Plant Physiol. (1979) 63, 704–708
0032-0889/79/63/0704/05/$00.50/0

Photocontrol of the Germination of *Onoclea* Spores

V. ANALYSIS OF GERMINATION PROCESSES BY MEANS OF TEMPERATURE

Received for publication June 9, 1978 and in revised form December 4, 1978

CHIN-YU CHEN AND HIROSHI IKUMA[1]
Department of Cellular and Molecular Biology, Division of Biological Sciences, University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

The physiological nature of photoinduced germination of *Onoclea sensibilis* L. spores was investigated by temporarily applying a range of temperatures, particularly 40 C, before and after short light treatment. Controls were germinated at 25 C.

The preinduction phase, during which photosensitivity is maximally developed in the dark, is sensitive to temperature. Treatment at 40 C for 8 or more hours reduces the developed photosensitivity to a minimal level, but the inhibition by 40 C treatment is reversed slowly after subsequent incubation at 25 C in the dark. The postinduction phase, in which dark processes lead to stain uptake and eventually to visible protrusion, is also sensitive to temperature. Inhibition by 40 C occurs shortly after photoinduction, but disappears 6 or more hours after irradiation. Postinduction spores whose germination is inhibited by 40 C treatment recover the ability to germinate after subsequent incubation at 25 C plus a second light treatment. The inhibition and recovery take place faster in postinduction spores than in preinduction spores. In addition, escape from 40 C inhibition is found in the postinduction phase but not in the preinduction phase. Temperatures lower than 25 C exert slow inhibition of both pre- and postinduction processes, and 30 to 35 C act to stimulate germination.

In comparison with our earlier work with anaerobiosis and cycloheximide, the postinduction step inhibited by 40 C can be located shortly after the step inhibited by anaerobiosis but before the cycloheximide sensitive step.

Onoclea spores germinate optimally in both light and darkness at an incubation temperature of 28 to 30 C, while incubation at 40 C is lethal to the spores (5, 12, 13). If a temporary treatment at 30 C is started within the first 12 h of dark soaking at 25 C, dark germination of *Onoclea* spores is maximal (12, 13). However, spores incubated at 25 C require light for maximal induction of germination (5, 14). Spore germination of many fern species, including *Onoclea*, is apparently controlled by phytochrome (6–12).

The germination processes of *Onoclea* spores have been operationally divided into the following three sequential phases with respect to short light treatment (15, 16): (*a*) a preinduction phase in which the spores imbibe water and develop maximal photosensitivity in the dark; (*b*) a photoinduction phase when light induces germination maximally; and (*c*) a dark postinduction phase in which photoinduced processes lead to unequal cell division and eventually to visible protrusion of rhizoidal and protonematal cells. The photoinduction phase is independent of temperature and anaerobiosis (15), but the two dark phases involve processes sensitive to anaerobiosis (15), cycloheximide (16), ethylene (2–4), and probably CO_2 (1). The physiological nature of the two dark phases was further investigated by treating spores at various temperatures, particularly at an elevated temperature of 40 C.

MATERIALS AND METHODS

The spores of *Onoclea sensibilis* L. were harvested in May 1977 from fertile fronds collected in March 1977, and stored in a desiccator in a refrigerator until use. The fertile fronds were collected from the same location and the spores were harvested by the same dehiscing method as described previously (14). This batch of spores required at least 24 h of dark presoaking at 25 C to develop maximal photosensitivity. Photoinduced germination reached about 70%, and dark germination was about 20%.

A small spatulaful of spores (about 10 mg) was sown on the surface of 10 ml distilled H_2O in a 5-cm Petri dish (14). After a given period of dark presoaking, spores were routinely illuminated with a 60-w incandescent lamp placed 30 cm away through a water heat filter (1.65×10^4 ergs cm^{-2} s^{-1}). Although less than 15 s were required to induce maximal germination, 5 min of irradiation were given to insure maximal effects. Final germination percentages were scored 5 days after the end of temperature or light treatment, using the acetocarmine-chloral hydrate stain uptake method (2, 15). The time course of stain uptake was followed at varying times after the light treatment.

