Seed Dormancy and Germination: Light and Nitrate

Kiemrust en kieming: licht en nitraat

Promotor: Dr. C. M. Karssen
persoonlijk hoogleraar bij de vakgroep Plantenfysiologie

Henk W. M. Hilhorst

Seed Dormancy and Germination: Light and Nitrate

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op woensdag 21 maart 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

Stellingen:

1. De oecologische betekenis van de "very low fluence response" ligt voor de licht-geinduceerde kieming van onkruidzaden eerder in een zeer korte blootstelling aan vol daglicht tijdens grondverplaatsing dan in een langdurige perceptie van zeer zwakke lichtintensiteit dicht onder het grondoppervlak.
   Hartmann and Nezadal (1990), Naturwiss. 77, In Press.

2. Het verschil tussen breken van kiemrust en stimuleren van kieming wordt onvoldoende onderkend en leidt daardoor vaak tot het onjuist opzetten van experimenten en interpreteren van de resultaten.
   Dit proefschrift.

3. Een verhoogde zuurstofopname van zaden na toediening van nitraat vormt geen bewijs dat nitraat de zuurstofopname beïnvloedt.
   Adkins *et al.* (1984), Physiol. Plant. **60**: 234-238.
   Hilton and Thomas (1986), J. Exp. Bot. **37**: 1516-1524.

4. Ondanks het feit dat is aangetoond dat bepaling van de nitraatreductase activiteit *in vivo* het meest betrouwbaar is wanneer deze gerelateerd wordt aan de afnamesnelheid van de hoeveelheid nitraat, worden activiteitsbepalingen nog steeds verricht door meting van de snelheid van toename van nitriet, hoewel goede nitraatbepalingsmethoden beschikbaar zijn.
   Soares *et al.* (1985), Physiol. Plant. **64**: 487-491.
   Dit proefschrift.

5. De discussie of verandering van de respons op plantehormonen het gevolg is van veranderingen in hormoongehalte of -gevoeligheid gaat voorbij aan de gedetailleerde en experimenteel onderbouwde modellen voor ligand-receptor interacties in dierlijke cellen.
   Trewavas and Cleland (1983), Trends Biochem. Sci. **8**: 354-357.
   Firn (1986), Physiol. Plant. **67**: 267-272.
   Weyers *et al.* (1987), Plant, Cell Environ. **10**: 1-10.

6. Het is waarschijnlijker dat de glycolyse een sleutelrol vervult bij de breking van kiemrust dan de pentose-fosfaatweg.
   Roberts (1973), *In*, W. Heydecker, ed, Seed Ecology. Butterworths, London. pp. 189-218.
   Come and Corbineau (1989), Abstr. 3rd Int. Workshop on Seeds, Williamsburg, USA.

7. Het feit dat de twee landen in Afrika die het economisch voor de wind gaat zowel een vrije pers als een parlementaire democratie hebben, zou de Afrikaanse bestuurders die hardnekkig blijven vasthouden aan hun eigen ontwikkelingsmodellen, moeten stimuleren deze modellen ter discussie te stellen.
   Sub-Saharan Africa. From crisis to sustainable growth. A long-term perspective study. The World Bank, Washington DC, October 1989.

8. De rol van vleerhonden bij de instandhouding van het tropisch regenwoud wordt, getuige de massale jacht op deze dieren, nauwelijks onderkend.

9. Er dienen eisen gesteld te worden aan de schriftelijke en mondelinge uitdrukkingsvaardigheid in het Engels van doctoraalstudenten.

10. De instelling van een rookverbod in overheidsgebouwen maskeert wellicht de onmacht van een overheid om grote milieuproblemen met even grote snelheid en daadkracht aan te pakken.

Stellingen behorende bij het proefschrift
"Seed dormancy and germination: light and nitrate".

Wageningen, 21 maart 1990 H. W. M. Hilhorst

"It is certain that all bodies whatsoever, though they have no sense, yet they have perception; for when one body is applied to another, there is a kind of election to embrace that which is agreeable, and to exclude or expel that which is ingrate; and whether the body be alterant or altered, evermore a perception precedeth operation; for else all bodies would be like one to another."

Francis Bacon
(about 1620)

voor Ingrid en Yowanka

CONTENTS

CHAPTER 1

General Introduction

General Introduction

The end and the beginning of the lifecycle of most higher plants is marked by the stage in which mature seeds are shed and dispersed. At this stage seeds may be in a condition to survive prolonged periods of unfavorable conditions, such as drought, unfavorable temperatures, poor soil conditions, and partial or absolute darkness. Seeds, either dry or imbibed, may remain in the soil for perhaps hundreds of years without losing their viability. The moment environmental conditions are favorable for the species and seeds are sensitive to these conditions many seeds of the soil seedbank will germinate and develop into new plants.

One implication of this survival mechanism is that seeds must be able to sense their environment in order to guarantee a high probability of a successful life cycle of the plant that will grow from the seed.

Until a few decades ago the main occupation of seed physiologists was to study the influence of environmental factors on germination. The wealth of information that has been gained clearly shows that seeds are indeed very sensitive to a great number of environmental factors. During the last 20 to 30 years more attempts have been made to translate the action of environmental stimuli into mechanisms that eventually lead to germination (*e.g.* Mohr, 1966; Roberts, 1969). This thesis is one of these attempts. The stimulating factors that were studied are light and nitrate. However, before presenting the state of knowledge about these factors the concept of dormancy and its definition that is used in this thesis will be introduced.

Dormancy

Viable seeds that do not germinate in an environment that is perfectly adequate for germination may be called dormant. The inability to germinate is the result of a block to germination in the seed itself. This block has to be removed before the seeds become sensitive to environmental factors that stimulate germination. The biological significance of dormancy must be seen in an ecological context. It may be regarded as a device for optimizing the distribution of germination in time and space. A thorough

description and discussion of the many aspects of dormancy can be found elsewhere (Bewley and Black, 1982; Karssen, 1982).

Over the years many definitions and forms of dormancy have been introduced. This has not contributed to unequivocal use of the term. Nikolaeva (1977) introduced no less than 15 types of dormancy while Lang *et al.* (1987) found 54 different terms in the literature. In an attempt to clarify the confusing situation they proposed three main classes of dormancy with at least 15 sub-classes. These classifications are based on the many different factors that impose dormancy. However, so long as the underlying mechanism(s) of dormancy breaking and induction are not known, it is premature to distinguish as many types of dormancy as factors that influence dormancy.

Together with the many forms of dormancy numerous definitions of dormancy can be found in the literature. Most of the definitions ignore the specific feature that distinguishes dormancy from inhibition of growth by inhibitors or unfavorable conditions, namely the fact that a dormant organism does not necessarily resume growth when placed in growth-favoring conditions.

In the present study the scheme of Fig.1 is used as a model for changes in dormancy and stimulation of germination.

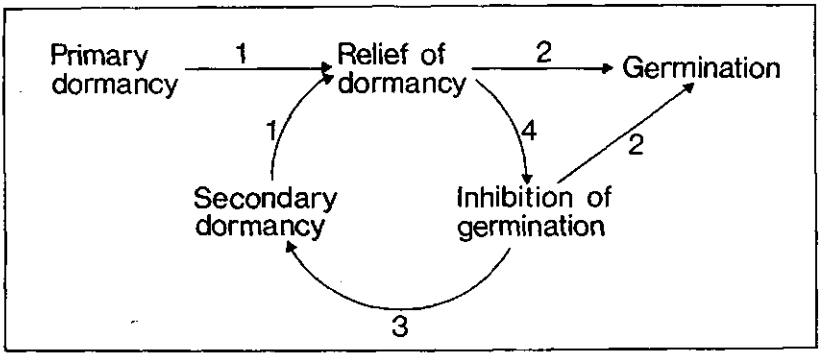


Fig. 1: Schematic presentation of changes in dormancy. For explanation see text. After Karssen (1982).

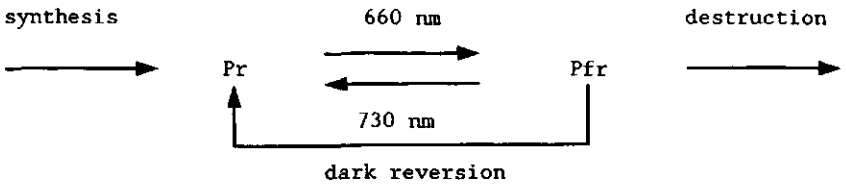
In many cases, newly formed mature seeds possess primary dormancy. This dormancy may gradually disappear upon storage of the dry seeds ('after-ripening'). Imbibed dormant seeds require incubation at a certain temperature for a certain period to relieve their primary dormancy (1). Only then seeds will become sensitive to factors that stimulate germination, but they will only germinate when the complete set of required factors is present (2). If this is not the case, germination will be inhibited (4). This state may be called quiescence. When germination is inhibited for extended periods, the seeds may enter a new state of dormancy called secondary dormancy that can be relieved by similar conditions as primary dormancy (3).

Temperature is the major regulating factor in this 'dormancy cycle'. The set of factors that is required for germination of non-dormant seeds contains an optimum temperature. This temperature is not necessarily similar to the optimal temperature for dormancy breaking. This is a strong indication that breaking of dormancy and stimulation of germination are separate processes.

