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FAR RED REVERSIBILITY OF THE INDUCTION OF RAPID CHLOROPHYLL ACCUMULATION IN DARK GROWN SEEDLINGS*

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Abbreviations: R = red light; FR = far red light; D = darkness; P = phytochrome; P_r = red absorbing form of P; P_{fr} = far red absorbing form of P.

SUMMARY

In leaves of dark grown bean, the degree of reversibility by far red of red-induced induction of rapid chlorophyll accumulation in white light depends upon the duration of a dark period between short red and far red irradiations. Reversibility reaches a maximum for a dark period of about 9 s. It is proposed that this can be explained by interaction of at least two first order processes. The first, with a rate constant at 20°C of 0.23 s^{-1}, is thought to represent migration of P_{fr} to sensitive sites. The second, with a rate constant of 0.1 s^{-1} is the escape from phytochrome control of one of the early steps in the induction process.

1. INTRODUCTION

The accumulation of chlorophyll *a* in seedlings of plants grown in complete darkness does not proceed at an appreciable rate upon transfer to white light during a lag period which may last for a few hours. This lag period can be abolished by exposing the plants to red light for a short period, some hours prior to the continuous exposure to white light. On the basis of action spectra this effect, called induction, has been shown to be mediated by phytochrome (see RAVEN 1973). Considerable controversy has existed as to the far red reversibility of this effect of red light. In contrast to the classical picture for responses mediated by phytochrome, in several plants the inductive effect of a red pre-irradiation could only be abolished to a small degree by a subsequent far red irradiation provided the plants had never been exposed to any light. RAVEN & SPRUIT (1972) and RAVEN (1973) have shown that the additional inductive effect of a second short red irradiation following the first one after a few hours, on the other hand was largely reversible by far red. This is accompanied, at least in pea seedlings, by a very marked increase in the fluence required for a standard induction (RAVEN & SHROPSHIRE 1975). This phenomenon has been called "de-etiolation". We have attempted to explain the change in photoreversibility accompanying this process by proposing that a very small quantity of P_{fr} initially formed migrates to sensitive centres where it is bound irreversibly. Thereby

* Dedicated to Prof. E. Havinga, on the occasion of his 70th birthday.

these centres become activated to develop the biosynthetic systems for rapid pigment production during the ensuing white light period (RAVEN & SPRUIT 1973). A consequence of this model is that the effect of a red irradiation applied to a completely etiolated plant should not be immediately reversible by far red since the latter maintains a level of P_{fr} sufficient to more or less saturate the centres. During migration the local concentration of P_{fr} increases leading to a P_{fr}/P_{tot} ratio in the centres, far above the value initially established outside. Consequently the FR reversibility should increase during the process and a certain inductive fluence of red, while not instantly reversible, should become increasingly so during a time period following the red irradiation. A study of this development of far red reversibility could provide information on the kinetics of the postulated migration process. Accordingly we have examined this process in dark grown bean and pea seedlings.

2. MATERIALS AND METHODS

Seeds of *Pisum sativum* L. cv. "Groene Krombek" were obtained form Nunhem's Zaden, Haelen, Holland. *Phaseolus vulgaris* L. cv. "Widuco" was obtained from C. Beemsterboer N.V., Warmenhuizen, Holland.

Plants were grown for 7 days at 20 °C in complete darkness, as reported previously by RAVEN (1973). All operations prior to the irradiation of the

