Photoperiodic Flowering Response of Biloxi Soybean in 72-Hour Cycles [1, 2]

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Recently, evidence has accumulated to indicate that the photoperiodic control of floral induction is mediated by an endogenous rhythm with periods of approximately 24 hours (9, 10, 11, 12, 13, 18, 19, 20, 21, 22). It has long been known that the floral response of Biloxi soybean varies with the length of the photoperiod (4, 5, 6, 7, 18). The optimum response with 24-hour treatment cycles is obtained with photoperiods between 6 and 10 hours and normally no flowering is produced with photoperiods longer than 14 or perhaps 14½ hours (1, 28). Hamner (18), however, has shown that if a dark period of 16 hours is given in each cycle after photoperiods of different lengths, flowering is obtained with photoperiods as long as 16 and 18 hours. In those experiments, however, the cycle duration was varied with the different lengths of photoperiods given. Since Nanda and Hamner (23) have demonstrated a periodic variation in floral response as a function of cycle durations, it was difficult to predict what results might be obtained with various photoperiods given in long cycles of standard duration. In the following experiments a 72-hour, or tridiurnal, cycle was used to further assess the effects of light breaks and differential photoperiod durations on photoperiodic and rhythmic flowering responses.

Materials and Methods

Biloxi soybean (*Glycine max* L. Merr.) seed, obtained from Dr. H. A. Borthwick (United States Department of Agriculture, Beltsville, Md.), were planted in 4 inch unglazed, earthen pots in the Plant Physiology greenhouse at the University of California, Los Angeles (UCLA). All experiments except experiment II were performed between February and August 1962 at UCLA. Experiment II was conducted between June and August of 1963 under similar conditions. The following standard procedures were used unless otherwise specified.

Seeds were sown in friable, sterilized soil mixed with vermiculite (2 parts soil to one part vermiculite). The soil used was Redondo Beach sand, a fine sandy loam soil, which had been supplemented with nutrients. Three to 5 seeds were planted in pots on the surface of the soil mixture which had been thoroughly moistened. Seeds were pressed firmly into the wet soil and covered with approximately 3 cm of fresh, dry soil. After germination of the seeds, 5 to 7 days after planting, the soil in each pot was inoculated with root nodule bacteria obtained from mature soybean roots and daily watering initiated. Seedlings were grown under long-day conditions in the greenhouse at 20 to 30°. The normal day length in the greenhouse was extended to approximately 20 hours by use of Mazda lamps which were turned on at 4 PM each evening and off at 2 AM to provide about 30 ft-c of illumination at the leaf surface. Air in the greenhouse was washed and filtered through activated charcoal to minimize smog damage.

After the first primary leaf had expanded, plants were selected for uniformity and thinned to leave 2 uniform plants in each pot. When the third trifoliate leaf was fully expanded (3 to 6 weeks after planting), the pots were moved to the experimental area and prepared for treatment. Twenty to 30 plants from each lot grown were left in the greenhouse as greenhouse controls.

A battery of G.E. power-groove, cool-white fluorescent tubes (F 96 PG 17/CW) were placed above the plants at a height which allowed approximately 1500 to 2000 ft-c of illumination, as measured with a Weston quartz meter, at the leaf surfaces for controlled photoperiod treatments. Normal temperature under these lights ranged from 27 to 30°. Temperature experiments in which the photoperiod temperature was decreased were conducted using similar lights in a refrigerated temperature control room maintained at 12°. Dark treatments were provided either by manually shifting the plants to dark chambers, or in some cases automatically in individual photocyclers (27) which were opened and closed electronically. The dark temperatures were maintained at approximately 22°. After 7 photoperiodic

[1] Received Feb. 11, 1964.

[2] This work was supported in part by National Science Foundation Grant G-23983 and also by subsidy from the UCLA Space Science Center under the National Aeronautics and Space Administration sustaining grant NsG 237-62.

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cycles, plants were returned to long day lengths in the greenhouse. Approximately 6 weeks later plants were removed from the greenhouse for dissection. Sufficient growing time was given to allow the extension of at least 8 internodes above the first node showing visible floral primordia on induced control plants. The axillary buds of each plant were dissected under a dissecting microscope and only those buds showing reproductive parts or primordia were scored as flowering. Excluding any secondary branches, the number of nodes which bore floral primordia were tabulated and the total number for each 10 plants of a treatment was recorded as the floral response. For simplicity, the floral response is generally referred to as nodes flowering or the number of flowering nodes. Since the floral response is designated as the total flowering nodes per 10 plants, the standard error of the response for each treatment was calculated from the mean and multiplied by 10. In the very few cases where there were more or less than 10 plants for a treatment, the recorded value was given as the mean multiplied by 10.