Consequently, the definition of seed dormancy used in this thesis is: ***dormancy is a temporary arrest of growth under otherwise growth-favoring conditions.***
Dormancy may be partial or virtually complete, in that the range and/or magnitude of conditions required for germination may vary.

Light

The stimulating effect of light on seed germination of many wild species has been known for a long time. Since the experiments of Borthwick *et al.* (1952), it has generally been agreed that red/far-red reversibility of a response is the result of the action of the plant pigment phytochrome. This pigment occurs in two forms: a stable inactive form with an absorption maximum at 660 nm (Pr) and an unstable form with an absorption maximum at 730 nm (Pfr) that is physiologically active. Schematically the phototransformations may be summarized as follows:



Many factors play a role in phytochrome-controlled germination: the amount of pre-existing Pfr, conversions of phytochrome intermediates in dry, partially hydrated and fully imbibed seeds, fluence and wavelength of the applied irradiation, number of irradiations, and interactions with other factors, especially temperature (see Bewley and Black, 1982; Frankland and Taylorson, 1983 for extensive reviews).

Despite the many reports on phytochrome-controlled germination, the role of phytochrome in the transduction chain that leads to germination is far from clear. In several species it was shown that gibberellins (GAs) could overcome the requirement for light (Taylorson, 1982). This by-pass of light requirement has led to the suggestion that Pfr plays an important role in the biosynthesis of GAs. It has indeed been shown that exposure to light increased levels of endogenous GAs in seeds of sitka spruce (*Picea sitchensis*) (Taylor and Wareing, 1979). Furthermore, enhancement of the promotive effect of GAs by light has been reported (Taylorson and Hendricks, 1976; Bewley, 1980). It was concluded that Pfr increased the sensitivity to GAs.

It is likely that he first step of the transduction chain between the transformation of Pr to Pfr and the final germination response is the binding of Pfr to a specific receptor, X, resulting in a phytochrome independent intermediate (I):

$$Pfr + X \longrightarrow PfrX \longrightarrow I$$

Evidence for this assumption comes from the phenomenon of escape from the antagonistic effect of far-red (FR) irradiation. When imbibed seeds are irradiated with red light (R) under conditions that favor germination, and

irradiated with FR after variable periods, the seeds escape from FR inhibition. The escape time is a measure of the reaction time of the binding of Pfr to X and subsequent transformation to the FR-insensitive intermediate. There is no conclusive evidence as to the nature of this Pfr binding component but it is generally assumed to be located in a membrane. Indeed, membrane fractions can be isolated to which phytochrome binds (Gallagher *et al.*, 1988). Also differential pelleting and sequestering of the Pr and Pfr forms with membrane-like structures has been reported (Pratt, 1986 and references therein). Sub-cellular distribution of phytochrome visualized by immunocytochemical labelling gave some indications of possible associations with plasma membrane, endoplasmatic reticulum and nuclear envelope in oat coleoptile parenchyma cells (Coleman and Pratt, 1974). Furthermore, factors known to influence membranes, such as temperature shifts and anesthetics, may influence phytochrome induced responses (Taylorson 1984, 1988).

Models

The monomeric model

There is little knowledge about the transduction chain after binding of Pfr to its receptor X that finally results in a response. Evidence is accumulating that calcium may play a role as secondary messenger (Roux, 1986). Nevertheless, several models have been described to explain responses to light.

Based on changing escape times during dark incubation of *Portulaca oleracea* and *Rumex crispus* seeds, and the calculated Pfr "activities" from the slopes of the escape curves, Duke *et al.* (1977) concluded that the germination response was a function of the level of interaction between Pfr and X and independent of the total level of phytochrome or the decay rate of Pfr to Pr. Based on a simple second-order reaction:

$$Pfr + X \underset{k_2}{\overset{k_1}{\rightleftharpoons}} PfrX \longrightarrow Response \qquad (1)$$

with $k_2/k_1 = K_{eq}$ = equilibrium constant, an expression was derived in which the response y was a function of the fluence, P_{tot}, μ (level of PfrX for 50 % germination), and σ (standard deviation of μ):

$$\text{probit } y = 5 + 1/\sigma \, (\ln[PfrX]_{eq} - \mu) \qquad (2)$$

with $[PfrX]_{eq}$ = level of [PfrX] at equilibrium =

$$1/2 \{ [Pfr]_i + [X]_i + K_{eq} - \sqrt{([Pfr]_i + [X]_i + K_{eq})^2 - 4[Pfr]_i[X]_i} \} \qquad (3)$$

$$\text{with } [Pfr] = (1 - e^{-(S1 + S2).a.Nt}).\phi.[P_{tot}] \qquad (4)$$

where S1 and S2 are photoconversion constants

a = attenuation factor based on seed coat transmission

ϕ = photoequilibrium = S1/(S1 + S2)

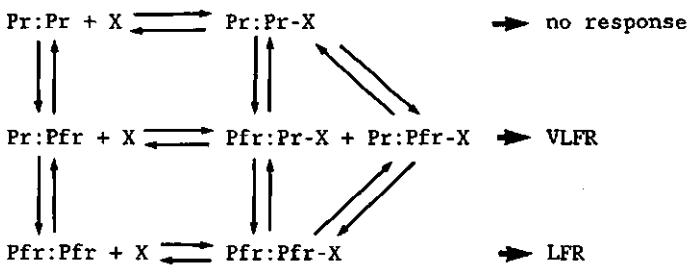
Nt = fluence

$[Pfr]_i$ and $[X]_i$ are initial concentrations

The expression of the response in probits is required because germination is an 'all-or-none' response. The tolerances of the individual seeds of the population are log-normally distributed around the dose for 50 % germination. With the appropriate values for the parameters of equation (2), Duke (1978) simulated the set of fluence-response curves obtained during dark-incubation of *Rumex crispus* seeds. An important limitation of the model is its use of whole-population parameters for the decreasing sub-population that responded to the light-stimulus. Normalization of the response is required to compare the curves (e.g. DePetter *et al.* 1985). Furthermore, the data presented by Duke (1978) lacked sufficient detail to assess the goodness of fit to the model. Nevertheless, it was concluded that the changing sensitivity to light during dark-incubation was independent of the levels of $[P_{tot}]$ and [X].

The dimeric model.

As early as 1972 (Briggs and Rice) a dimer structure for the phytochrome molecule was proposed. It has been shown that the molecule exists *in vitro* as a dimer of two identical sub-units each containing one chromophore (Hunt and Pratt, 1980; Jones and Quail, 1986). Evidence is now accumulating that phytochrome also exists as a dimer *in vivo* (Brockmann *et al.* 1987). With one exception, all attempts to correlate final responses with the state of the phytochrome system have so far been based on the simple Pr-Pfr scheme. The exception is the work of VanDerWoude (1985) who adopted the dimer concept to explain biphasic fluence-response curves. Many R-induced responses, including those of seedling growth (Mandoli and Briggs, 1981) and seed germination (Blaauw-Jansen, 1983; Kendrick and Cone, 1985; DePetter *et al.*, 1985), occur in response to two distinct fluence ranges. The two response types are termed very low fluence response (VLFR) and low fluence response (LFR). The fluence requirements of these response types differ approximately 10,000 fold. In the dimer model of VanDerWoude (1985) phytochrome acts as a dimer that exists in three interphototransformable species. The primary elements of the proposed reaction scheme were:



The VLFR results from the specific association of Pr:Pfr with a receptor X, while the LFR results from the establishment and action of Pfr:Pfr-X. VanDerWoude tested this model by comparing the fluence ranges required for the VLFR and LFR responses with those required for the formation of Pr:Pfr-X and Pfr:Pfr-X. The relative contributions of each complex of the

reaction scheme to the total amount of phytochrome (P:P) could be calculated from an equation derived from equation (4). Assuming stoichiometric association of Pr:Pfr and Pfr:Pfr with X, the fluence range over which the formation of the two active complexes occurred was determined. It followed from the model that the level of [X] in the seeds used (*Lactuca sativa* cv. Grand Rapids) was about one thousandth of the total amount of phytochrome dimers and occupation of less than one percent of [X] by Pr:Pfr would result in a detectable response. The model could explain the occurrence of biphasic fluence-response curves and the fluence ranges over which the responses occurred. Moreover, since fluences required to maximize the responses were generally similar between different sensitization treatments (chilling, ethanol) it was concluded that the magnitude of the responses was related to the activity and not the formation of the phytochrome-receptor complexes. This conclusion was supported by the differences in the calculated binding curves of X and the phytochrome dimers and the shape of the observed germination response curve.

DePetter (1987) introduced kinetic parameters to this model and also assumed the existence of an equilibrium between inactive and active receptors. An equation to describe the biphasic response was derived by applying the general drug theory to the reaction scheme. Complete biphasic fluence-response curves could be simulated by giving the rate constants the appropriate values. An allosteric receptor model gave the best simulations. In contrast with VanDerWoude (1985) it was concluded that both VLFR and LFR responses were essentially related to the formation of Pr:Pfr- X and Pfr:Pfr-X complexes and not their activities. It was also concluded that the degree of dormancy mainly depended on the level of activated receptors X.

As stated by the author himself (DePetter, 1987) this model has its limitations. The measured response (germination) is the end of a large number of transductions, initiated by the phototransformations. It is therefore not likely that the final response is a direct reflection of the formation of active phytochrome-receptor complexes. An indication for this may be that values given to some dissociation constants are not realistic. In the model these dissociation constants were equivalents for μ (fluence value for half-maximal response) and σ (standard deviation of μ).The values of μ and σ calculated from the experimental curves were orders of

magnitude lower than the arbitrary values used in the model.