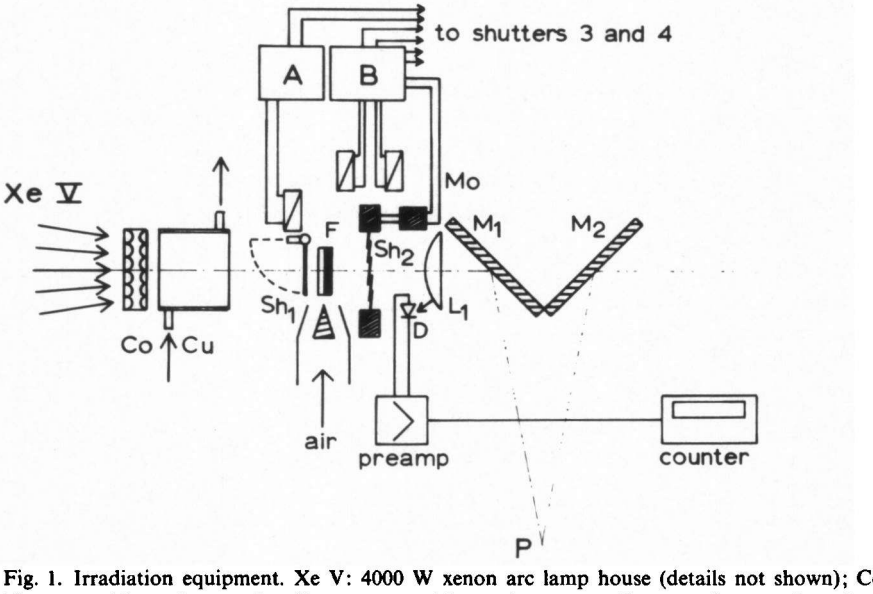

Fig. 1. Irradiation equipment. Xe V: 4000 W xenon arc lamp house (details not shown); Co: "honeycomb" condenser; Cu: filter cuvette with running water; $Sh_{1....4}$: shutters; L_1: plano-convex lens; M_1, M_2: mirrors; Mo: shutter winding motor (a second one operates Sh_4); D: photodiode; A, B: shutter commands; F: interference filter; P: plants to be irradiated. A second lamp house and associated equipment is on the right. Baffles and other arrangements to keep out stray light not shown.

seedlings were done in complete darkness. After the inductive irradiation the plants were returned to darkness for 16 h (beans) or 24 h (peas), after which they were placed under white fluorescent light at 20 °C for 5 h (beans) or 2 h (peas) at an intensity of 1.1–1.5 W m^{-2}. Chlorophyll was estimated as reported previously (RAVEN 1973) by the method of BRUINSMA (1963). Amounts of chlorophyll were based on a constant number of leaves. This circumvents problems arising from formative effects of the inductive radiation resulting in changes in dry or fresh weight. All data are expressed as per cent of the amount of chlorophyll extracted from controls that received a standard dose of red only.

The irradiation equipment (*fig. 1*) consisted of two 4000 W high pressure xenon lamps mounted in commercial housings ("Xenosol V", Zeiss). A 10 cm layer of running water (Cu) removes a large part of the long wave far red. In addition, the interference filters (F) are cooled by an intense current of air. The filters are Baird Atomic, half-with about 15 nm, max. transmission about 60%. For red irradiation, a filter with transmission max. at 659 nm was used, far red was 731 nm. The light intensities measured at the level of the leaves with a wavelength corrected photodiode meter ("Optometer 80X", United Detector Technology Inc.) were: red, 26–30 W m^{-2}, far red 24–26 W m^{-2}. They were kept constant throughout a series of experiments, different fluences being obtained by varying the exposures.

3. RESULTS

Notwithstanding the high irradiances available, red fluences approaching saturation of the inductive effect could only be obtained for beans in periods of more than 4 s. Since this proved to be comparable to the time constants of the reactions

