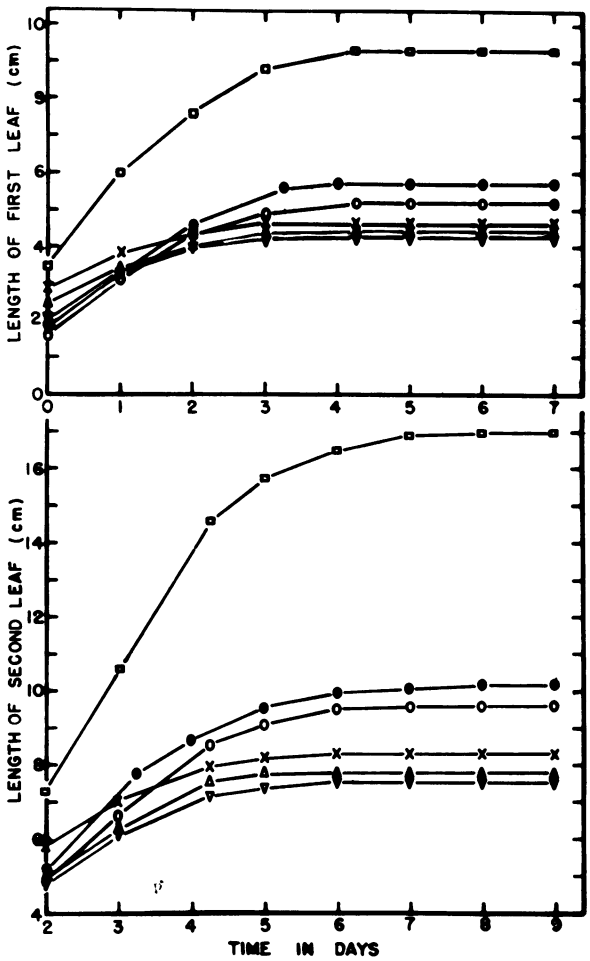


FIG. 7. Bioassay of extracts from light-treated and dark-imbibed seeds (30 gm each) using dwarf-5 corn seedling. Zones of chromatogram of Rf = 0.6 to 0.8 and 0.8 to 1.0 eluted with acetone-water (1 : 1), and 10 % of each eluate applied to individual plants. (Zones of lower Rf not shown, because they caused no growth promotion.) —□—□—, tall sibling plant; —×—×—, dwarf-5 control treated with Tween-80 water; —○—○—, eluate (Rf = 0.8–1.0) from light-treated seeds; —●—●—, eluate (Rf = 0.8–1.0) from dark-imbibed seeds; —△—△—, eluate (Rf = 0.6–0.8) from light-treated seeds; —▽—▽—, eluate (Rf = 0.6–0.8) from dark-imbibed seeds. Above: growth of the first leaf. Below: growth of the second leaf.

DISCUSSION

IMPORTANCE OF FULL WATER SUPPLY. It has been known for some time that lettuce seeds vary in their sensitivity to red and far-red light according to the preceding period of dark imbibition. Borthwick et al (2) reported that in their system the germination reached a near-maximum value when Grand Rapids seeds were exposed at from 12 to 20 hours after the beginning of imbibition. Evenari and Neumann (4) reported that with 500 ft-c-minutes irradiation the maximum germination was obtained when the seeds were exposed after 16 minutes at 22° C or 30 minutes at 26° C, while with shorter periods of irradiation the maximum was reached only after 8 hours' imbibition. Flint (8), using the Big Boston variety and 600 ft-c-minutes' illumination found maximum germination (90.5 %) when the illumination came after soaking 100 minutes at 25° C. Neither of the above time schedules for var. Grand Rapids agrees with the results presented in this paper, in which the highest sensitivity to red light falls at about 1¼ hours. The discrepancies may in part be due to the different amounts of water available to the seeds. In preliminary experiments in which moist filter paper was used as wetting medium, the time relations at the start were similar to figure 1C, but divergent later. Figure 8 shows a typical result with double layers of filter paper and 1 ml of distilled water in 5 cm Petri dishes; the largest change from figure 1C is at 4 and 8 hours, where the presence of GA_3 maintains the germination at high levels.