Nitrate

Nitrate has long been known to promote germination in many species (Roberts and Smith, 1977; Vincent and Roberts, 1977). In the recent past two proposals for the mechanism of action of nitrate have been described (Roberts, 1969; Roberts, 1973; Hendricks and Taylorson, 1975; Roberts and Smith, 1977). The hypothesis of Roberts was based on observations that inhibitors of glycolysis, the citric acid cycle and the terminal oxidation reactions of the mitochondrial electron transport chain could promote germination. Supposition of the model was that dormant seeds were deficient in some oxygen-requiring processes required for germination while non-dormant seeds were not. The alternative oxygen-requiring process would be depleted of oxygen because of a lower affinity for oxygen than the cytochrome pathway of respiration. Inhibition of the latter by inhibitors, such as azide and cyanide would direct the flow of oxygen to the alternative pathway, thus breaking dormancy. The pentose phosphate pathway was suggested to be the alternative oxygen-requiring process essential for germination. Germination stimulants like nitrate and nitrite were assumed to re-oxidize $NADPH_2$ to NADP, thus stimulating the operation of the pentose phosphate pathway (Fig. 2a). For this purpose their $NADPH_2$ dependent reductases had to be induced.

The other hypothesis (Hendricks and Taylorson, 1975) also acknowledges the importance of the pentose phosphate pathway but assumes that re-oxidation of $NADPH_2$ proceeds through peroxidase action (Fig. 2b). This assumption was based on the observation that germination in lettuce and pigweed (*Amaranthus albus*) seeds was correlated with inhibition of catalase activity. It was hypothesized that inhibition of catalase activity by nitrite, thiourea and hydroxylamine provided peroxidase with substrate for the $NADPH_2$-requiring reduction of H_2O_2 to H_2O.

Both models have been criticized extensively (Bewley and Black, 1982). The main objection to the models is the lack of evidence that the pentose phosphate pathway plays a role in the breaking of dormancy.

The hypothesis that a shift in respiratory metabolism is essential for

the transition from the dormant to the non-dormant state has been tested in great detail in *Avena fatua* (Adkins *et al.*, 1984a/b). It was shown that azide, an inhibitor of cytochrome oxidation, could stimulate the uptake of oxygen and also induce germination. Oxygen uptake and germination were

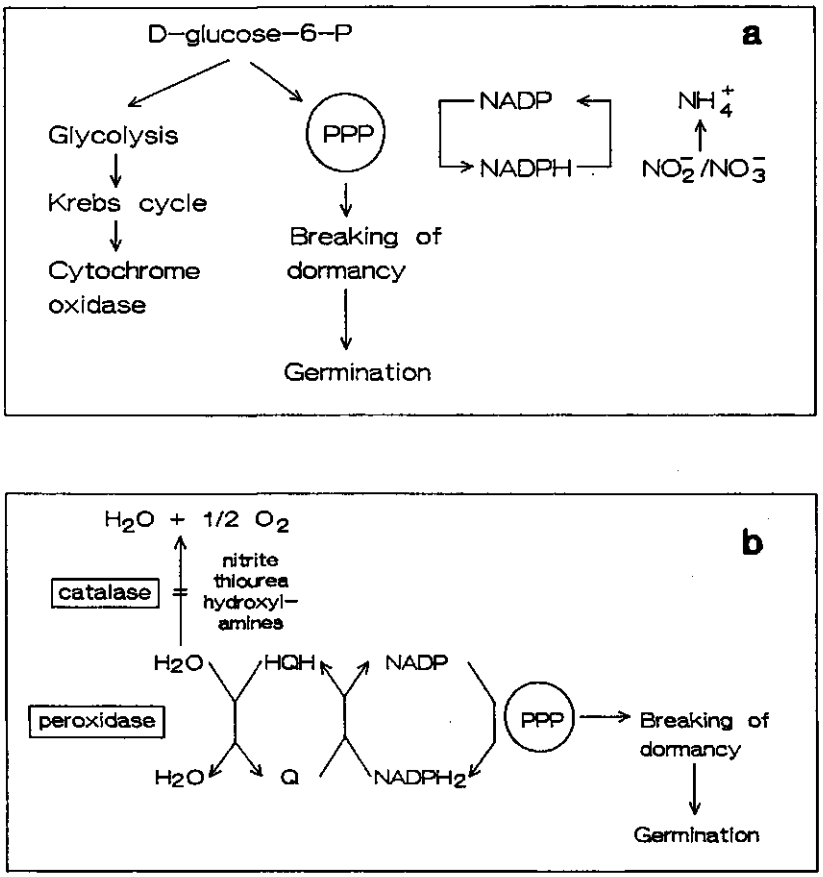


Fig. 2: Hypotheses for the role of hydrogen acceptors in breaking of dormancy through activation of the pentose phosphate pathway (PPP). (a) after Roberts (1973), (b) after Hendricks and Taylorson (1975)

inhibited when, besides azide, SHAM was applied. SHAM alone did not inhibit germination. It was concluded that either the cytochrome pathway of respiration or the alternative pathway was required for germination. Similarities between the effects of azide and nitrate, especially with respect to oxygen uptake, lead to the conclusion that azide and nitrate overcame the same block to germination, in that both components were able to induce re-oxidation of NADH. However, a causal relationship between uptake of oxygen and breaking of dormancy remains to be proven.

Interaction between light and nitrate

For a number of wild species seed germination has been shown to depend on the simultaneous presence of Pfr and nitrate (*e.g.* Vincent and Roberts, 1977; Karssen and de Vries, 1983; Hilton, 1985; Probert *et al.*, 1987). In other species nitrate stimulated germination in the dark, but the possible effect of pre-existing Pfr was not excluded (Roberts and Smith, 1977). the role of pre-existing nitrate in light-promoted germination has been considered in a few studies only (Goudey *et al.*, 1988). Thus, the question whether the simultaneous presence of Pfr and nitrate is obligatory in the species that are light- and/or nitrate-requiring remains to be answered. Generally, the interaction between light and nitrate was reported to be synergystic, but only a few studies have been undertaken to characterize the interaction in more detail. In seeds of *Ranunculus sceleratus* the promotive effect of nitrate depended on the level of Pfr in the seeds and increased the sensitivity of the seed population to temperature shifts (Probert *et al.*, 1987). Light-promoted seed germination in *Kalanchoë blossfeldiana* could be modulated by nitrate (DePetter, 1985). From the allosteric receptor model for the action of phytochrome in these seeds it was concluded that nitrate could increase the level of active Pfr:Pfr-X by influencing the dissociation constant of this complex.

Ecological significance of light and nitrate

In seeds, phytochrome is a detector for light quantity and quality. It enables germination of seeds that are at or close to the soil surface, provided that other factors are not limiting. Phytochrome provides seeds with a device by which germination may be induced by only a very short pulse of light. This is the case when the soil is disturbed and seeds are exposed to light for perhaps less than a second. Germination may proceed even when seeds are returned beneath the soil surface to a depth of up to 10 cm (Hartmann, 1990). However, seeds that are less sensitive to light need to be on the soil surface for considerable longer periods. A flush of germination of weeds in a crop field after disturbance of the soil also indicates that light may be the sole limiting factor for germination. Seeds in a soil seed bank often go through successive (seasonal) cycles of dormancy. When seeds enter a period of non-dormancy, only burial in the soil prevents them from germinating. Light-requirement, therefore, seems to be a tool to maintain a seed bank over a great length of time; only those seeds that perceive light will germinate (Karssen, 1980/81; Bewley and Black, 1982; Frankland and Taylorson, 1983).

Seeds that are shaded by foliage often fail to germinate. Chlorophyll absorbs red light more effectively than far-red light. Therefore, shade light will have a lower ratio of red to far-red light than sun light, establishing Pfr/Ptot levels which may be below the threshold level for germination (Frankland and Taylorson, 1983). In this way seeds may detect their proximity to other plants and germination beneath established plants may be prevented. However, light conditions during maturation on the motherplant, such as light quality (Hayes and Klein, 1974) and photoperiod (Karssen, 1970) may be of equal importance. Also the properties of the extra-embryonic tissues, like chlorophyll content, may influence the quality of the light that is received by the embryo (Creswell and Grime, 1981).

As stated before, seed germination may depend on the simultaneous presence of Pfr and nitrate. Thus, a non-dormant, light-requiring seed may fail to germinate after exposure to light when its nitrate level is below its threshold. It may be argued that a sensing mechanism for nitrate may provide the seed with information about the nitrogen status of the soil.

This information may be provided directly by interaction with soil components, or indirectly via the motherplant. Plants grown on high nitrate levels will produce seeds with increased nitrate content (Saini *et al.* 1985; Bouwmeester, 1990). It may also be argued that the nitrate levels sensed by the seeds in the soil give information about the proximity of established plants. These plants take up nitrate from the soil, thus depleting nitrate of seeds in their vicinity (Pons, 1989).

Scope and outline of this study

It is clear that light and nitrate are both natural environmental factors that play a crucial role in the ecological behavior of many weed species. Although the promotive action of these factors has been known for a long time, little is known about the transduction pathways between perception of the environmental signals and the final germination response.