All experiments were performed in duplicates and repeated at least twice. Necessary manipulations in the dark were carried out under dim green light (14). The standard incubation temperature was 25 C. Incubators (Precision Instruments) were used to obtain other incubation temperatures (5, 10, 15, 20, 30, 35, 38, 40, 43, and 50 C). Incubator temperatures were maintained within 1 C fluctuations.

During the course of the present investigations, it was important to distinguish whether treatments with high temperatures killed spores or simply suppressed their ability to germinate. Since all viable spores were capable of taking up the acetocarmine-chloral hydrate stain and proceeding to visible protrusion under diffuse light at 25 C, all spores subjected to temperature treatments were subsequently left on the laboratory bench for at least 1 week or as long as 1 month to examine their ability to germinate. Those spores that lost the ability to germinate under diffuse light are referred to in this paper as not viable or killed.

RESULTS AND DISCUSSION

Preliminary Experiments. In order to determine a general pattern of temperature effects on *Onoclea* spore germination: (*a*) spores were incubated at a given temperature throughout the entire period; (*b*) temperature treatment was limited to the preinduction period, then spores were irradiated and subsequently incubated at 25 C; and (*c*) spores were incubated at 25 C during

[1] To whom request for reprints should be addressed.

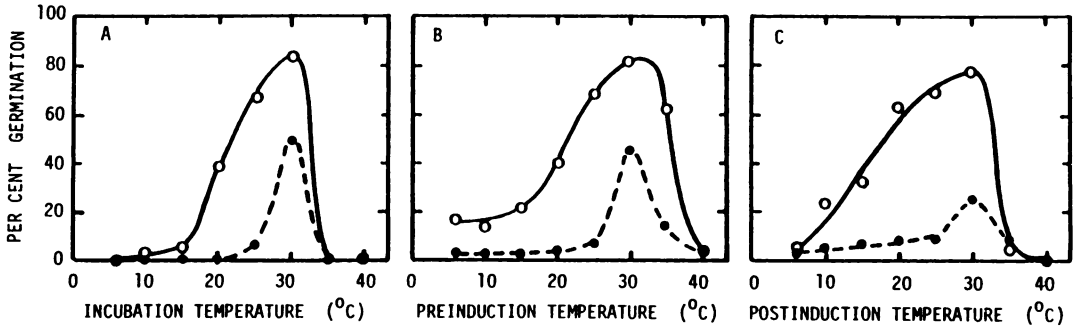

FIG. 1. Effects of temperature on the germination of photoinduced (○) and dark-incubated (●) *Onoclea* spores. Spores were kept at a given temperature throughout the entire incubation period of 6 days (A), or temperature treatment was limited to either the preinduction period of 1 day (B) or the postinduction period of 5 days (C). Spores were incubated at 25 C during the postinduction period for Figure 1B, and during the preinduction period for Figure 1C.

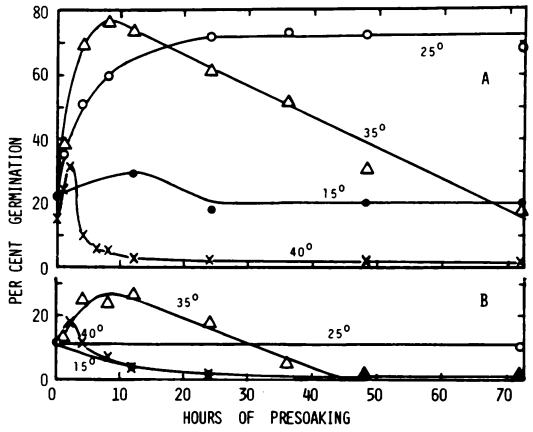

FIG. 2. Effects of temperature on the development of photosensitivity. Spores were soaked at a given temperature for various periods (abscissa), irradiated for 5 min, and incubated at 25 C for 5 days in the dark. A: germination of irradiated spores. B: germination of dark controls. Experiments at 40 C were conducted separately from those at 15, 25, and 35 C, and irradiation at 0 time gave somewhat lower per cent germination.

the preinduction period, then irradiated, and transferred to a given temperature.