The presence of filter paper also changes the effectiveness of far-red in the presence of GA_3, for in figure 8 far-red light appears partially to reverse the GA_3 effect. In this experiment exposure was only for 1 minute, while in table II, with 5 minutes exposure to the same light, but without filter paper, there was no reversal. Even 50 minutes exposure (table III) gave much less reversal than in figure 8. It is possible that GA_3 becomes adsorbed on the paper so that the amount entering the seeds is reduced, but subsequently it is slowly released, giving the seeds a delayed treatment. The method used in the present experiments, without filter paper, thus has several advantages.

WATER UPTAKE AND SENSITIVITY OF SEED. It is extremely interesting that maximum germination resulted from exactly the same duration of water imbibition prior to the treatments, whether the germination was caused by red light or by GA_3 (fig 6). A similar finding has recently been reported by Yamaki et al (30) for light-sensitive tobacco seeds. This observation indicates that the metabolism of the lettuce seed is most ready for induction when its water content reaches about 40 %. At this time saturation with water is still far from complete. However, some experiments in which the seed was separated into fruit-coat, cotyledons, and hypocotyl + root, indicate that the curves of figures 1A and 1B mainly represent the behavior of the cotyledons, and the hypocotyl + root unit in fact reaches its maximum water content before the 2nd hour. The maximum sensitivity of the seed to red light may, therefore, be attained as soon as the uptake of water by the hypocotyl + root region has been completed. Presumably the water uptake makes the seeds ready for the induction of germination by initiating their metabolism.

A POSSIBLE MECHANISM OF GA_3 ACTION ON LETTUCE SEED GERMINATION. The light sensitivity of the seed evidently depends upon the integrity of the

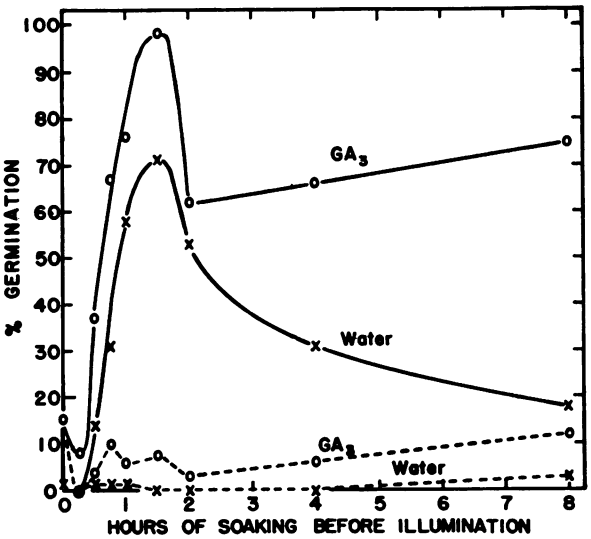


FIG. 8. Effect of 1 minute red (*solid lines*) or 1 minute far-red (*dotted lines*) after various periods of presoaking on germination of lettuce seeds imbibed in 1 ml of GA_3 solution (100 ppm) or water in presence of double layers of filter paper.

endosperm layer, the innermost covering of the seed, since it disappears when this is removed (1, 11). But the photosensitive site resides in the hypocotyl region, where active elongation takes place (11, 12). The actual elongation of the radicle of seeds in which the endosperm no longer restricts germination appears not to be appreciably influenced by light (11). Klein and Preiss (15) have shown too that the photosensitivity of the seeds does not change even when they are turned over in the interval between red and far-red irradiation.

These facts suggest that the postulated pigment system is in the hypocotyl, rather than in the endosperm, and that on red light treatment this pigment activates one or more chemical reactions which lead to breaking the mechanically restricting endosperm layer.

That GA_3 so closely mimics the effect of red light in its action and timing strongly suggests that it acts at the same site as red light, namely in the embryonic hypocotyl. Yamaki et al (30) reported that substances which induce germination of tobacco seeds in darkness diffuse out from leaf discs which have been illuminated with red light, and that one of these substances has the same Rf value as that of the gibberellins on chromatograms developed with several different solvent systems. Although this observation strongly suggests that a gibberellin is produced in tobacco seeds by red light, our extractions have failed repeatedly to demonstrate that even a trace of gibberellin is produced by light treatment of lettuce seeds. This applies both to GA_3 and to GA_5. One must conclude, therefore, that it is most improbable that light induces germination of lettuce seeds by liberating a gibberellin. Haber and Tolbert (10) have come to a similar conclusion.