With seeds of *Sisymbrium officinale* as a model system the action of these factors and their mutual interactions were studied. After a first characterization of the system by classical germination experiments (Chapter 2), the interactions between light, nitrate and gibberellins were studied in more detail with the aid of co-factor analysis (Chapter 3). The possible involvement of phytochrome-induced nitrate reduction was studied (Chapter 4). General dose-response theory was used to study the changing sensitivity to light and nitrate during induction of secondary dormancy (Chapters 5 and 6). Finally, an integral, descriptive model of the involvement of light and nitrate in seed germination is presented and discussed (Chapter 7).

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CHAPTER 2

Gibberellin-biosynthesis and -sensitivity mediated stimulation of seed germination of *Sisymbrium officinale* by red light and nitrate

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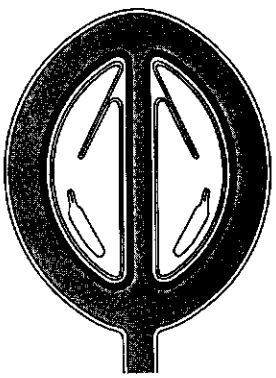
Gibberellin-biosynthesis and -sensitivity mediated stimulation of seed germination of *Sisymbrium officinale* by red light and nitrate

H. W. M. Hilhorst, A. I. Smitt and C. M. Karssen



Hilhorst, H. W. M., Smitt, A. I. and Karssen, C. M. 1986. Gibberellin-biosynthesis and -sensitivity mediated stimulation of seed germination of *Sisymbrium officinale* by red light and nitrate. – Physiol. Plant. 67: 285–290.

Light stimulated seed germination of *Sisymbrium officinale* (L.) Scop. (hedge mustard) by means of two different mechanisms. Light effect I was absolutely dependent on the simultaneous presence of nitrate. Without nitrate, red (R) irradiated seeds did not escape from the antagonizing action of far-red (FR) irradiation. The data indicated that nitrate acted as a cofactor at the level of the FR absorbing form of phytochrome (P_{fr}). The combined action of R and nitrate could be replaced by addition of the gibberellins 4 and 7 (GA_{4+7}). This action could be inhibited by the growth retardant tetcyclacis, an inhibitor to GA biosynthesis in cell free systems and intact plants. The action of tetcyclacis was fully neutralized by GA_{4+7}. It is concluded that the combination of R and nitrate stimulated GA biosynthesis. Omission of nitrate from the incubation medium enabled the study of light effects apart from GA biosynthesis. In such conditions R stimulated the sensitivity to GA_{4+7} (light effect II). The two light effects could also be distinguished by their different reactions to the temperature of a pre-treatment in water and darkness. The sensitivity to R and nitrate was subject to breaking and induction of dormancy. Both processes were stimulated at rising temperatures. Due to a different optimum, breaking of dormancy prevailed at lower temperatures and induction of secondary dormancy at more elevated temperatures. The sensitivity to GA_{4+7} rose and fell in a comparable way during dark incubation at a broad range of temperatures. The capacity of light to stimulate GA_{4+7} action did not diminish at higher temperatures, it even tended to rise. The study indicated that seed germination is regulated by an increase in both the levels of GAs and the sensitivity to GAs.

Additional key words – Dormancy, phytochrome, tetcyclacis.

H. W. M. Hilhorst, A. I. Smitt and C. M. Karssen, Dept of Plant Physiology, Agricultural Univ., Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

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Introduction

Sisymbrium officinale (hedge mustard) is a common ruderal plant on the Eurasian continent. During burial experiments it was found that seeds only passed through a seasonal pattern of change in dormancy when the soil contained sufficient nitrate (Karssen 1980/81b). Experiments under controlled conditions showed that nitrate was not required during the period that the changes in dormancy occurred, but had to be present during actual germination in combination with red light (R) (Karssen and De Vries 1983). When either R or nitrate was missing, induction of secondary dormancy occurred and the responsiveness to R and nitrate was lost.

The promotive action of nitrate on the germination of seeds has been described for several species (Roberts and Smith 1977). Also the interaction between light and nitrate is well known in many species (Vincent and Roberts 1977, 1979, Roberts and Benjamin 1979). Little is known about the mechanisms of nitrate action and its

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interaction with light (Roberts and Smith 1977, Taylorson 1982).

Interactions of nitrate and growth regulators have been reported for a number of species. Egley (1984) postulated that ethylene increased the sensitivity of *Portuclacca oleracea* seeds to nitrate, whereas Saini et al. (1985) suggested that in *Chenopodium album* seeds nitrate stimulated the responsiveness to GA_{4+7} and ethylene combinations. In wild oat (*Avena fatua*) (2-Chloroethyl) trimethylammoniumchloride (chlorocholine chloride, CCC), an inhibitor of GA-biosynthesis, inhibited the nitrate and nitrite induced germination (Adkins et al. 1984).

Growth regulators, in particular GAs, have often been found to substitute for light in the stimulation of germination, suggesting a P_{fr} controlled GA-biosynthesis. However, in *Lamium amplexicaule* (Taylorson and Hendricks 1976) and *Kalanchoë blossfeldiana* (Fredericq et al. 1983), GA action was found to be still dependent on P_{fr}, albeit at very low levels. Therefore, Bewley and Black (1982) hypothesized that if a P_{fr} stimulated increase of GAs were to happen, it would break dormancy only when it interacted with P_{fr}.

To investigate a possible relationship between the promotive action of R and nitrate on germination and the biosynthesis of GAs, a recently developed growth retardant, tetcyclacis, was used. Tetcyclacis is a norbornenodiazentine derivative which shows a remarkably high biological activity in the retardation of shoot growth in plants (Rademacher and Jung 1981). The effect of tetcyclacis on plant growth could almost completely be alleviated by the simultaneous application of GA_3. The new compound lowered the endogenous GA content in higher plants (Rademacher et al. 1984) and inhibited the oxidative reactions from *ent*-kaurene to *ent*-kaurenoic acid in a cell-free system from the endosperm of immature pumpkin seeds (Graebe 1982). It also blocked GA production in fungi (Rademacher et al. 1984).

Abbreviations – GA, gibberellin; R and FR, red and far-red irradiated; P_r and P_{fr}, red and far-red absorbing form of phytochrome.

Materials and methods

Seeds

Ripe seeds were collected from wild plants growing in natural places in the vicinity of Wageningen. A batch collected in 1979 was used. Seeds were cleaned and stored dry at 2°C in the dark. Under these conditions no changes in dormancy were observed.

Germination conditions

Triplicates of 50 seeds were sown in 5 cm Petri dishes on one layer of filter paper (Schleicher and Schüll no. 595) moistened with 1.5 ml of distilled water or the test solution. If a solution was applied temporarily, the seeds were then rinsed with 100 ml distilled water on a Büchner funnel and transferred to a new Petri dish, moistened and further incubated.

Light doses were always saturating. Red light (620–700 nm) was obtained from 6 red fluorescent tubes (Philips TL 20W/15) filtered by 3 mm red plexiglass (Red 501, Röhm & Haas, Darmstadt, GFR). Far-red light (>690 nm) came from 6 incandescent tubes filtered by 1 layer Red 501 and 2 layers Blue 627 plexiglass (Röhm & Haas). The light irradiances for R and FR were 2.6 W m^{-2} and 3.0 W m^{-2}, respectively. Sowing and all further handling of the seeds was performed in green light obtained by filtering one green fluorescent tube (Philips TL 40W/17) with two layers of yellow (no. 46) and two layers of blue (no. 62) Cinemoid filters (Strand Electric, London, U.K.).

Solutions

All compounds were dissolved in 0.01 *M* phosphate-citrate buffer pH 3.0. Tetcyclacis was dissolved in a small volume of acetone and then mixed with a large volume of the buffer solution. The solution was stirred until no further increase of the absorbance at 240 nm could be observed. The solution was filtered and kept in the dark at room temperature. Buffer composition did not affect germination.

The following substances were used: 5-(4-chlorophenyl)-3,4,9,10-pentaazatetracyclo-5,4,1,$0^{2,6}$,$0^{8,11}$-dodeca-3,9-diene (tetcyclacis = NDA = BAS 106 W, LAB 102883) and GA_{4+7}, a mixture of gibberellins 4 and 7.

Results

Interaction of red light and nitrate

The seeds of *Sisymbrium officinale* which were used in the present study only germinated to a high percentage at the optimal temperature of 24°C if they were incubated in a nitrate solution and were irradiated with a saturating R dose (Tab 1). A pretreatment at 2°C was required to raise the sensitivity to the stimulative condi-

Tab. 1. The effect of a pretreatment at 2°C in water and darkness on the germination at 24°C. At 24°C seeds were incubated in water or 10 m*M* KNO_3 and kept in darkness (D) or irradiated for 5 min with red light (R). When seeds were directly sown at 24°C, R was given after 1 h of dark imbibition.