Experimentation and Results

The basic tridiurnal treatment, consisting of 7 consecutive cycles with an 8-hour photoperiod followed by 64 hours of continuous darkness during the experimental period, served as the control for comparison with modified tridiurnal treatments in the following experiments. In preliminary experiments it was found that there was a direct linear relationship between the number of tridiurnal cycles and the floral response of Biloxi soybean (18). This linear relationship was also maintained for modified tridiurnal cycles in which single or multiple light interruptions were applied during the 64-hour experimental period. In the following experiments, the 72-hour cycle was selected because it was long enough to allow long durations of darkness following most of the light treatments given in the experimental period.

Experiment I. Four-Hour, High Intensity Light-Break. (See 15, 21.) Plants were removed from the greenhouse and prepared for light-dark exposures in the experimental treatment area. Treatments with 4-hour, high intensity (1500 ft-c) light interruptions initiated at one hour intervals for every hour of the 64-hour experimental period were given for 7 consecutive tridiurnal cycles and the resulting floral response of each treatment was compared to that of control treatments which received continuous darkness during the last 64 hours of each cycle. Diagrams of a few selected treatments are presented at the bottom of figure 1 for illustration. Each point of the graph in figure 1 represents the floral response, per 10 plants, resulting from 7 cycles of the specified treatment. Experimental treatments are plotted here to correspond with the time at which light interruptions were initiated in the cycle.

Additional control treatments with cycle lengths of 24 and 48 hours, each receiving 8-hour photoperiods given in 7 consecutive cycles, produced the same level of flowering as that resulting from a standard treatment of tridiurnal cycles. These results agree with the findings of Blaney and Hamner (1) and Nanda and Hamner (23) and demonstrate that the 48 hours of darkness following each short day (8 hours light: 16 hours dark) in a tridiurnal cycle is innocuous to the short day treatment.

The results presented in figure 1 indicate a rhythmic floral response to light interruptions with alternating phases of stimulation and inhibition. A complete alternation of phase occurs approximately each 24 hours. At the top of figure 1 is shown an interpretation of the results indicating that light given during the first 12 hours, or photophil phase, of each 24-hour period in the 72-hour cycle is stimulatory and light occurring during the second 12 hours, or photophobe phase is inhibitory. Although these results are consistent with the Bunning hypothesis (8), our interpretation may differ slightly in detail. It may be noted that the inhibition by light in the photophobe zones, occurring at the beginning and the end of the experimental dark period, is much greater in magnitude than that resulting from light applied in the middle of the cycle.

In figure 1, the beginning of the first inhibitory zone in the cycle might be more accurately defined by plotting the curve using the end of the light interruption period rather than the time of initiation, for it appears that light extending into the second 12-hour period is inhibitory even though it might be initiated at some earlier time. Interruptions begun at the 22-hour point and extending to the 26-hour point are innocuous, presumably because the inhibition caused by light from 22 to 24 hours is counterbalanced by the light stimulation from 24 to 26 hours. Similar problems exist in attempts to clearly define each photophobe and photophil phase. On the other hand, responses showing the definition of inhibition zones at the beginning and the end of the experimental period seem consistent with the concept of light interaction between the light break and the nearest main light period, for the light break appears to act as an extension of the photoperiod. However, this relationship holds only if the intervened duration of darkness is less than a certain critical dark period length (approximately 10 to 14 hours). Since the zone of inhibition in the middle of the cycle is sufficiently far removed from the main light periods, and is bounded on each side by zones of stimulation, the effects of such light interaction can be completely discounted for that region.

The floral response in zones of stimulation (photophil phases) following the 24 and 48-hour points are highly variable. Responses in the stimulation zones, however, are at a level which approaches the limits of resolution of our assay tool. With the exception of the deflection at the 30-hour point, differences from stimulation optima cannot be statistically demonstrated and the data from experiment I might leave