The results (Fig. 1) show that: (*a*) under all conditions 30 C is the optimal temperature for germination of both irradiated and dark spores; (*b*) 25 C gives the largest difference in germination between light treatment and dark incubation; and (*c*) extreme temperatures of 5 and 40 C caused very low or no germination even with light treatment. These results agree in part with the data reported by Towill for the temperature range of 20 to 36 C (13). Spores treated at 35 and 40 C throughout the incubation period and during the postinduction period lost their viability, while the spores treated at 35 and 40 C only during the preinduction period showed full viability.

These data prompted us to examine further the physiological nature of the pre- and postinduction periods by temporarily incubating spores at various temperatures. In these studies, 25 C was used as the standard incubation temperature, because at this temperature dark germination was low and photoinduction of germination was optimal.

Effects of Temperature on Preinduction Processes. When spores were incubated in the dark at 15, 25, 35, and 40 C for various durations, and transferred to 25 C for irradiation and subsequent dark incubation at 25 C for 5 days, the following results (Fig. 2A) were obtained: (*a*) at 15 C, photosensitivity did not develop to a high level even after dark soaking for a long period; (*b*) at the standard temperature of 25 C, photosensitivity developed to a maximal level after 24 h of dark soaking and maintained it for at least 72 h (14); (*c*) at 35 C, photosensitivity reached a maximum at 8 h of dark soaking, then decreased gradually after longer presoaking; and (*d*) at 40 C, photosensitivity developed only during the first 2 h, but decreased very rapidly thereafter. Presoaking at 30 C (data not shown) allowed the spores to develop photosensitivity with a rate between that of 35 C and that of 25 C, reaching a near maximum at 8 h, and maintained it for at least 48 h.

The data of dark germination under different temperature pretreatments are shown in Figure 2B. In general, prolonged presoaking at either 15, 35, or 40 C led dark germination to a minimal level (about 2%). The decrease caused by 15 C proceeded slowly as the temperature pretreatment was prolonged. Short pretreatment at 35 or 40 C acted to increase dark germination slightly, but long pretreatment reduced dark germination. These patterns of dark germination appear to influence the photosensitivity curves at 35 and 40 C (Fig. 2A). Under these experimental conditions, all spores treated at 35 C and lower temperatures were viable, but spores treated at 40 C had decreased viability after presoaking periods longer than 24 h, and had completely lost their viability after 72 h at 40 C.

At the standard incubation temperature of 25 C, the photosensitivity curve (Fig. 2A) suggests two successive stages: the first 24 h when photosensitivity develops to a maximal level, and a subsequent stage when the maximal photosensitivity is maintained. The first developmental period is different in length among spore batches (*cf.* 12–16), but the maintenance of maximal photosensitivity lasts at least 2 days in all spore batches tested. The initial 2 h of photosensitivity development is insensitive to high temperatures (Fig. 2A) and anaerobiosis (15); initially dehydrated spores probably take up water during this stage, but their metabolism which is sensitive to high temperature and anaerobiosis is apparently not activated. From microscopic observations, Fisher and Miller (4) stated that their *Onoclea* spores attained the fully imbibed size within 30 min of soaking, but they did not present the time course of water uptake.

The time course of stain uptake (Fig. 3) by the spores presoaked at 15, 25, and 35 C before irradiation clearly show that: (*a*) half-maximal germination is attained at practically the identical time of 22 to 24 h after irradiation for all temperature treatments; and (*b*) the degrees of the final germination attained are influenced by the preinduction temperature.