Pretreatment at 2°C	Germination, % (± SD)			
	Water		10 m*M* KNO_3	
days	D	R	D	R
0	0	6±1	0	16±3
5	1±1	8±3	2±2	90±2

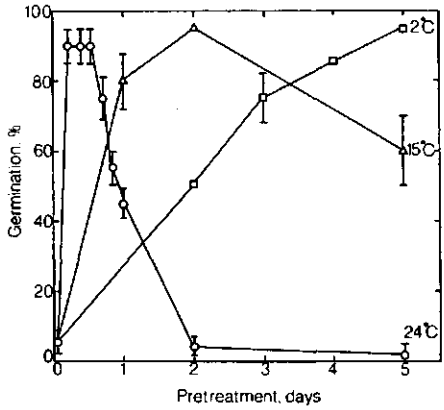

Fig. 1. The effect of a pretreatment at 2, 15 and 24°C in darkness in water on the germination at 24°C in 50 m*M* KNO_3. At the transfer to the second set of conditions the seeds were irradiated with red light for 5 min. Vertical bars indicate SD.

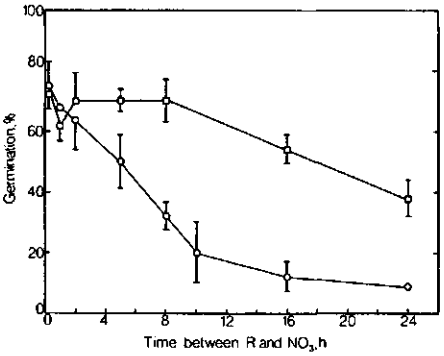

Fig. 2. The effect of a delay in the application of nitrate (10 m*M* KNO_3) on the germination at 24°C. All seeds were pretreated for 40 h in water in the dark at 15°C and subsequently transferred to 24°C and irradiated with red light for 5 min (t = 0). Nitrate was applied at the indicated times with (□) or without (○) an extra 5 min dose of red light. Vertical bars indicate SD.

tions. Application of R or nitrate alone had hardly any effect. Dormancy could be broken at a much wider range of temperatures than the chilling conditions only (Fig. 1). At higher temperatures the breaking of primary dormancy occurred at a much higher rate. However, the same was true for the induction of secondary dormancy. Therefore, the period of optimal sensitivity to R and nitrate occurred markedly earlier at higher temperatures but also lasted a much shorter time. In the present study a dark incubation of 2 days at 15°C in water was chosen as the standard treatment for breaking primary dormancy.

When the application of nitrate did not occur simultaneously with the transfer from 15 to 24°C and with R, germination was decreased (Fig. 2). A second R dose at the moment nitrate was applied prevented the decrease, but only during the first 8 h, thereafter secondary dormancy caused a general insensitivity to both factors. These data suggest that P_{fr} and nitrate have to be present simultaneously to stimulate germination. A FR-escape experiment confirmed this conclusion. The escape from the R antagonizing action of FR is generally accepted as an indication that the result of P_{Fr} action is established and therefore P_{fr} can be reversed to P_r without affecting germination. When R and nitrate were applied simultaneously at the transfer from 15 to 24°C, the escape from FR inhibition started immediately (Fig. 3). However, an 8 h delay of the nitrate application also delayed the escape from FR and, as was seen in Fig. 2, decreased the final germination percentage. Thus, nitrate seems to be an essential cofactor for the action of P_{Fr}.

Interaction of red light, nitrate and gibberellins

The effect of GA_{4+7} at a range of concentrations on the germination of *S. officinale* seeds was studied in combination with R, with and without nitrate, and with FR (Fig. 4). R + KNO_3 caused 90% germination, leaving little function for GA_{4+7}. When nitrate was omitted from the incubation medium germination was prevented both in the presence of high and low P_{fr} levels. GA_{4+7} fully replaced both the requirement for nitrate (R curve) and for the combination of R and nitrate (FR curve). Thus, high P_{fr} levels were not an indispensable factor for the action of applied GA_{4+7}, as it was for nitrate. Nevertheless, R still had a function, in decreasing the required concentration of GA_{4+7} (Fig. 4).

At a concentration of 100 μ*M*, tetcyclacis completely inhibited the stimulatory action of R + KNO_3 (Fig. 4); at 10 μ*M* it was ineffective (data not shown). The inhibi-

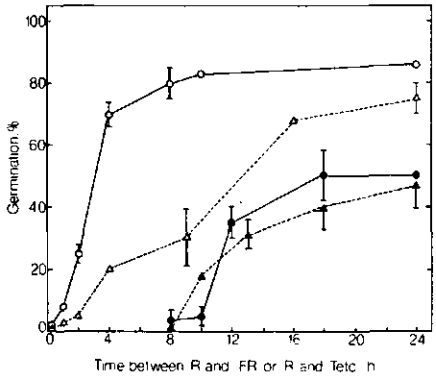

Fig. 3. The effect of a delay in the application of nitrate (10 m*M* KNO_3) on the escape from the inhibitory effect of a far-red irradiation (○, ●) or tetcyclacis (100 μ*M* △, ▲). Seeds were pretreated as in Fig. 2 and nitrate was applied at t = 0 (○, △) or at t = 8 (●, ▲). FR and tetcyclacis were applied at indicated times. Germination was counted 3 days after the transfer to 24°C, and seeds incubated in tetcyclacis were transferred to water after 1 day. Vertical bars indicate SD.

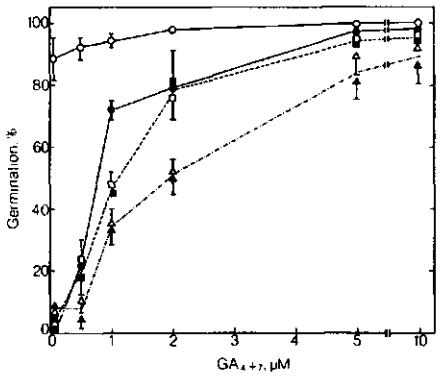

Fig. 4. The effect of a 5 min red (R) or far-red (FR) irradiation, with or without the application of 10 m*M* KNO_3 or 100 µ*M* tetcyclacis, on the germination at 24°C in a range of GA_{4+7} concentrations, buffered at pH 3.0. The seeds were pretreated as in Fig. 2. All irradiations and treatments at t = 0. Tetcyclacis-treated seeds were transferred to water after 1 day at 24°C. Treatments were: R + NO_3^- (○), R + NO_3^- + Tetc. (●), R (□), R + Tetc. (■), FR (△), and FR + Tetc. (▲). Vertical bars indicate SD.

tory effect of the growth retardant on germination was fully reversed by 5 µ*M* GA_{4+7}. Therefore, the results favour the conclusion that tetcyclacis inhibits GA biosynthesis. It is noteworthy that tetcyclacis did not change the sensitivity to GA_{4+7} in combination with R nor with FR (Fig. 4). The responses to GA_{4+7} in combination with R + KNO_3 + tetcyclacis and R + tetcyclacis were similar. Thus, nitrate was not required for the action of GA_{4+7} after a FR irradiation (data not shown).

The escape from FR was compared with the escape from tetcyclacis (Fig. 3). Similar escape phenomena were observed. When R and nitrate were applied simultaneously at the transfer from 15 to 24°C the escape from tetcyclacis started immediately like the FR escape, albeit at a much lower rate. An 8 h delay of the nitrate application also delayed the tetcyclacis escape, which then occurred at a similar rate as the FR escape. Evidently, both R and nitate were required to escape from FR and from tetcyclacis.

Two light effects

The foregoing results suggest that red light had two different effects in *S. officinale*. The first effect depended absolutely on nitrate and could be inhibited by tetcyclacis. The second light effect shifted the GA_{4+7} requirement to lower concentrations, and was independent of nitrate and insensitive to tetcyclacis. The two light ef-

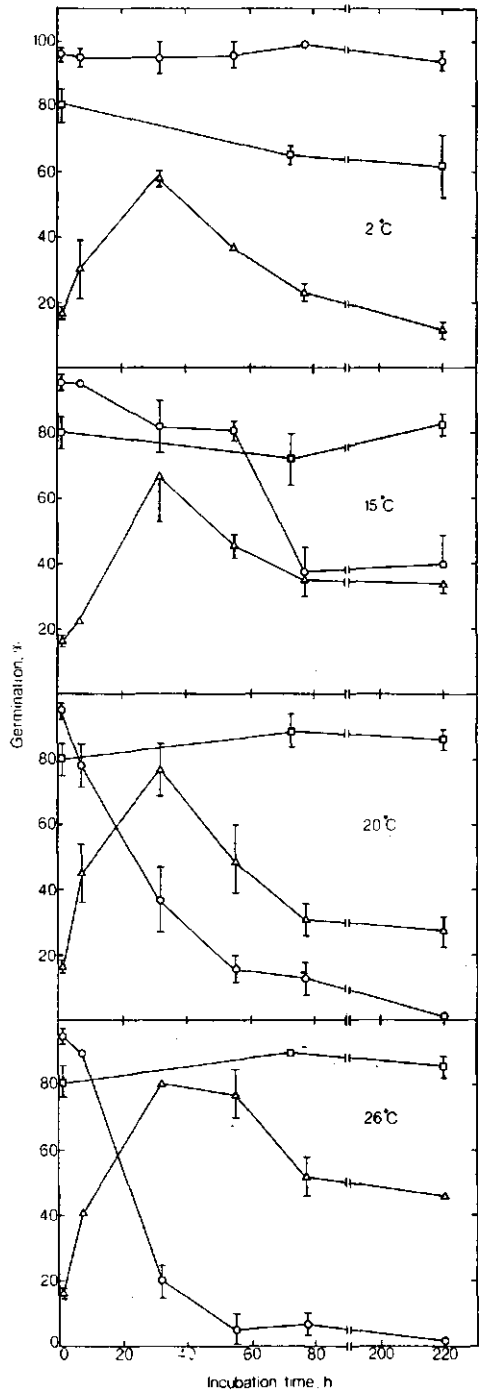

Fig. 5. The effect of temperature and duration of a pretreatment on the sensitivity to germination-stimulating conditions. All seeds were incubated for 40 h at 15°C in darkness in water and subsequently the incubation in water and darkness was prolonged at the indicated temperature and time. Finally all seeds were transferred to 24°C and to a medium containing 10 m*M* KNO_3 (○) or 2 µ*M* GA_{4+7} (□, △) and irradiated with red light (○, □) or kept in darkness (△). Vertical bars indicate SD.