The most reasonable explanation of the action of gibberellic acid, therefore, is that it somehow initiates, independently of light, one of the series of chemical reactions leading to germination, thus producing the same final product for breaking the mechanical restriction of the endosperm layer. This would account both for the non-reversibility of gibberellin action by far-red and for the identity of timing of its effect with that of red light. Thus, of the three possible alternatives listed in the Introduction, the second one appears nearest to the truth.

SUMMARY

I. The effect of gibberellic acid (GA_3) on the germination of Grand Rapids lettuce seeds has been compared with the action of red light, in the hope of elucidating the mode of action of these factors.

II. The work was carried out by shaking the seeds directly with a GA_3 solution or water throughout the period of imbibition and germination. This method was found to yield more uniform and more repeatable results than the ordinary one using moist filter paper.

III. During the course of imbibition, the seeds gradually become sensitive to red light, and reach their maximum sensitivity 1¼ hours after the beginning of imbibition, when water uptake is still not complete; thereafter the sensitivity to red light gradually decreases.

IV. In seeds which have been induced to germinate by red light, the inhibitory effect of far-red light gradually decreases with increasing time interval between red and far-red treatments. Far-red light no longer inhibits the germination process after the first sign of visible germination occurs (8–9 hrs after red light treatment at 25° C).

V. GA_3 at 60 ppm induces maximal germination; germination is almost linearly proportional to concentration below this level. However, if the solution is injected directly into the seed, concentrations as low as 1 ppm are fully effective.

VI. The action of GA_3 is not to control the initial rate of growth of the hypocotyl and the root, since this rate is increased only at about 16 hours after the first sign of visible germination.

VII. The time of greatest sensitivity for the induction of germination by gibberellin occurs after about 1¼ hours of presoaking in water; this timing is identical with the most sensitive period of the seeds to red light.

VIII. Far-red light does not inhibit the germination of seeds which have been treated with GA_3 so as to produce 90 % germination. If the time of GA_3 treatment is adjusted to give only 50 % germination, and the far-red exposure increased, then far-red does partially inhibit, but this inhibition is exerted to the same extent whether the exposure is made *before or after* the GA_3 is applied. Thus, heavy doses of far-red desensitize the seeds to gibberellin.

IX. Repeated attempts to detect a gibberellin as the end-product of the action of light in the seeds failed completely, whether the assay was for GA_3 or

GA_5. Small amounts of gibberellin were detected in the seeds, but the quantity was not increased by a light dosage sufficient to cause maximum germination.

X. It is deduced that GA_3 acts by initiating one of the chemical reactions which result from the light reaction, so that its end product is the same as that produced by light.