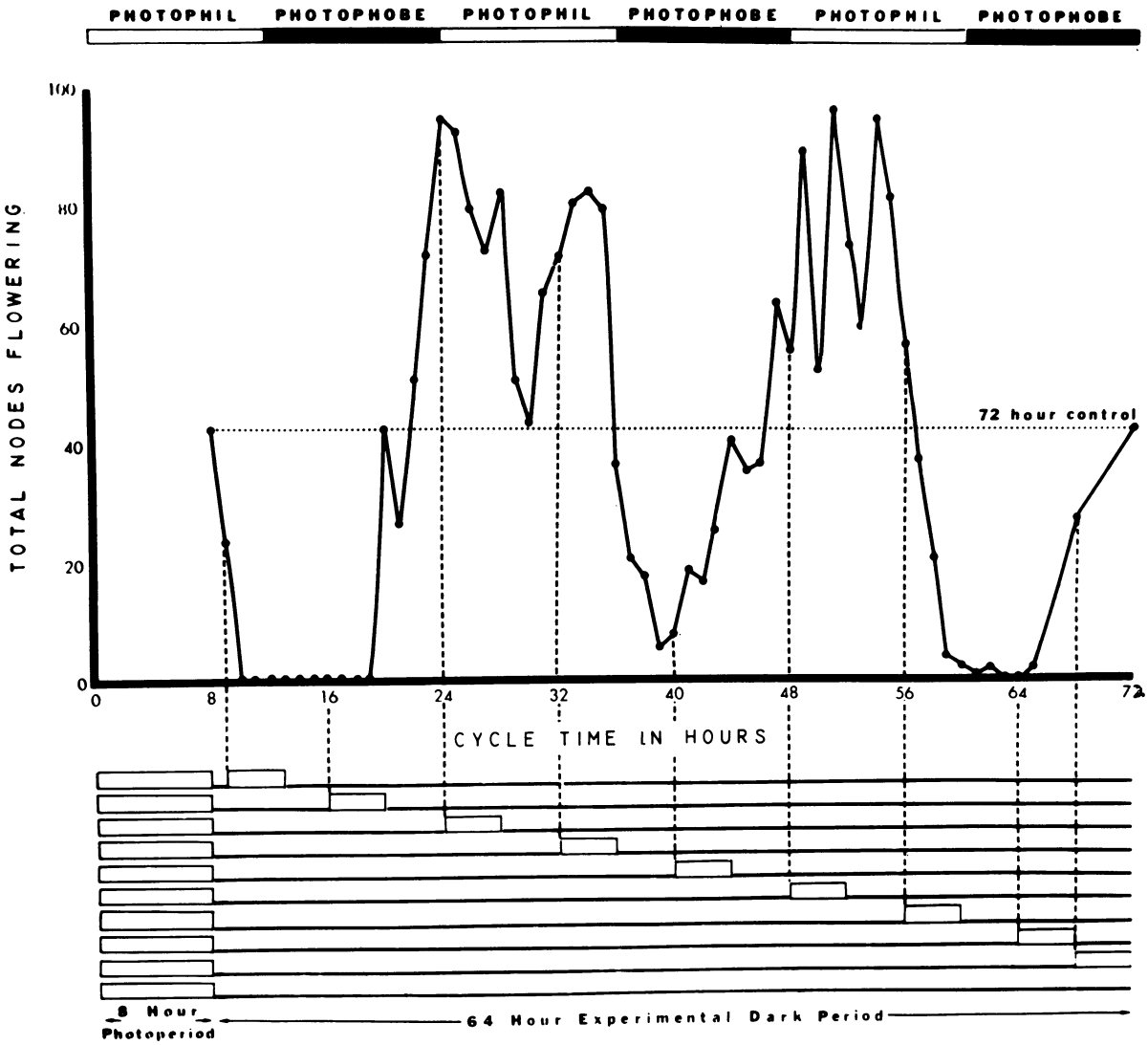

FIG. 1. Four-hour high intensity light breaks applied during the experimental dark period of a 72-hour cycle. Responses shown are those resulting from 7 cycles of the designated treatment. Points are plotted to correspond with the beginning of the light break interval. The 72-hour control level indicated is that level of flowering produced by 7 basic tridiurnal cycles in which no light interruptions were given during the 64-hour dark period. Below is a treatment diagram of selected treatments represented in a single cycle.

some serious question as to the reliability of the dip at the 30-hour point. In view of these reservations, a separate experiment was designed to quantitatively determine the level of floral stimulation caused by supplementary light applied during the second photophil phase of the tridiurnal cycle. Such an experiment should resolve any statistical fluctuations and accurately define deflections which might occur due to secondary reactions within the fundamental rhythm displayed.

Experiment II. Quantitative Determination of Photophil Light Stimulation during the Second 24 Hours of a Tridiurnal Cycle. Plants were moved from the long day conditions of the greenhouse to the experimental area. One hundred pots were separated into 20 treatment lots and the remaining 50 were retained for use as short day and tridiurnal cycle controls. The treatment lots were set up to duplicate treatments given between the 20 and 40-hour points in experiment I. Each treatment lot contained 3 experimental groups of 10 plants to which 5, 6, and 7 cycles, respectively, of the specified treatment was applied. Since it had been shown that there is a direct linear relationship between the level of flowering obtained and the number of individual cycles applied to soybean (15, 18), each treatment lot represents the equivalent of a 3 point assay for the effect of light applied at that particular time

interval. In figure 2, therefore, the solid points represent the regressed value for a 6-cycle response to a treatment in which a 4-hour light interruption was initiated at the designated point in a tridiurnal treatment cycle. The regressed 6-cycle response was calculated from the treatment responses to 5, 6, and 7 cycles of treatment and the maximum standard error for each point rarely exceeds twice the diameter of the point designations in the figure.