fects could be distinguished more clearly by means of a different reaction to the temperature of a pretreatment (Fig. 5). Subsequent to standard dormancy breaking treatment at 15°C, the incubation in water and darkness was prolonged for different periods of time at a range of temperatures. Thereafter the sensitivity to either R + KNO_3, GA_{4+7} or R + GA_{4+7} was tested at 24°C. It is shown in Fig. 5 that the sensitivity to R + KNO_3 was strongly influenced by the temperature of the pretreatment. At 2°C the seeds retained the maximum sensitivity for 220 h. The sensitivity decreased more rapidly at higher temperatures of the pretreatment, diminishing to almost zero within 60 h at 26°C. For the sensitivity test to GA_{4+7} a suboptimal concentration of 2 μM was chosen. After the preceding 40 h at 15°C the response to 2 μM GA_{4+7} in the dark was still low. At all temperatures tested the sensitivity rose during the first 30 h and decreased thereafter. R increased the response to GA_{4+7} in all temperature-time combinations. The response to GA_{4+7} in the dark tended to increase at higher temperatures.

Discussion

The influence of environmental factors on plant morphogenesis in certain cases is mediated through plant hormones. It is a matter of debate whether those factors influence the levels of hormones or the sensitivity to hormones (Trewavas and Cleland 1983). We conclude from the present results that light stimulates the germination of *S. officinale* seeds through both mechanisms. Light effect I promotes germination in absolute dependency on the simultaneous presence of nitrate (Tab. 1). Light effect II expresses itself in the absence of nitrate as a lower requirement for applied GA_{4+7}.

Light effect I

The promotive effect of nitrate (and nitrite) on seed germination has been examined intensively in recent years, and has prompted the formulation of hypotheses concerning dormancy mechanisms, but there is no general agreement as to its mechanism of action or its relationship to phytochrome action (Roberts and Smith 1977, Taylorson 1982). The present results clearly show that the action of nitrate in the seeds of *S. officinale* is directly linked to phytochrome action. Neither of the factors could promote germination on its own (Tab. 1). Without nitrate the escape from FR did not occur (Fig. 3) and P_{fr} disappeared (Fig. 2). It is concluded that nitrate acts as an indispensable cofactor to P_{fr} action. It has been argued that the primary site of action of P_{fr} is a membrane function ofter referred to as X (see Karssen 1980/81a). The decreasing availability or level of X at higher temperatures is held responsible for the induction of secondary light-irresponsiveness (skotodormancy). The data shown in Fig. 2 indicate that addition of nitrate cannot reverse this induction. Therefore, nitrate cannot replace the requirement for X.

The inhibition by tetcyclacis of the R + KNO_3 induced germination suggests a link between the action of these two factors and GA biosynthesis (Fig. 4). However, besides an inhibition of GA-stimulated cell elongation tetcyclacis also inhibits, at high concentrations, cell division, probably by influencing sterol biosynthesis (Grossman et al. 1985, Nitsche et al. 1985). Still, we accept our data as evidence for a promotive effect of R + nitrate on GA biosynthesis since (1) GA_{4+7} completely neutralized the inhibition by tetcyclacis, whereas the latter compound did not influence the promotive action of GA_{4+7} (Fig. 4) and (2) radicle protrusion of seeds is believed to occur through cell elongation, whereas cell division is not essential at this stage (Haber and Luippold 1960).

The difference in rates of escape from FR and tetcyclacis when nitrate was added simultaneously with R (t = 0, Fig. 3) indicates that P_{fr} is not needed throughout GA biosynthesis. Thus, at certain times, P_{fr} can be reversed to P_r without affecting germination, whereas tetcyclacis still inhibits.

The sensitivity of the seeds to light effect I strongly depended on the lenght and the temperature of the incubation period preceding the transfer to 24°C, R and nitrate (Fig. 1, 5). The temperature-time relations in the breaking and induction of dormancy showed a pattern which was very similar to observations made in *Arabidopsis thaliana* (Cone and Spruit 1983) and *Rumex crispus* and *R. obtusifolius* (Totterdell and Roberts 1979). It is shown that breaking of primary dormancy and induction of secondary dormancy are simultaneous processes which both increase in rate at rising temperatures. Due to a different temperature optimum of both processes dormancy breaking dominates at lower temperatures and dormancy induction at higher temperatures.

Light effect II

Because the stimulation of GA biosynthesis depended on both light and nitrate, omission of the latter factor opened up a unique possibility to study light effects on seeds apart from GA biosynthesis. In this way it was shown that light also influenced the sensitivity to GA (light effect II). Light may do so by stimulating the activity or formation of GA receptor sites. The sensitivity to GA_{4+7} showed a peculiar pattern, in particular because it was hardly influenced by temperature (Fig. 5). In contrast to the sensitivity to R + KNO_3, the effect of R + GA_{4+7} was maintained at higher temperatures, it even tended to increase. The characteristics of the photocontrol of light effect II and its reaction to temperature is under current investigation.

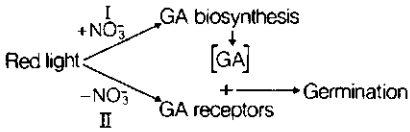

Fig. 6. Schematic presentation of the conclusions concerning light effects I and II.

Light effects I and II

The results of our study are summarized schematically in Fig. 6. Evidently, light effect I is the primary event since germination depends on GAs. Nevertheless, light effect II may occur before I. It is shown in Fig. 3 that after a delay in the nitrate application by 8 h, the escape from tetcyclacis occurred at a higher rate than at t = 0. Since reaction II did not require nitrate, it could proceed normally during the 8 h delay. Therefore, the seeds required a lower level of endogenous GA after 8 h at 24°C, and this caused a faster escape from tetcyclacis.

In a parallel study with seeds of *Arabidopsis thaliana*, the use of GA-deficient mutants made it possible to distinguish two similar light effects (C. M. Karssen, unpublished results). In this species nitrate played no role.

If indeed these two light effects occur more generally in seeds, it might explain why in certain species GA application could not replace the requirement for light. Light effect II could be the limiting factor in those seeds (Taylorson and Hendricks 1976, Fredericq et al. 1983).

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CHAPTER 3

Dual effect of light on the gibberellin- and nitrate-stimulated seed germination of *Sisymbrium officinale* and *Arabidopsis thaliana*

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Dual Effect of Light on the Gibberellin- and Nitrate-Stimulated Seed Germination of *Sisymbrium officinale* and *Arabidopsis thaliana*

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HENK W. M. HILHORST* AND CEES M. KARSSEN
Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

ABSTRACT

Red light (R) has a dual effect on the seed germination of the two related species *Arabidopsis thaliana* and *Sisymbrium officinale*. The two species provide different means to separate the light-effects. In *S. officinale*, stimulation of germination by R depends on the simultaneous presence of nitrate (light-effect I). The effect of both factors is completely blocked by tetcyclacis, an inhibitor of gibberellin (GA)-biosynthesis. Addition of a mixture of gibberellins A_4 and A_7 (GA_{4+7}) antagonizes the inhibition. In the absence of nitrate, R shifts germination to lower GA-requirement (light-effect II). In *A. thaliana* a similar second light-effect is seen on the GA-requirement of GA-deficient *ga-1* mutant seeds. R stimulates germination of wild type seeds in water (light-effect I). For both species, light-effect I shows a fluence threshold value of approximately 10^{-5} moles per square meter, which is independent of the nitrate concentration. Increasing nitrate concentrations narrow the fluence-range required for maximal germination whereby the product of nitrate concentration and fluence value determines the germination level, indicating a multiplicative interaction between R and nitrate. Fluence-response curves for light-effect II are similar for both species. Germination occurs in the range of 10^{-6} to 10^{-2} moles per square meter fluence. The maximal level of germination is determined by the level of dark-germination and light-effect II. Increasing GA_{4+7} concentrations induce a shift to lower fluence values. It is shown that in the second effect the co-action of R and exogenous GA_{4+7} is clearly additive. It is concluded that light-effect I induces a chain of events leading to GA biosynthesis. Light-effect II seems to enhance the sensitivity of the seeds to GAs.