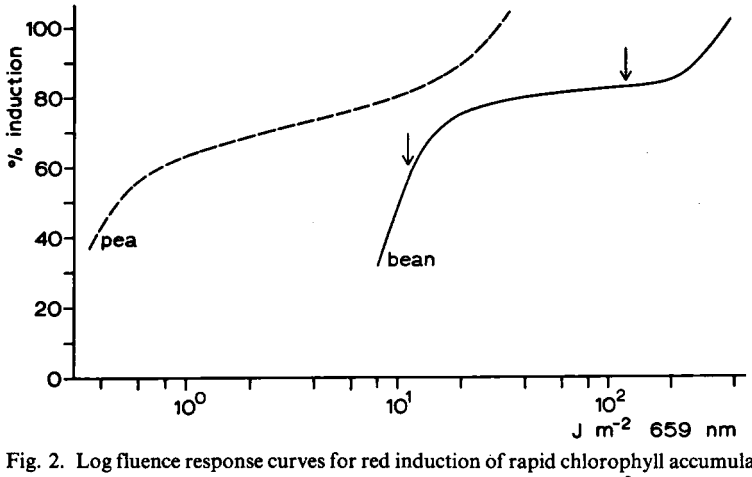

Fig. 2. Log fluence response curves for red induction of rapid chlorophyll accumulation in pea and bean. Fluences were obtained at constant irradiances of 26–30 W m^{-2} by variation of the exposure time. Arrows indicate fluences applied in the experiments of *fig. 3a* and *3b*.

involved, we tried three strategies. In a first series of experiments, the following irradiation schedule was adopted: 0.38 s R followed by a variable dark period after which 3 s FR was given. This whole sequence was then immediately repeated 11 times, so that a total of 4.2 s (126 J m^{-2}) R and 33 s (858 J m^{-2}) FR was given to the plants. These red fluences fall on the first, quasi saturated, induction level (*fig. 2*) (see discussion). The effect of various dark periods between R and FR is shown in *fig. 3b*. Since the interpretation of these repetitive irradiation experiments might pose problems, in another series only one R-D-FR cycle was given. The fluence of R applied in this case, 11.4 J m^{-2}, was decidedly below saturation and as *fig. 2* shows, the level of induction in this case is about 66% of that of the previous case. The results of this type of experiments are shown in *fig. 3a*. Third, since pea seedlings are more sensitive to red than beans by a factor of more than 10 (*fig. 2*), they were treated with a single exposure of 0.38 s (11.4 J m^{-2}) R, followed by the usual dark periods and terminated by 30 s (750 J m^{-2}) FR. The results were qualitatively similar to those of *fig. 3a*, though the reproducibility left to be desired. We return to this point in the discussion.

4. DISCUSSION

The experiments of both *fig. 3a* and *3b* show some interesting features. First, we observe a range of dark periods between a R and a FR exposure for which the FR reversibility of R is decidedly enhanced. In the experiments of RAVEN (1973) the red irradiation usually lasted for 60 s. It is clear that effects such as those shown in *fig. 3* should completely escape notice under such conditions. Second, at the shortest dark periods applied in the present study, reversibility sharply decreases and it appears that, at least in the one-shot experiments (*fig. 3a*), far red immediately following red has no reverting capacity.

The data in *fig. 3* can be interpreted as resulting from a combination of two first order processes:

$$\text{Ind.} = a.e^{-k_1 t} + b(1-e^{-k_2 t})$$

Curves calculated from this equation are shown for the following parameters:
Fig. 3a, full line: $a = 1.00$; $b = 0.70$; $k_1 = 0.23\ s^{-1}$; $k_2 = 0.095\ s^{-1}$,
Fig. 3b, full line: $a = 0.80$; $b = 0.80$; $k_1 = 0.22\ s^{-1}$; $k_2 = 0.10\ s^{-1}$.
In *fig. 3a*, a slighly better fit is obtained with $a = 1.16$; $b = 0.72$; $k_1 = 0.26\ s^{-1}$; $k_2 = 0.09\ s^{-1}$ (broken line). This however, seems implausible since it would imply that FR immediately following R would lead to 16% more induction than R alone. Though this may not be impossible, it would require considerably higher irradiances (i.e. shorter exposures) to establish this with certainty. Since it does not appear to be particularly relevant to the present problem, we will leave this question open for the moment.