It was presumed that a 6-cycle response to stimulatory treatments would be more accurate than that obtained for 7 cycles because of the reduced variability in that range of flowering. The hollow circles in figure 2 represent a single point analysis for the 6-cycle response similar to that used for the 7-cycle response in figure 1. These are simply mean values given as total flowering nodes per 10 plants for each point. The dotted lines at the beginning and the end of the curve indicate that intermediate treatment points were omitted. Since the deflection at the 30-hour point below the optimum level of stimulation was significant in the single point analysis of the 6-cycle response as well as in the 7-cycle response shown in figure 1, it is obvious that the resolving power employed by the 3 point regression analysis for each treatment in experiment II far exceeds that required to test the statistical significance of the dip. Other fluctuations in this photophil interval which were found in experiment I are not substantiated by these results. Certainly this lack of floral stimulation at the 30-hour point suggests a secondary interaction with the fundamental rhythm, but thus far we have no explanation for its occurrence.

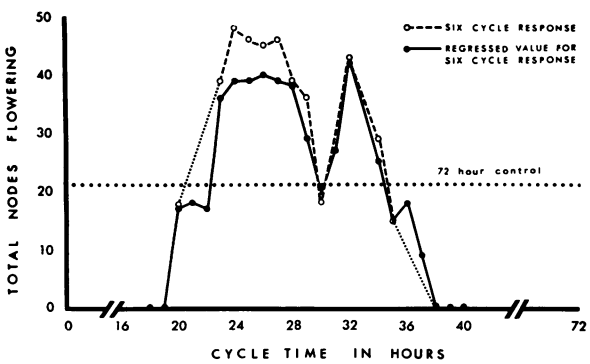

FIG. 2. Quantitative determination of photophil light stimulation due to 4-hour high intensity light breaks given during the second 24 hours of a tridiurnal cycle. Each point shown represents the beginning of the light break interval for the specific treatment as in figure 1. Hollow points designate the response of 10 plants to 6 cycles of treatment. Dotted lines connecting points indicate omission of intermediate treatments. Solid points designate the regressed value for a 6-cycle response obtained from analysis of responses obtained from 5, 6, and 7 cycles of the specified treatment. The 72-hour control level represents that level of flowering obtained from 6 basic tridiurnal cycles in which the 8-hour photoperiod is followed by 64 hours of continuous darkness.

The results of these 2 experiments not only support previous findings that an endogenous rhythm participates in the photoperiodic flowering response, but also provide some insight into the mechanism of the operation of that rhythm. It is clear from experiment I that the 8-hour high intensity light period which initiates each 72-hour cycle controls the basic oscillation of the rhythm and that these oscillations proceed through 3 complete 24-hour cycles, each cycle of which has a 12-hour phase during which light inhibits flowering. It is also clear that the endogenous oscillation initiated by the 8-hour photoperiod is not completely overcome by interruption of the cycle with a 4-hour photoperiod. Specifically this is demonstrated by the fact that a 4-hour photoperiod between the 36 and 48-hour points of the cycle while inhibitory does not completely inhibit flowering. On the other hand numerous experiments (1, 23, 24, 26) have shown that 8-hour photoperiods given each 32 to 36 hours are completely inhibitory. In the latter case, presumably each 8-hour photoperiod initiates an endogenous circadian rhythm. The rhythm initiated by each photoperiod is completely out of phase with that initiated by the previous photoperiod and no flowering results. In experiment I, however, the 4-hour photoperiod coming half way between two 8-hour photoperiods which are 72 hours apart is not sufficient to overcome the basic endogenous oscillation caused by the 8-hour photoperiod. Furthermore, it appears from experiments I and II that stimulation of flowering in Biloxi soybean results when high intensity photoperiods are distributed in time so that the period of their frequency is 24 hours or multiples thereof. Successive photoperiods coming in phase with one another with respect to the endogenous circadian rhythm increase the amplitude of that rhythm to the extent that flowering results.

It is not understood why there is irregularity in stimulation of flowering by light during photophil phases. Our results led us to carry out additional studies of the effects of the photoperiod on initiating and maintaining the rhythm and to study the effects of temperature on this particular aspect of the problem.

Experiments III and IV. Differential Photoperiods. In experiment III different lengths of photoperiod were applied in a 72-hour cycle. The effect of different photoperiod durations was also assessed in experiment IV by applying these different lengths of photoperiod as intervening treatments during the last 48 hours of a tridiurnal cycle which had been initiated by standard donor or short day treatments during the first 24 hours. The temperature sensitivity of the light period was also assessed by applying the light treatments at a reduced temperature. These experiments were run simultaneously to eliminate, or to minimize, interexperimental variation.