Inasmuch as a relatively short treatment at 40 C effectively inhibits the development of photosensitivity (Fig. 2A) without damaging spore viability, spores were subjected to 40 C for various durations after the development of maximal photosensitivity but before irradiation. Figure 4A shows the effect of the length of 40 C treatment on the final germination attained. Germination starts to decrease after 2 h with a half-maximal inhibition being about 5 h. Dark germination increases significantly for 2 h at 40 C, but

decreases to a minimal value thereafter. The maximal inhibition is reached after 8 or more h of treatment at 40 C. Similar observations were made when spores were pretreated at 25 C for 2 h and 11 h, instead of 24 h (data not shown).

In order to examine if the inhibition at 40 C was reversed by subsequent incubation at 25 C, the spores treated at 40 C for 8 h were transferred back to 25 C for various periods before irradiation. The results in Figure 4B show that the recovery does take place, but with a lag period of about 10 h, and proceeds slowly. The half-time of recovery is about 30 h. These results indicate that the developed photosensitivity can be inhibited by 40 C, but the inhibition can be reversed gradually after subsequent incubation at 25 C. The time course of recovery was faster when 38 C was used, but it was very slow when spores were treated at 43 C (data not shown).

Possible alterations of the established photosensitivity by other temperatures were also examined with the spores presoaked at 25 C for 24 h. Temperature treatments were given for 8 h immediately before illumination. The results (Fig. 5) show that: (*a*) treatments at 30 and 35 C induce germination higher than 25 C controls both with light and in darkness; (*b*) temperatures lower than 25 C slightly inhibit photoinduced germination but cause practically no effect on dark germination; and (*c*) temperatures above 35 C markedly inhibit germination. Spores treated at 40 and 43 C still retained their viability, while those at 50 C were killed.

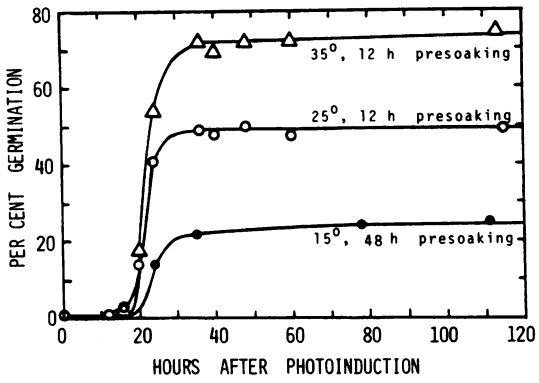

FIG. 3. Time course of stain uptake of spores presoaked at 15, 25, and 35 C. Spores were presoaked in the dark at 15 C for 48 h, at 25 C for 12 h, and at 35 C for 12 h, then irradiated with 5-min light, and subsequently incubated in the dark at 25 C. After varying periods from irradiation (abscissa), spore samples were taken for examination of percentages of spores that took up acetocarmine stain (expressed as per cent germination on the ordinate). Results of presoaking at 25 C for 24 h were the same as the 35 C curve.

The effects of temperature on the preinduction processes of spore germination can be summarized as follows: (*a*) the processes of photosensitivity development and its maintenance are sensitive to temperature with 25 C being the optimal temperature; (*b*) 30 C is capable of inducing dark germination when given from the beginning of soaking (13) and of elevating the germination of both photoinduced and dark spores when given after 24 h at 25 C; (*c*) the photosensitivity established by incubation at 25 C for 24 h is abolished at 40 C for 8 or more h; and (*d*) the photosensitivity once abolished by 40 C can be slowly restored after subsequent incubation at 25 C. Anaerobiosis and cycloheximide also cause inhibition of developed photosensitivity, and spores subsequently recover the ability to germinate after the inhibitory conditions are removed (15, 16).

Effects of Temperature on Postinduction Processes. Since high temperature markedly inhibited germination when applied during the postinduction period (Fig. 1C), the nature of this inhibition was further examined. Photoinduced spores were incubated at 40 C for various durations with the incubation starting from 0, 1, 3, and 6 h after irradiation (Fig. 6). The germination of spores subjected to 40 C immediately after irradiation was inhibited after a lag period of 2 h. This lag shortens for spores treated at 40 C 1 and 3 h after irradiation. However, the germination of spores subjected to 40 C after 6 h from photoinduction is much less inhibited even with 12 h of 40 C treatment. All treated spores retained their full viability.