The agreement between measured values and calculated curves appears satisfactory, the more so since the kinetic constants for the two types of irradiation schedules are close together. We conclude, then, that the time course of reversibility is governed, on a time scale of less than a few minutes, by two

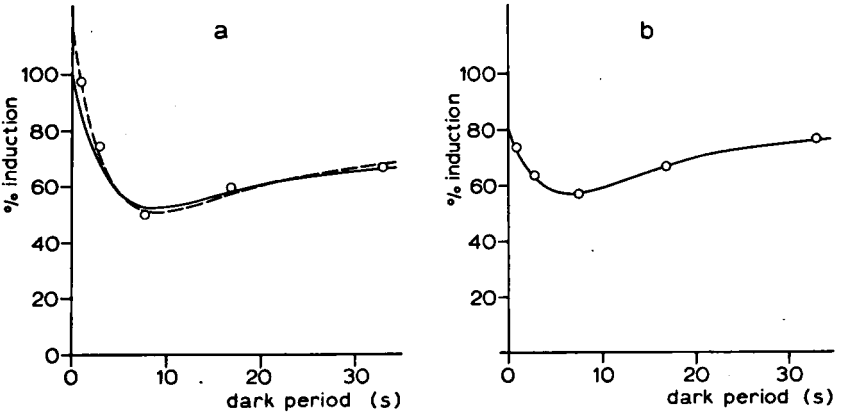

Fig. 3. Induction of rapid chlorophyll accumulation in bean by an irradiation schedule: R-D-FR, as a function of the dark interval.
a. One-shot experiment, 0.38 s R (11.4 J m^{-2}), 3.2 s FR (83 J m^{-2}). Full line: a = 1.00; b = 0.70; k_1 = 0.23 s^{-1}; k_2 = 0.095 s^{-1}. Broken line: a = 1.16; b = 0.72; k_1 = 0.26 s^{-1}; k_2 = 0.090 s^{-1}. The 100% level represents the induction by 0.38 s R only.
b. The same irradiation schedule as in a., repeated 11 times in immediate succession. Full line: a = 0.80; b = 0.80; k_1 = 0.22 s^{-1}; k_2 = 0.10 s^{-1}. The 100% level represents the induction by one continuous exposure to 4.2 s R (126 J m^{-2}). Note that the 100% level in fig. a corresponds to an induction that is about 66% of that in fig. b (cf. *fig. 2*). Circles represent experimental data.

processes. The first leads to a rapidly increasing reversibility, with a half life of about 3 s. The second is a first order "escape" from photoreversibility, with a half life of about 7 s.

In the experiments with pea, in which plant the reversibility after 1 min R exposures is low, we obtained evidence for a similar though less pronounced maximum in the reversibility in one-shot experiments at a dark period of 2–3 s. We tentatively ascribe this to a more rapid and more complete escape from reversibility in this plant. These factors make pea, notwithstanding its far higher light sensitivity, rather unsuitable for this type of experiment.

The question arises as to the nature of reactions 1 and 2. One possibility is that reaction 1 represents the decay of an intermediate preceding P_{fr}, since as long as no P_{fr} has been formed, FR obviously cannot phototransform it. After completion of the irradiation this intermediate would have time to form P_{fr} during a dark period, reversibility increasing with time. At closer examination this explanation, however, appears unattractive for the following reason. The slowest step in the reaction sequence leading to P_{fr} is:

$$\text{meta-}R_b \longrightarrow P_{fr}.$$

(KENDRICK & SPRUIT 1973, 1977: SPRUIT & KENDRICK 1977). The reaction constants for this step are incompletely known. LINSCHITZ et al. (1966) and SMITH (1973) have determined them for phytochrome in solution. They fall in the range 0.26–0.38 s^{-1} ($t_{1/2}$ = 2.7–1.8 s) at 0.6° and 2°C. Extrapolating this to

20 °C with a (hypothetical) $Q_{10} = 2.5$, we obtain $t_{1/2} = 0.4$–0.3 s. The reaction constants have not been determined *in vivo*. However, in experiments with seedlings of *Amaranthus caudatus* and pea we observed that at 22 °C the decay of meta R_b *in vivo* was more rapid than the writing speed of the recorder (KENDRICK & SPRUIT, unpublished) which brings $t_{1/2}$ in this case to well below 0.5 s. There can be little doubt that the rate of this reaction is strongly dependent upon temperature. It appears, therefore, that reaction 1 is about ten times slower that the decay of meta-R_b *in vivo* as well as *in vitro*.