Plants for experiment III were separated into 13 treatment groups and given photoperiods of different durations at normal temperature (28°) followed by a continuous dark period at 22° for the

remainder of the 72-hour cycle. Another 2 treatment groups were run at 24 and 48-hour cycles as controls in which standard 8-hour photoperiods were applied at normal temperatures. Additional plants were separated into 9 treatment groups which were given different lengths of photoperiods at a reduced temperature of 12°. The dark temperature for these treatments was maintained at 22° so that only the temperature of the photoperiod was changed for this second lot of treatments. At normal temperatures (curve 1, fig 3), the optimum photoperiod occurs for a light duration of between 6 and 10 hours, as is the case for photoperiods given in a 24-hour cycle. The critical photoperiod, however, exceeds 18 hours and the shape of the floral response curve has a positive skew as compared to that which would be obtained in a 24-hour cycle where the critical photoperiod is approximately 14 hours. A reduction of the temperature during the photoperiod, as designated in curve 2 of figure 3, results in a spread of the floral response curve with a floral optimum occurring with photoperiods between 12 and 16 hours in duration. The critical photoperiod apparently exceeds 18 hours. Although the range of response is significantly spread by reduced temperature during the photoperiod, the magnitude at the optimum in each case is approximately the same. This shift in photoperiodic response indicates that there is a temperature sensitive component involved in the high intensity light effect and the differential has a temperature coefficient of approximately 2.

Plants set aside for experiment IV were given treatments in which different lengths of photoperiod were applied as intervening treatments in tridiurnal cycles. Each cycle was introduced by a donor treatment (28) consisting of an 8-hour photoperiod followed by 16 hours of darkness. The different lengths of photoperiod, therefore, were initiated at the 24-hour point in the cycle and were followed by continuous darkness at 22°. Curve 1 of figure 4 shows the floral response of intervening photoperiods given at normal temperatures of 28°. Since the control level of flowering for 7 consecutive donor cycles, or 7 consecutive tridiurnal cycles, was approximately 40 flowers per 10 plants, any response above this level represents additive stimulation to the donor treatments, whereas responses below the control level indicate photoperiods inhibitory to the donor treatments in the cycle. It is apparent, therefore, that photoperiods less than 12 hours in duration are highly stimulatory to the donor treatments. Longer photoperiods do not appear to be significantly inhibitory until they reach durations of approximately 20 hours. It was presumed, in drawing this curve, that photoperiods exceeding 20 hours in duration would completely nullify the inductive effects of donor treatments. Longer photoperiods at normal temperatures, however, were not tested. Curve 2 of figure 4 indicates the floral response obtained with different lengths of intervening photoperiods at 12°. Only the temperature of the intervening photoperiod was decreased. Donor photoperiods and dark periods were given at the same temperature used in previous experiments (28° and 22°, respectively). As was the case in experiment III, the floral response curve is shifted by a temperature reduction and a temperature coefficient of approximately 2 may be calculated. It may be noted that there is a distinct bimodal character to the curves presented in figure 4. The majority of this disposition is undoubtedly caused by the imposition of the donor response on that occasioned by the experimental, or intervening, photoperiods. There is some indication, however, that the responses have dual character for other reasons as well. The curves presented in figure 3 tend to confirm this view since their responses are not complicated by the inclusion of donor treatments.

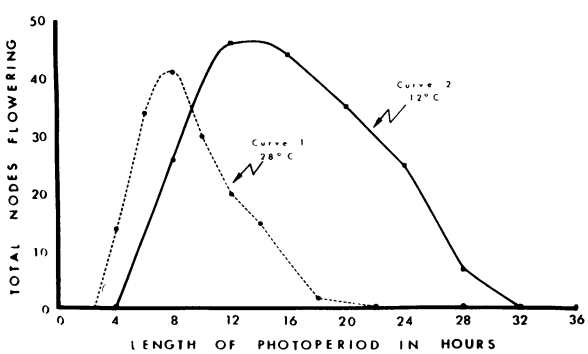

FIG. 3. Floral response to different lengths of photoperiod given in 7 repeated 72-hour cycles. Curve 1 represents the response to photoperiods given at a normal temperature of 28°. Curve 2 shows the response with the temperature reduced to 12° during the photoperiod. In both curves, each photoperiod was followed by continuous darkness at 22° for the remainder of the cycle.

Discussion

It is clear that organisms throughout the plant and animal kingdoms have the ability to meter the passage of time with great accuracy. The mechanism whereby this time measurement is accomplished has been called the biological clock (14). Many organisms exhibit circadian (approximately diurnal) rhythms in behavior or activity, and it is generally assumed by biologists that these rhythms are more or less a direct manifestation of the biological clock. Since these rhythms in activity or behavior proceed even when the organism is placed under constant environmental conditions, they have been called endogenous rhythms. It is apparent in plant photoperiodism, that the organism is using some kind of a biological clock to measure the length of the day. The results presented here as well as many other recent results have indicated that the measurement of day length involves an endogenous circadian rhythm. The similarities of the influence of or lack of influence of environmental variables on the endogenous rhythms of various organisms leads one to suspect