Photoinduced spores were next incubated at 40 C for a fixed duration of 3 h after various periods from irradiation. Figure 7 clearly shows that: (*a*) the first 3 h of postinduction is highly sensitive to 40 C; (*b*) spores start escaping from the 40 C inhibition after 3 h in the postinduction, with a half-time of escape at about 4.5 h; and (*c*) spores incubated at 25 C for 6 or more h after irradiation escape from the inhibitory action of 40 C completely. Practically the same results as above were obtained, when spores were treated at 40 C for 4 h (data not shown).

The postinduction pattern of recovery from 40 C inhibition was

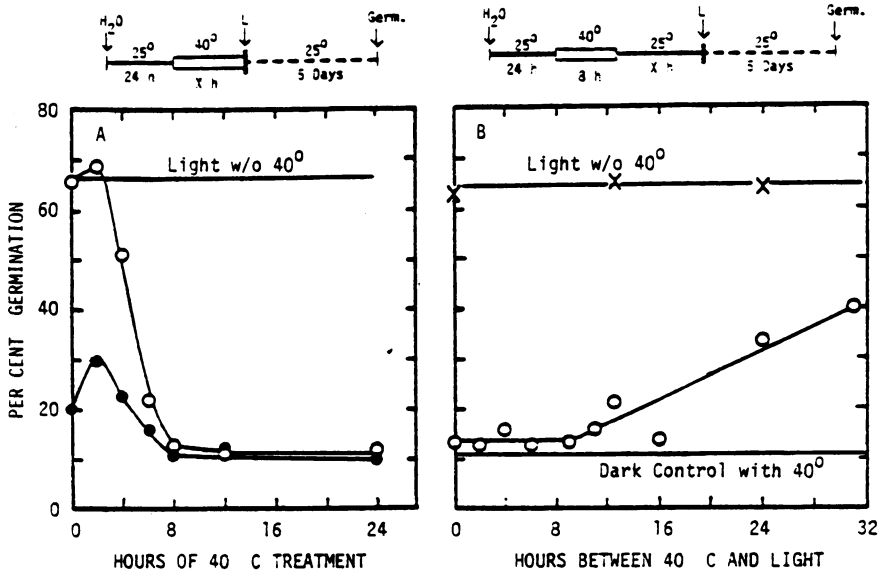

FIG. 4. Inhibitory action of 40 C (A) and reversal of the inhibition by subsequent incubation at 25 C (B) in the spores whose photosensitivity had been fully developed by initial dark soaking at 25 C for 24 h. Experimental protocols are shown above this and later figures; L signifies 5-min irradiation and X in the diagrams is plotted on the abscissa of the figures. (○): Irradiated spores; (●) (Fig. 4A): dark-incubated spores.

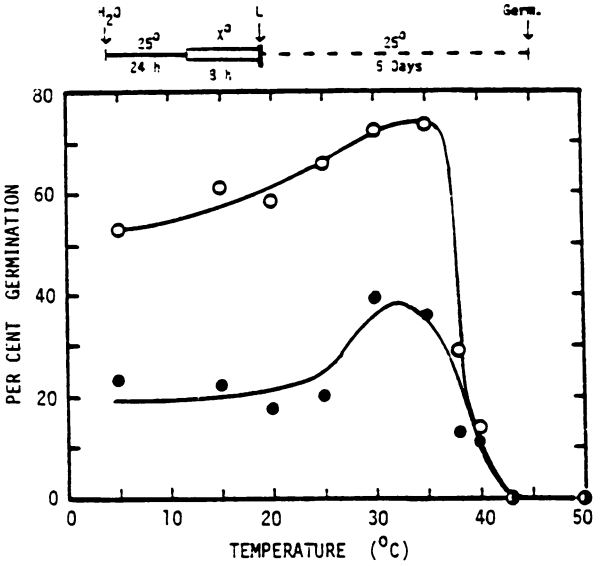

FIG. 5. Effect of temperature on preinduction spores. (○): Irradiated spores; (●): dark-incubated spores.