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Literature Cited

1. Borthwick, H. A. and W. W. Robbins. 1928. Lettuce seed and its germination. Hilgardia 3: 275–304.
2. Borthwick, H. A., S. B. Hendricks, E. H. Toole, and V. K. Toole. 1954. Action of light on lettuce seed germination. Bot. Gaz. 115: 205–225.
3. Brian, P. W. 1959. Effects of gibberellins on plant growth and development. Biol. Rev. 34: 37–84.
4. Evenari, M. and G. Neumann. 1953. The germination of lettuce seeds. III. The effect of light on germination. Bull. Res. Counc. Israel 3: 136–145.
5. Evenari, M. and G. Neumann. 1953. The germination of lettuce seeds. IV. The influence of relative humidity of the air on light effect and germination. Palestine Jour. Bot., Jerusalem Ser., 6: 96–100.
6. Evenari, M., G. Neumann, S. Blumenthal-Goldschmidt, A. M. Mayer, and A. Poljakoff-Mayber. 1958. The influence of gibberellic acid and kinetin on germination and seedling growth of lettuce. Bull. Res. Counc. Israel 6D: 65–72.
7. Flint, L. H. 1934. Light in relation to dormancy and germination in lettuce seed. Science 80: 38–40.
8. Flint, L. H. and E. D. McAlister. 1935. Wave lengths of radiation in the visible spectrum inhibiting the germination of light-sensitive lettuce seed. Smithsonian. Misc. Coll. 94(5): 1–11.
9. Flint, L. H. and E. D. McAlister. 1937. Wavelengths of radiation in the visible spectrum promoting the germination of light-sensitive lettuce seed. Smithsonian Misc. Coll. 96 (2): 1–8.
10. Haber, A. H., and N. E. Tolbert. 1959. Effects of gibberellic acid, kinetin, and light on the germination of lettuce seed. In: Photoperiodism and Related Phenomena in Plants and Animals. Amer. Assoc. for Advancement of Science, Washington, D. C.
11. Ikuma, H. and K. V. Thimann. 1958. The mechanism of germination in light-sensitive lettuce seed. Plant Physiol. 33 suppl.: xxiv.
12. Ikuma, H. and K. V. Thimann. 1959. The photosensitive site in lettuce seeds. Science 130: 568–569.
13. Kahn, A., J. A. Goss, and D. E. Smith. 1957. Effect of gibberellin on germination of lettuce seeds. Science 125: 645–646.
14. Kahn, A. 1960. Promotion of lettuce seed germination by gibberellin. Plant Physiol. 35: 333–339.
15. Klein, S. and J. W. Preiss. 1958. Reversibility of the red-far-red reaction by irradiation at different sites. Nature 181: 200–201.
16. Kribben, F. J. 1957. Die Abkürzung der Samenruhe bei Arabidopsis durch Gibberellinsäure. Naturwiss. 44: 313.
17. Lona, F. 1956. L'acido gibberellico determina la germinazione dei semi di *Lactuca scariola* in fase di scoto-inhibizione. L'Ateneo Parmense. 27 (4): 641–644.
18. MacMillan, J. and P. J. Suter. 1958. The occurrence of gibberellin A_1 in higher plants: Isolation from the seed of runner bean (*Phaseolus multiflorus*). Naturwiss. 45: 46.
19. Murakami, Y. 1959. A paper chromatographic survey of gibberellins and auxines in immature seeds of leguminous plants. Bot. Mag. Tokyo 72: 36–43.
20. Murakami, Y. 1959. The occurrence of gibberellins in mature dry seeds. Bot. Mag. Tokyo 72: 438–442.
21. Neely, P. M. 1959. The development and use of a bioassay for gibberellins. Ph. D. Thesis, University of California at Los Angeles.
22. Nitsch, J. P. 1958. Présence de gibbérellines dans l'albumen immature du Pommier. Bull. soc. bot. France 105: 479–482.
23. Ogawara, K. and K. Ono. 1957. Tabako-shushi no hikari-hatsuga ni oyobosu gibberellin no eikyo (the effect of gibberellin on the light-induced germination of tobacco seeds). (In Japanese). Pp. 9–10. Abstr. 1st Meeting Jap. Gibberellin Res. Assoc.
24. Phinney, B. O., C. A. West, M. Ritzel, and P. M. Neely. 1957. Evidence for "gibberellin-like" substances from flowering plants. Proc. Nat. Acad. Sci. 43: 398–404.
25. Radley, M. 1958. The distribution of substances similar to gibberellic acid in higher plants. Ann. Bot., N. S. 22: 297–307.
26. Stowe, B. B. and T. Yamaki. 1957. The history and physiological action of the gibberellins. Ann. Rev. Plant Physiol. 8: 181–216.
27. Sumiki, Y. Occurrence of GA_1 in fruits of Japanese citrus. Proc. Intern. Cong. Plant Growth Substances, Yonkers, N. Y. (in press).
28. Toole, V. K. and H. M. Cathy. 1959. Germination of Grand Rapids Lettuce and *Lepidium virginicum* seeds as affected by gibberellin. Plant Physiol. 34 suppl: xvi.
29. West, C. A. and B. O. Phinney. 1959. Gibberellins from flowering plants. I. Isolation and properties of a gibberellin from *Phaseolus vulgaris* L. Jour. Amer. Chem. Soc. 81: 2424–2427.
30. Yamaki, T., T. Hashimoto, T. Ishii, and M. Yamada. 1958. Some physiological effects of gibberellin on seed germination, leaf expansion, dehydration of mitochondria, and probable formation of gibberellin in leaf. Pp. 51–52. Abstr. 2nd Meeting Jap. Gibberellin Res. Assoc.