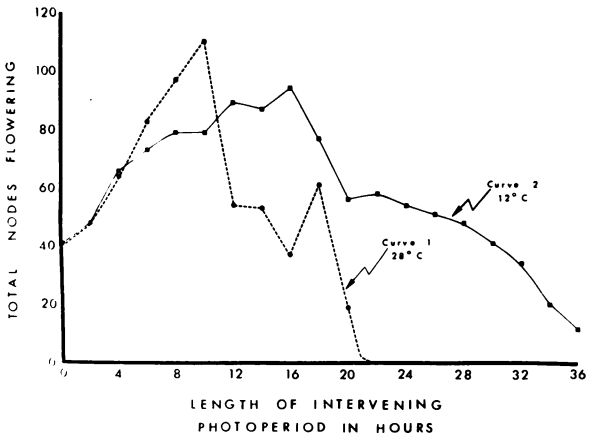

FIG. 4. Floral response to different lengths of photoperiod applied as intervening treatments in a tridiurnal cycle. Intervening photoperiods were given during the last 48 hours of a 72-hour cycle following a donor treatment (8L: 16D). Intervening photoperiods, therefore, were initiated at the 24-hour point in the cycle so that the point in the figure designated as an intervening photoperiod of zero duration represents the basic donor and tridiurnal control level produced by 8 hours light and 64 hours of darkness. Stimulation and inhibition to the basic donor treatments are indicated by responses above and below this control level of flowering. Responses in curve 1 were obtained with intervening photoperiods given at a normal temperature of 28°. Responses in curve 2 resulted when the temperature of the intervening photoperiods was reduced to 12°. In both cases above, donor photoperiods were applied at 28° and all dark periods at 22°. Each tridiurnal cycle of treatment was repeated 7 times.

that there is a single biological clock involved in all of these phenomena.

Since the amount of flowering in Biloxi soybean plants is determined by when the plant receives light in relation to an endogenous circadian rhythm, the possibility is presented of using the magnitude of flowering to measure the status of the rhythm at any particular point when the plant is illuminated. In other words, study of photoperiodism might provide an insight into the mechanism of the biological clock.

The experiments described here indicate that the endogenous circadian rhythm of sensitivity which determines the photoperiodic response may be initiated by an 8-hour photoperiod of high intensity light, and that the strength of the oscillation thereby induced cannot be completely overcome by a 4-hour photoperiod coming out of phase with this rhythm some thirty-six hours later. Sirohi and Hamner (28) have shown that a 12-hour day is essentially innocuous with respect to the flowering response of Biloxi soybean, and this in spite of the fact that Biloxi soybean plants exposed to consecutive short days of 12 hours will flower. It appears that this plant fails to flower on day lengths longer than its critical (about 14 hours) because such day lengths are actively inhibitory to flowering and that the critical day length is determined by the length of the day beyond 12 hours which is sufficiently inhibitory to overcome the plant's natural tendency to flower. The oscillations of an endogenous rhythm induced by an 8-hour photoperiod gradually decrease in amplitude during the successive phases of the rhythm to the extent that illumination during the first photophobe phase is extremely inhibitory to flowering while illumination during the second photophobe phase is less inhibitory and during the third photophobe phase still less inhibitory, as is indicated by the control curve of Nanda and Hamner (23). On the other hand, in the present experiments the third photophobe phase showed a great deal of inhibition caused by 4 hours of illumination. Presumably, therefore, the marked inhibition caused by 4 hours of illumination toward the end of the long dark period in the 72-hour cycle was an interaction between that light period and the succeeding photoperiod of the next 72-hour cycle. It seems possible that the 4-hour photoperiod initiated some sort of a rhythm of its own and that if the photophobe phase of that newly induced rhythm coincided with the photoperiod of the next cycle a marked inhibition resulted. Since the only measure we have of the status of the rhythm with periodic illumination is the ultimate flowering response and since this response is both stimulated and inhibited by light, the problems of interpretation are not simple.

Due to the impressive and almost overwhelming evidence for the involvement of phytochrome in the photoperiodic response as a photoreceptor (16), it would seem appropriate that some attempt be made to interpret our results in relation to the phytochrome pigment. It has been shown that the application of red or white light causes the conversion of the red absorbing phytochrome (P_r) to its far-red absorbing form (P_{fr}) and that subsequent radiation with far-red light will convert the pigment back to its P_r form (3). P_{fr} may also revert in darkness to P_r via another pathway which is purported to be temperature sensitive (2, 16). Hendricks (16) has estimated the half life of this dark reversion to be about 2 to 3 hours for most plants, and perhaps 4 hours for Biloxi soybean. It has been proposed that a light break in the middle of the night, or after 4 hours of darkness, causes the conversion of the reverted P_r to P_{fr} and that P_{fr} results in the inhibition of flowering in short-day plants. This explanation proves inadequate for the experiments presented in this paper. In experiments I and II, the light break for every experimental treatment is more than sufficient to totally convert phytochrome to its active P_{fr} form. Much the same reasoning holds for experiments III and IV in that the long dark periods following the light treatments are in every case sufficient to allow complete reversion of P_{fr} to the P_r form. If phytochrome is the critical component the results clearly indicate that the effects of P_{fr} may be inhibitory, innocuous, or stimulatory to flowering depending upon its time of occurrence in the cycle. It seems optimistic to suppose that phytochrome should be enzymatically responsible for such a