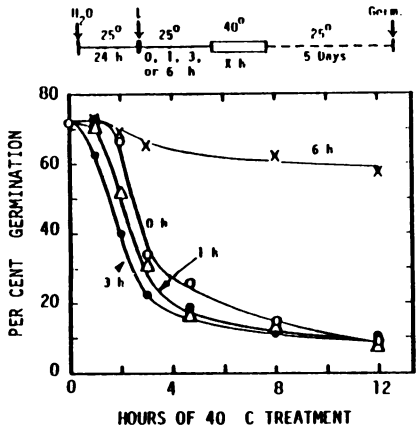

FIG. 6. Effects of various periods of 40 C treatment given 0, 1, 3, and 6 h after irradiation.

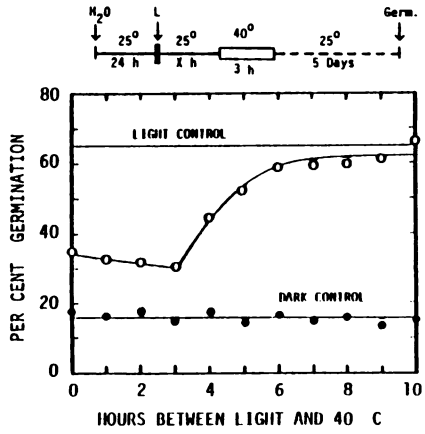

FIG. 7. Effect of 3-h treatments with 40 C given various periods after light.

examined, as follows: photoinduced spores were subjected to 40 C for 4 h, between 2 and 6 h of postinduction. After varying periods at 25 C, spores were irradiated with the second light. The recovery curve (Fig. 8) show that: (*a*) there is no noticeable lag period; (*b*) the half-time or recovery is about 7 h; (*c*) the maximal recovery reached at about 16 h is less than the germination level of the light control; and (*d*) the ability to recover gradually decreases as the interval between 40 C and the second light is prolonged beyond 20 h. In comparison with the preinduction recovery curve (Fig. 4B), the postinduction recovery is much faster with no noticeable lag.

The temperature sensitivity of the postinduction phase as examined with 3-h treatments at various temperatures (Fig. 9) is similar to the temperature curve for the preinduction processes (Fig. 5), with slight stimulation at 30 C and slight inhibition at low temperatures. Temperatures higher than 35 C cause strong inhibition of germination. Spores at 50 C were found not viable as in the case of the preinduction study.

The postinduction phase has thus an early step which is strongly inhibited by a short treatment at 40 C. Spores treated at 40 C recover from the inhibition much faster than the preinduction spores, but the maximal recovery does not reach the level of controls and a long recovery period decreases the ability of spores to germinate. In addition, postinduction spores can escape from 40 C inhibition if the start of the treatment was delayed, but no escape was detected in preinduction spores. With 40 C treatments, escape and recovery patterns are clearly different between pre- and postinduction spores.

Germination Steps of *Onoclea* Spores. Not only with 40 C treatment (this report), but with anaerobiosis (15) or cycloheximide (16) treatment, postinduction spores, but not preinduction spores, are capable of escaping from inhibitory conditions. The

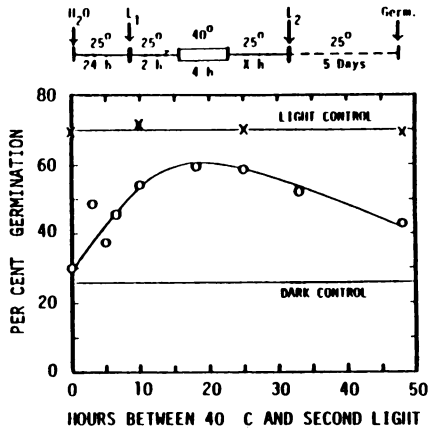

FIG. 8. Recovery from 40 C inhibition by subsequent incubation at 25 C. In the diagram of experimental protocols L_1 signifies the first 5-min irradiation and L_2 the second 5-min irradiation.