Seed germination of a large number of species is promoted by Pfr. However, most light-requiring seeds do germinate in the dark when exogenous GAs[1] are applied (25). This by-pass of light requirement has led to the suggestion that Pfr plays an essential role in the biosynthesis of GAs. Indeed, it has been observed that in some light-requiring species exposure to R increases endogenous levels of GAs, as measured by bioassay (15, 24). In several species light also enhances the stimulation of germination by exogenous GAs whereby the effects of light and GA are mostly additive. Therefore it was suggested that Pfr also increases the sensitivity of the seeds to GAs (3, 26). Previous studies with seeds of *Sisymbrium officinale* (11) and *Arabidopsis thaliana* (12), two closely related wild species, presented arguments for a dual light-effect on GA-biosynthesis and the sensitivity to GAs. Both species provided different means to separate the light effects. Seed germination of *S. officinale* depends on the simultaneous availability of Pfr and nitrate. The effect of both factors can be completely blocked by tetcyclacis, an inhibitor of GA-biosynthesis (19). Addition of GA_{4+7} antagonized the inhibition. Besides its effect on GA-biosynthesis in higher plants (20) where tetcyclacis may inhibit the oxidative reactions from *ent*-kaurene to *ent*-kaurenoic acid (10), inhibition of sterol synthesis has been reported (18). However, sterol synthesis is mainly connected with cell-division while it is widely accepted that during the first stages of germination cell-elongation is the principal growth process. Since nitrate could not stimulate dark germination in *Sisymbrium* it was concluded that Pfr was the essential factor for the initiation of GA-biosynthesis (light-effect I) (11). In the absence of nitrate, light shifted germination to a lower GA-requirement than in darkness (light-effect *II*). Tetcyclacis did not inhibit this second light-effect.

In *A. thaliana* dwarf mutants have been isolated that are absolutely dependent on GAs for germination (16). The mutants (gene symbol *ga-1*) lack endogenous GAs. This was demonstrated by fractionation of acidic fractions of entire plants by HPLC and subsequent testing of all the fractions in the *d-5* corn bioassay. No GA-like activity was found in the *ga-1* mutant while in the wild type several zones with GA-like activity were present (JAD Zeevaart, personal communication). The *ga-1* mutant has a strongly reduced *ent*-kaurene synthesizing capacity (2). Feeding studies have demonstrated that the *ga-1* block is prior to *ent*-kaurene (JAD Zeevaart, personal communication). Irradiation with R shifts the requirement for GAs of *ga-1* seeds to a lower level (light-effect II) (12). Wild type seeds only require GA in darkness, light induces germination in water (light-effect I).

It is the aim of the present study to further characterize the light effects in seeds of both species in the absence and presence of GA-biosynthesis. Fluence response experiments and subsequent analysis of the dose-response relations with the aid of models for different types of co-action (17, 23) will be used to answer the questions as to the nature of the light effects.

MATERIAL AND METHODS

Seeds. Ripe seeds of *S. officinale* were collected from wild plants growing in natural habitats in the vicinity of Wageningen. A batch collected in 1984 was used. Seeds were cleaned and stored dry at 2°C in the dark. Under these conditions no changes in dormancy were observed during the experimental period.

Seeds of *A. thaliana* were provided by Dr. M. Koornneef (Department of Genetics, Agricultural University Wageningen, The Netherlands). In this study seeds of wild type and of the

[1] Abbreviations: GA, gibberellin; GA_{4+7}, mixture of gibberellins A_4 and A_7; (V)LF, (very) low fluence; R, red light; LDP, log dose-probit.

GA-deficient *ga-1* (NG5) mutant were used. The seeds were stored dry at room temperature.

Germination Conditions. Triplicates of 50 or 50 to 100 seeds of *S. officinale* and *A. thaliana*, respectively, were sown in 5 cm Petri dishes on one layer of filter paper (Schleicher and Schüll No. 595) and moistened with 1.5 ml of distilled water or the test solution. At the start of each experiment primary dormancy of the seeds was broken by dark incubation for 40 h at 15°C for the *Sisymbrium* seeds and 7 d at 10°C for the *Arabidopsis* seeds. After this period seeds were kept at 24°C for 1 h in the dark, were irradiated and then returned to 24°C in the dark. Germination of the *Sisymbrium* seeds was counted after 3 d, and germination of the *Arabidopsis* seeds after 5 d at 24°C.

Broadband red light (620–700 nm) was obtained from six red fluorescent tubes (Philips TL 20W/15) filtered by 3 mm red Plexiglas (Red 501, Röhm & Haas, Darmstadt, FRG). Fluence at seed level was 1.5×10^{-3} mol·m^{-2}. Fluence-response curves were obtained by irradiating the seeds with a custom-build projector with a 250 W quarts-iodine lamp (Philips) equipped with a narrow waveband interference filter of 660 nm (B40; Balzers, Liechtenstein) with approximately 10 nm bandwidth at 50% of the transmission maximum. The fluence rate was varied by inserting neutral glass filters (NG; Schott u Gen., Mainz, FRG) behind the interference filter. The fluence rate was calculated from the transmission characteristics of the neutral filters. During irradiation of the seeds the lids of the Petri dishes were removed. Irradiation time was 20 s except when fluence values higher than 1.06×10^{-3} mol·m^{-2} were required. In those cases the fluence rate was varied by prolonging the irradiation time to maximally 5 min. Reciprocity was not affected at these irradiation times (HWM Hilhorst, unpublished results). Every experiment was repeated at least once with qualitatively similar results. Sowing and all further handling of the seeds were performed in dim green light obtained by filtering one green fluorescent tube (Philips TL 40W/17) with two layers of yellow (No. 46) and two layers of blue (No. 62) Cinemoid filters (Strand Electric, London, U.K.). Dark controls were conducted in absolute darkness and germination results were always similar to those obtained by seeds that were manipulated in dim green light and irradiated with red light of the lowest fluence values used (10^{-10} mol·m^{-2}).

Test Solutions. All compounds were dissolved in distilled water. Tetcyclacis was dissolved in a small volume of acetone and then mixed with a large volume of distilled water. The solution was stirred until no further increase of the absorbance at 240 nm could be observed. The solution was filtered and kept in the dark at room temperature. Final concentration of a saturated solution was approximately 10^{-4} M. The following substances were used: 5-(4-chlorophenyl)-3,4,9,10-pentaazatetracyclo-5,4,1,$0^{2,6}$,$0^{8,11}$ dodeca-3,9-diene (tetcyclacis = NDA = BAS 106W, LAB 102 883); GA_{4+7}, a mixture of gibberellins A4 and A7 (ICI, Yalding, U.K.); KNO_3 of highest available purity (BDH, Poole, U.K.).

Calculations. For the analysis of interaction types it is necessary to compare the subpopulations that react to the light stimulus. Germination is a quantal or 'all-or-none' response. For every individual seed of a population there is a level of intensity (tolerance) of the stimulus below which germination does not occur. This tolerance varies from seed to seed and may be assumed to be normally distributed around the logarithm of a mean level of Pfr required for 50% germination (μ) and with standard deviation σ (7). Response expressed in percentage germination results in a cumulative normal distribution which can be linearized with germination in terms of probits (8) to a log-dose probit (LDP) relation:

$$\text{probit } y = 5 + (\ln[\text{Pfr}] - \mu/\sigma)$$

where μ is a function of those factors which affect the Pfr tolerance in individual seeds and σ is a function of changes in the range of Pfr requirement of the seed population. In this work it is assumed that the Pfr requirement of the individual seeds of the seed population is normally distributed around a mean level of Pfr required for half-maximal germination, instead of 50% germination (21). With this approach it is possible to compare dose-response relationships of Pfr and the applied growth regulators independent of the response of zero dose (zero response) and the maximal response at the experimental conditions (maximal response), leaving a nongerminating fraction). Also the population parameters can be calculated and from the weighted regression line (LDP line) the sigmoid curve of germination *versus* log fluence can be generated which fits the original data points (5, 6). The data obtained from the fluence-response experiments were subjected to probit analysis with the aid of a computer program developed by De Petter *et al.* (6). The population parameters and the log dose-probit line were calculated and subsequently the best fitting sigmoid curve through the data points was produced. The automated calculation procedure included variance calculations with standard formulae.

RESULTS

Germination under Non-GA-Producing Conditions. Following a dormancy breaking preincubation both *ga-1* seeds of *Arabidopsis* and *Sisymbrium* seeds deprived of nitrate failed to germinate in water, neither in darkness nor after a saturating dose of R (Fig. 1, A and B). There was an absolute dependency on the presence of exogenous GA_{4+7}. In the dark the reaction to a range of GA_{4+7} concentrations was similar. A saturating dose of R induced the seeds to germinate at lower GA_{4+7} levels: In the *ga-1* mutant of *Arabidopsis* to about one-third (Fig. 1A) and in *Sisymbrium* to one-tenth of the GA_{4+7} concentration required in the dark (Fig. 1B). In the suboptimal segment of the dose-response curves this shift was parallel in both species. This indicates an additive effect of R to the GA_{4+7} response.

Germination under GA-Producing Conditions. It was shown before that wild-type *Arabidopsis* seeds germinated readily in water after saturating R, whereas the response of *Sisymbrium* seeds to R was dependent on nitrate (11, 12). The reaction of *Sisymbrium* seeds to a range of KNO_3 concentrations is shown in Figure 1C. It is seen that light hardly had a stimulative effect without KNO_3 and KNO_3 was ineffective in the dark. It should be noted that active nitrate concentrations were 1000 times higher than the active GA_{4+7} concentrations. In *ga-1 Arabidopsis* seeds also the combination of saturating R and KNO_3 could not replace the requirement for application of GA_{4+7} (data not shown).