diversity of metabolic reactions. One might presume, however, that the action of phytochrome was dependent upon interaction with its available substrate (16, 17). This might indeed be true, but at this point it would seem more appropriate to discuss the response in terms of substrate rather than phytochrome. There is good evidence that phytochrome is the photoreceptive pigment involved in the photoperiodic flowering response, but a rhythmic change in physiological state should be related to the primary energy source, light, rather than phytochrome. In experiment I, therefore, one would say that light was stimulatory, innocuous, or inhibitory to flowering depending on its time of occurrence with respect to an endogenous rhythm with periods of approximately 24 hours. An endogenous circadian rhythm appears to regulate the photoperiodic flowering response, and in spite of the fact that light may serve to phase or even initiate the rhythm, there is no evidence that phytochrome has any direct function in the time measuring capacity exhibited by the rhythm. Our results are in good agreement with the Bunning hypothesis in that light given during the photophil, or first 12 hours, of any 24-hour interval in the cycle is indeed stimulatory. The second 12 hours on the other hand is apparently photophobe, rather than scotophil as Bunning suggested (8, 10). Light is obviously inhibitory during the second 12-hour interval. However, darkness in this interval is innocuous rather than stimulatory.

Sirohi and Hamner (28) have shown that light given in photoperiods shorter than the critical but exceeding 12 hours in duration may be inhibitory to flowering even though they may themselves be inductive. In experiments III and IV a study was made of the effect of photoperiod length on flowering in cycles long enough so that the photoperiod, regardless of its length, was always followed by long dark periods. In both these experiments flowering occurred at photoperiods much longer than would be obtained at cycle lengths of 24 hours. In both experiments, lowering the temperature during the photoperiod had a marked effect upon the length of the photoperiod required to produce the maximum flowering, indicating that the stimulation of flowering by high intensity photoperiod has a high temperature coefficient. In experiment IV where the variable photoperiod was given in an intervening cycle of 48 hours in length between 7 donor cycles, the stimulation of flowering by the intervening photoperiod at normal temperatures occurred during the first 12 hours of the photoperiod only. On the other hand, at lower temperature the stimulation occurred over a period of nearly 24 hours. It appears, therefore, that the basic rhythm induced by the photoperiod may be influenced by temperature.

In both experiments with differential photoperiods, the dark periods following each photoperiod are longer than any possible critical dark duration. The critical photoperiod at a normal temperature of 28° in experiment III, therefore, is not due to a limitation of a dark requirement. Experiment IV demonstrates that the decreased floral response resulting from a photoperiod of 20 hours is not simply due to lack of floral stimulation, but rather an active inhibition in that the floral response falls below that of the donor control treatment.

Although the interaction between stimulation and inhibition in the control of the photoperiodic flowering response is apparent, there is little evidence that would indicate the number of these processes which might be in operation. A major question concerning floral rhythmicity involves whether the rhythm is initiated with the onset or cessation of a light or dark treatment. In experiments by Blaney and Hamner (1) and Nanda and Hamner (23) standard durations of photoperiod have been applied in cycles of different lengths. Since the initiation of the dark period in each cycle is uniformly removed from the beginning of the light period, only the time of light initiation is varied in this type of treatment. For experiments using different lengths of photoperiod in a cycle of a standard duration, as was the case in experiment III, the time of light initiation in each case is identical, whereas the onset of darkness varies. The former experiments, therefore, indicate that the observed rhythmicity is associated with the initiation of light (or cessation of darkness) independent of any light cessation or dark initiation. In light break experiments such as that represented in experiment I, interruptions of the experimental dark period constitute a simultaneous variation both of the time of light initiation and dark initiation in the cycle. The resulting rhythmicity diverges from that obtained in cycle duration experiments sufficiently to suggest that a second rhythm associated with the initiation of darkness is interposed upon the light rhythm.

The positive skew of floral responses found in experiments III and IV are further suggestive of 2 separate rhythms. The curves presented in figure 4 are particularly suggestive of a bimodal distribution. One might speculate that this could be a result of 2 independent and overlapping response curves.