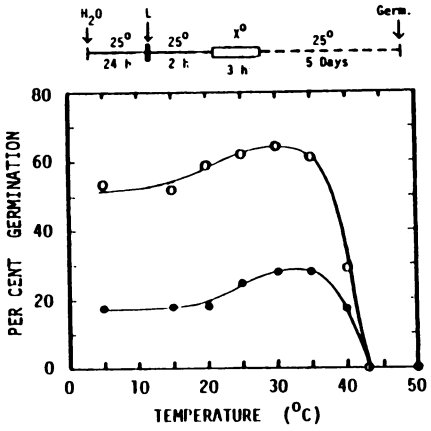

FIG. 9. Effect of temperature on postinduction spores. (○): Irradiated spores; (●): dark-incubated spores.

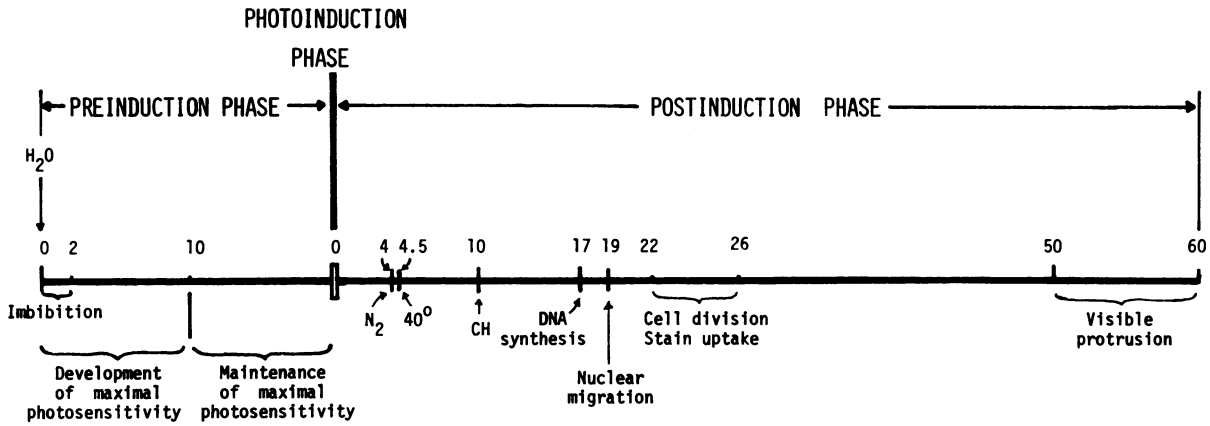

FIG. 10. Summary diagram showing the germination processes of *Onoclea* spores, using our standard germination procedure (with incubation at 25 C and a short exposure to light). Numbers on the time course line are all in h measured either from the start of soaking (for preinduction) or from the light treatment (for postinduction). Although the development of maximal photosensitivity is indicated here to occur 10 h after the onset of imbibition, this timing varies widely among spore batches. Timing for inhibition by anaerobiosis (N_2, taken from ref. 15), 40 C (taken from Fig. 7), or cycloheximide (CH, taken from ref. 16) corresponds to the half-maximal escape from respective inhibition. Timing for maximal DNA synthesis, that for the half-time of nuclear migration, and that for the half-time of asymmetrical cell division are taken from the data of Fisher and Miller (4) upon the assumption that their timing for cell division corresponds to that of our acetocarmine-chloral hydrate stain uptake (Fig. 3).