Another possibility is that the reaction represents migration of phytochrome. We will suppose that P_{fr} formed by light moves to acceptor sites by a first order process with $k_1 = 0.23\,s^{-1}$. Activation of these sites is essential for induction but there is another phytochrome-dependent process involved which partially escapes phytochrome control by another first order process with $k_2 = 0.10\,s^{-1}$. PRATT & MARMÉ (1976) have measured the time course of pelletability of P in *Avena* after R irradiation *in vivo*. At 10 °C, the lowest temperature used, this process was observed to be pseudo first order with $t_{1/2} = 4$–5 s. In maize coleoptiles, LEHMANN & SCHÄFER (1978) observed more complicated kinetics that could be resolved into three first order components. The most rapid of these had a half life of about 5 s at 25 °C. The authors suggest that this reaction represents decay of an intermediate to P_{fr}. For the reasons stated above, this interpretation must be considered improbable. It appears more likely that these rapid reactions indeed are migrations of P_{fr}. Since in pelletability studies no information has as yet been obtained about the nature of the receptor(s), at this time it cannot be excluded that, within a single plant organ or cell, a number of binding sites exist, with different kinetics for the binding reaction, explaining non-first order kinetics of pelletability of bulk P. On the other hand, it appears well established now that a fraction of the P in dark grown plants exists already bound to certain membranes. We propose that our reaction 1 represents a rapid migration of P_{fr} to an organelle involved in the induction process. Reaction 2 could then be related to the pre-existing P fraction. The nature of these two components is as yet unknown. They may even be located in different parts of the plant.

The difference between the initial reversibility ($t_D = 0$) in *fig. 3a* and *3b* can be understood by observing that in the repetitive irradiation experiments, which last for a total period of 5 or 6 seconds (as well as in one-shot experiments of longer R exposures), a fraction of the P_{fr} repeatedly formed during the R exposures, will have had time to partially occupy the centres. It is unfortunate that even our shortest exposures, 0.38 s, are not really very short compared with the time constants of the reactions. It is desirable to repeat these experiments with higher irradiances which, however, were not available to us.

Little can be said about reaction 2. Obviously a chain of reactions initiated by P_{fr} at some (early) stage will escape the control by light. FREDERICQ (1964, 1965) e.g. has observed that the reversibility of the effect of a red night break upon the flowering response in *Pharbitis* and *Kalanchoë* is lost on a time scale of minutes.

It is remarkable that in bean (and considerably less so, in pea) no complete escape can be observed, 20–30% of the induction remaining reversible after dark periods of from several minutes to hours (see also RAVEN 1973). We are therefore tempted to suppose that in this stage operation of a third phytochrome dependent process becomes apparent. The suggestion that more than one phytochrome controlled process is involved has also been made for the induction of lettuce seed germination (BLAAUW-JANSEN & BLAAUW 1976a, 1976b; SMALL et al. 1979). The fluence response curves in *fig. 2* also show similarity with those for lettuce seed germination in the presence in both of two response regions, one with a high, the other with a considerably lower light sensitivity. This has been interpreted as evidence for two phytochrome controlled reactions operating in series (SMALL et al. 1979).

Finally, the above remarks may be relevant to the interpretation of fluence response curves like those in *fig. 2*. Often, as in the present study, such curves are obtained by varying the exposure at constant irradiance. Even at the relatively high irradiances available in our experiments, the higher fluences in *fig. 2* were obtained at exposures comparable in time to or exceeding the time constant of reaction 2. It is not unlikely, therefore, that the apparent transition from a light sensitive to a less sensitive response, as shown in *fig. 2* is linked in some way to the progress of reactions 1 and 2 during long exposures. If, as suggested, a third phytochrome process is indeed involved, becoming the controlling factor after dark periods of more than 20 s, its light requirement may be predicted to be considerably higher than that of reaction 1. With our standard inductive fluences this reaction is already partly saturated. These considerations form an argument for measuring fluence response curves for the induction of rapid chlorophyll accumulation, as well as for other phytochrome-controlled reactions, at exposures not exceeding 0.5 s. Also, reversibility should be studied at both higher as well as lower levels of induction. Unfortunately, sufficiently high irradiances are hard to obtain.

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