There are good indications, therefore, that there are at least 3 separate processes involved in the photoperiodic responses of Biloxi soybean which apparently interact to determine the time measuring capacity of the organism. The first process might be referred to as the dark requirement or light interaction effect. This component acts like an internal timer or hourglass and would tend to mask the other components when its effects are limiting. If phytochrome plays any direct role in time measurement, it would probably be limited to this component which is only one facet of the clock mechanism. The second component, as illustrated by response to cycles of various duration (23), appears to be a rhythmic process associated with the beginning of the light period. The rhythmic periods of this oscillator are insensitive to temperature, but the magnitude of the resulting floral response may be damped by a reduction in temperature and a temperature shift may

cause a shift in the phase (25). The third component, as revealed by experiments III and IV with differential photoperiods, is an oscillator associated with the light duration or time of light extinction. In this case, the magnitude of the optimum floral response is not affected by temperature change, but the duration of the optimum photoperiod is strongly dependent on the temperature and is increased with a temperature decrease. Correspondingly, the critical photoperiod is affected by temperature. Interaction between these rhythmic components might well illustrate the complexity of temperature compensation associated with photoperiodism and the biological clock. Certainly the quality of temperature compensation does not mean that temperature sensitive processes are not involved in the clock mechanism, but more probably that various components of the clock respond to temperature in opposing ways so that the effect is homeostatic.

Summary

Photoperiodic flowering responses of Biloxi soybean (*Glycine max* L. Merr.) were investigated in a 72-hour, or tridiurnal, cycle using high intensity light breaks and differential photoperiods. Four-hour high intensity light breaks given during the 64-hour experimental dark period of a tridiurnal cycle (each cycle initiated with an eight-hour high intensity light period) may be stimulatory, innocuous, or inhibitory to flowering depending on the time at which the interruption is applied. The application of such light breaks at various times during the cycle results in a rhythmic pattern of floral responses with alternate phases of stimulation and inhibition in periods of approximately 24 hours. Light applied during the first 12 hours, or photophil phase, is stimulatory whereas light which occurs during the second 12 hours or photophobe phase is inhibitory. The results indicate that the 8-hour high intensity photoperiod given at the beginning of each 72-hour cycle initiates a fundamental oscillation which persists through 3 complete 24-hour periods. High intensity light breaks produce oscillations which may damp the fundamental if their frequencies fall out of phase with it. On the other hand, if secondary oscillations fall in phase with the fundamental, the amplitude of the rhythm is increased to the extent that flowering results.

The application of differential light durations both as initial and intervened photoperiods in a tridiurnal cycle shows that the critical photoperiod is much longer than that obtained in 24-hour cycles. Since 72-hour cycles provide long dark periods after each photoperiod, the photoperiodic response cannot be ascribed to a critical dark requirement. Long photoperiods may inhibit and completely nullify floral stimulation, however, indicating that photoperiodic responses result from an interaction between stimulatory and inhibitory processes. Temperature during the photoperiod has a marked influence both on the critical photoperiod and the duration of light required for a maximum flowering response indicating that the high intensity light reaction has a high temperature coefficient. The results denote that the basic endogenous rhythm has separate components which interact to produce a temperature compensated clock mechanism which mediates photoperiodic time measurement.

Acknowledgments

The authors are indebted to Mr. John J. O'Connor for technical assistance and to Dr. Giraj S. Sirohi and Dr. Bruce H. Carpenter for help during this investigation. Thanks are also due Mr. William H. Shumate and Mr. Paul H. Moore for help in correcting the manuscript.

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The Role of Glycolic Acid Metabolism in Opening of Leaf Stomata [1]

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The initial observation (23) that α-hydroxysulfonates, which are effective competitive inhibitors of glycolate oxidase (20), prevent the opening of stomata in the light stimulated further experiments on the physiology of stomatal movement (15). It seemed reasonable to suspect that the enzymic oxidation of glycolic acid was connected with the process of stomatal opening, and hence that any interference with either the synthesis or further oxidative metabolism of this substrate might also result in an inhibition of stomatal opening.

In a previous report (15), we presented evidence, obtained from a standard leaf disk assay of stomatal closure, that the processes of opening and closing are controlled by different and independent mechanisms. By use of biochemical inhibitors as well as by other means, it was found possible to inhibit opening and thereby induce closing. In other related investigations, the effect of the chemical control of stomatal opening on the rates of transpiration and photosynthesis have been investigated in the laboratory, the greenhouse, and outdoors (10, 11, 14, 25, 27, 28).

Further experiments have now been carried out on the mechanism of action of α-hydroxysulfonates on stomatal movement, and of the effect of the concentrations of CO_2 and O_2 in the atmosphere surrounding the leaf tissue on the synthesis of glycolic acid and on stomatal movement. The results clearly demonstrate the close relation between the synthesis and metabolism of glycolic acid and the process of stomatal opening that takes place in the light. A preliminary account of these experiments has been published (24).

Materials and Methods

Tobacco leaves (*Nicotiana tabacum*) were kept in the dark for an hour before the start of the experi-

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