pattern and timing of escape are different for different inhibitory conditions. A half-time of escape signifies that 50% of spores have passed through an inhibitory step on their way toward germination. Comparison of half-times of escape would thus indicate a time sequence of postinduction processes. With the three inhibitory conditions studied thus far, the postinduction phase can be temporally sequenced as a step sensitive to anaerobiosis at 4 h, a step inhibited by 40 C treatment at 4.5 h, and a cycloheximide-sensitive step at 10 h. These steps are further followed by microscopically detectable steps: *i.e.*, a half-time of the stain uptake and cell division step at 22 to 28 h (Fig. 3 and ref. 15) and that of visible protrusion at about 50 to 60 h (14, 15). Fisher and Miller (4) recently reported the following timing: DNA synthesis peaking at about 17 h, a half-time of nuclear migration at about 18 to 19 h, and a half-time of cell division at about 22 to 26 h. Although their method of photoinduction of germination is different from ours, their cell division curve is practically identical with our stain uptake curve. The sequential events in postinduction is schematically summarized in Figure 10, along our standard procedure of germination study (dark incubation at 25 C interrupted by a short light treatment), which also includes preinduction events described earlier.

Preinduction spores respond differently to inhibitory effects of 40 C (this report) and anaerobiosis (15) from postinduction spores. But the pattern of cycloheximide inhibition is practically identical between pre- and postinduction spores; these observations are interpreted to indicate that cycloheximide inhibits the synthesis of a short lived protein that is needed in the postinduction phase (16). Which specific processes in preinduction would 40 C and anaerobiosis inhibit is not clear from the physiological studies thus far performed. From the data available it can be concluded that the preinduction phase is involved in preparation for maximal photosensitivity, while the postinduction phase is directed toward germination through specific steps.

LITERATURE CITED

1. EDWARDS ME 1977 Carbon dioxide and ethylene control of spore germination in *Onoclea sensibilis* L. Plant Physiol 59: 756–758
2. EDWARDS ME, JH MILLER 1972 Growth regulation by ethylene in fern gametophytes. III. Inhibition of spore germination. Am J Bot 59: 458–465
3. FISHER RW, JH MILLER 1975 Growth regulation by ethylene in fern gametophytes. IV. Involvement of photosynthesis in overcoming ethylene inhibition of spore germination. Am J Bot 62: 1104–1111
4. FISHER RW, JM MILLER 1978 Growth regulation by ethylene in fern gametophytes. V. Ethylene and the early events of spore germination. Am J Bot 65: 334–339
5. HARTT C 1925 Conditions for germination of spores of *Onoclea sensibilis*. Bot Gaz 79: 427–440
6. MILLER JH 1968 Fern gametophytes as experimental material. Bot Rev 34: 361–440
7. MOHR H 1956 Die Beeinflussung der Keimung von Farnsporen durch Licht und andere Faktoren. Planta 46: 534–551
8. MOHR H, U MEYER, K HARTMANN 1964 Die Beeinflussung der Farensporen-Keimung (*Osmunda cinnamonea* L. und *O. claytoniana* L.) über das Phytochromsystem und die Photosynthese. Planta 60: 483–496
9. RAGHAVAN V 1971 Phytochrome control of germination of the spores of *Asplenium nidus*. Plant Physiol 48: 100–102
10. RAGHAVAN V 1973 Blue light interference in the phytochrome-controlled germination of the spores of *Cheilanthes farinosa*. Plant Physiol 51: 306–311
11. SUGAI M, M FURUYA 1967 Photomorphogenesis in *Pteris vittata*. I. Phytochrome mediated spore germination and blue light interaction. Plant Cell Physiol 8: 737–748
12. TOWILL LR 1977 Light and temperature control of *Onoclea* spore germination. Plant Physiol 59: S-33
13. TOWILL LR 1978 Temperature and photocontrol of *Onoclea* spore germination. Plant Physiol 62: 116–119
14. TOWILL LR, H IKUMA 1973 Photocontrol of the germination of *Onoclea* spores. I. Action spectrum. Plant Physiol 51: 973–978
15. TOWILL LR, H IKUMA 1975 Photocontrol of the germination of *Onoclea* spores. II. Analysis of germination processes by means of anaerobiosis. Plant Physiol 55: 150–154
16. TOWILL LR, H IKUMA 1975 Photocontrol of the germination of *Onoclea* spores. III. Analysis of germination processes by means of cycloheximide. Plant Physiol 55: 803–808