Fluence-Response Curves under GA-Producing Conditions. In order to quantify the effect of R on germination under GA-producing conditions fluence-response curves were made for the germination of *Sisymbrium* seeds in different concentrations of nitrate (Fig. 2) and of wild type *Arabidopsis* seeds in water and nitrate (Fig. 3).

Sisymbrium. In water, germination of *Sisymbrium* seeds was only slightly stimulated by R (Fig. 2). At the nitrate concentrations used germination was stimulated by R at fluences higher than 10^{-5} mol·m^{-2} (Fig. 2). This threshold value was the same for all concentrations and below this value no germination occurred. The slope of the LDP line increased significantly with the nitrate concentration (Table I). Addition of tetcyclacis to 5 mM nitrate shifted the fluence-response curve to higher fluence values and reduced maximal germination levels (Fig. 2). The slopes of the LDP lines were similar to the slope of the curve of 1 mM nitrate (Table I) and this is a lower value than could be expected for 5 mM nitrate. Although maximal germination was strongly decreased at increasing concentration of tetcyclacis, both LDP lines were similar. A good correlation was found between the product of fluence and nitrate concentration, expressed as the sum of the logarithms, and the probit of the corresponding

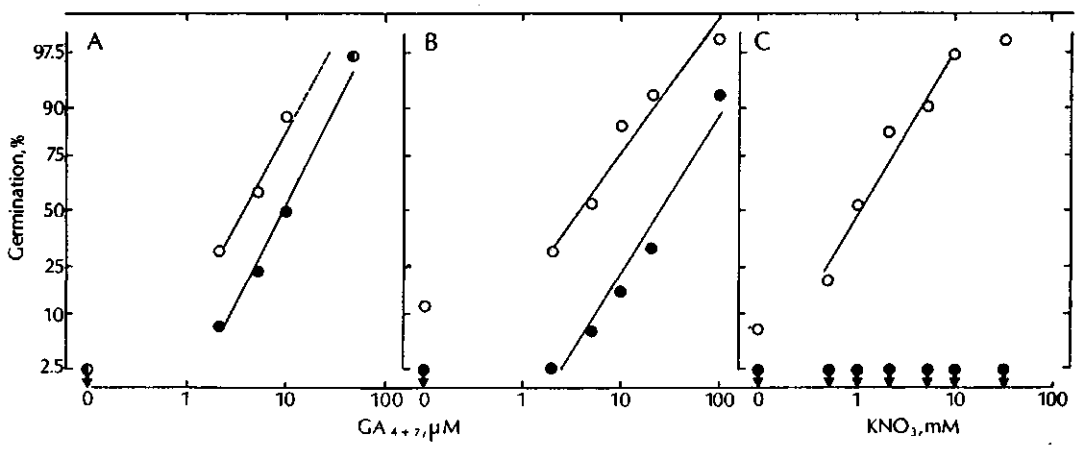

FIG. 1. Effect of a 10 min R irradiation on the germination at 24°C of seeds of the *ga-1* mutant of *A. thaliana* (A) and *S. officinale* (B, C) in a range of concentrations of GA_{4+7} (A, B) or nitrate (C). (○), R; (●), dark; (●→), no germination.

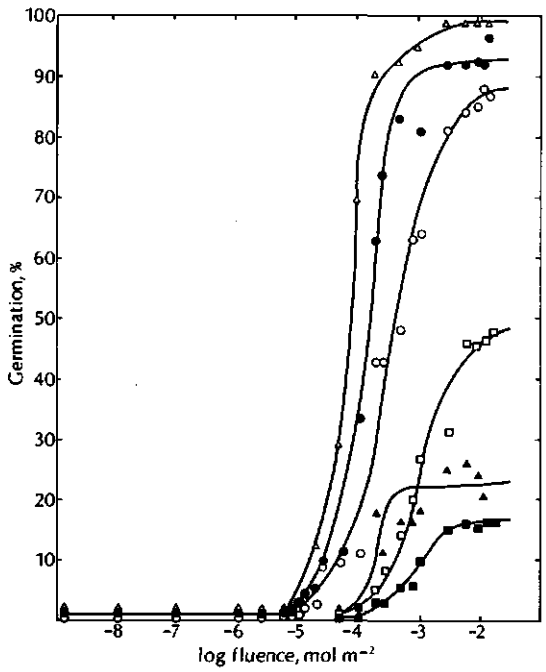

FIG. 2. Fluence response curves for the germination at 24°C of seeds of *S. officinale* in water (▲), 1 (○), 2 (●), 32 (△) mM KNO_3 and 5 mM KNO_3 with 5 (□) or 10 (■) μM tetcyclacis. Curves are calculated from the population parameters.

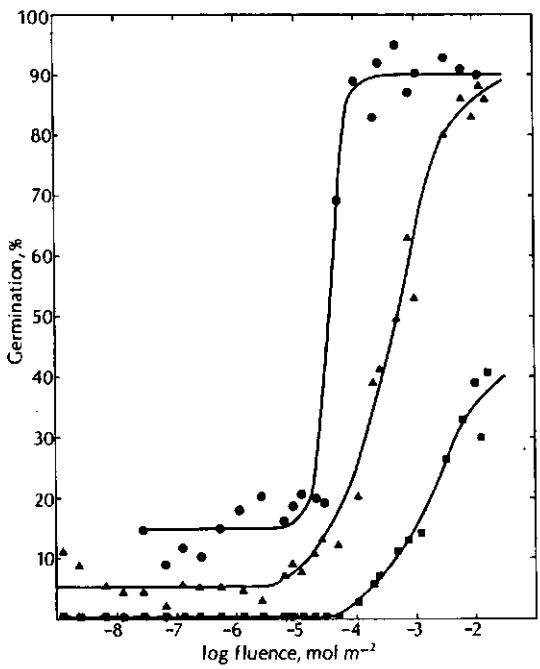

FIG. 3. Fluence-response curves for the germination at 24°C of wild type seeds of *A. thaliana* in water (▲), 10 mM KNO_3 (●) and 2μM tetcyclacis in water (■). Curves are calculated from population parameters.

germination response (Fig. 4A), indicating a multiplicative interaction between Pfr and nitrate. The data from the 32 mM nitrate fluence-response curve were not included since this concentration was highly saturating.

Arabidopsis. Germination of wild type *Arabidopsis* seeds did not depend on nitrate. Nearly all seeds germinated in water at saturating fluence values (Fig. 3). R stimulated in the range of 10^{-5} to 10^{-2} mol·m^{-2}. Addition of 10 mM KNO_3 narrowed the fluence range to 10^{-5} to 10^{-4}, but the fluence threshold did not change. Dark germination (fluences < 10^{-5} mol·m^{-2}) was some-

Table I. *Population Parameters and Standard Deviations(s) Obtained after Weighted Regression of the Data Points of Figures 2, 3, 5, and 6*

Species	Medium	Concentration	R⁺[a]	s(R⁺)	R⁺[b]	s(R⁺)	m[c]	s(m)	B	s(B)
		mol·L⁻¹	%	%	%	%	*log mol · m⁻²*			
S. officinale	H_2O		0.55	0.13	21.47	1.27	−3.75	0.04	4.99	0.26
(Fig. 2)	KNO_3	$1*10^{-3}$	1.70	0.59	86.07	1.77	−3.45	0.04	1.47	0.10
	KNO_3	$2*10^{-3}$	1.49	0.32	89.41	1.05	−3.88	0.03	2.07	0.19
	KNO_3	$3.2*10^{-3}$	1.20	0.19	96.12	0.40	−4.14	0.02	3.00	0.16
	KNO_3	$5*10^{-3}$								
	+ Tetcy-	$5*10^{-6}$	0		50.53	2.19	−2.94	0.05	1.44	0.11
	clacis	$1*10^{-5}$	0		16.98	1.02	−3.03	0.06	1.53	0.20
(Fig. 5)	GA_{4+7}	$2*10^{-6}$	2.34	0.23	28.64	1.99	−3.31	0.10	1.19	0.14
	GA_{4+7}	$5*10^{-6}$	5.81	0.56	52.74	3.48	−3.56	0.12	0.96	0.12
	GA_{4+7}	$1*10^{-5}$	18.67	1.43	70.96	1.90	−4.57	0.08	0.91	0.09
	GA_{4+7}	$2*10^{-5}$	35.76	1.33	57.26	1.14	−4.62	0.06	1.27	0.12
A. thaliana	H_2O		5.62	0.44	84.62	2.20	−3.36	0.05	1.21	0.08
Wild type	KNO_3	$1*10^{-2}$	15.65	0.81	73.72	1.10	−4.40	0.02	4.66	0.56
(Fig. 3)	Tetcy-	$2*10^{-6}$	0.40	0.10	45.33	5.93	−2.66	0.15	1.16	0.01
	clacis									
A. thaliana	GA_{4+7}	$2*10^{-6}$	7.56	0.43	21.50	2.33	−3.43	0.17	1.00	0.20.
ga-1 (Fig. 6)	GA_{4+7}	$5*10^{-6}$	20.27	0.87	40.27	6.39	−4.34	0.25	1.01	0.25
	GA_{4+7}	$1*10^{-5}$	49.45	1.53	41.59	5.98	−4.46	0.26	0.83	0.18

[a] R^-, minimum response. [b] R^+, response range. [c] m, dose for half-maximal response. [d] B; slope = $1/\sigma$.

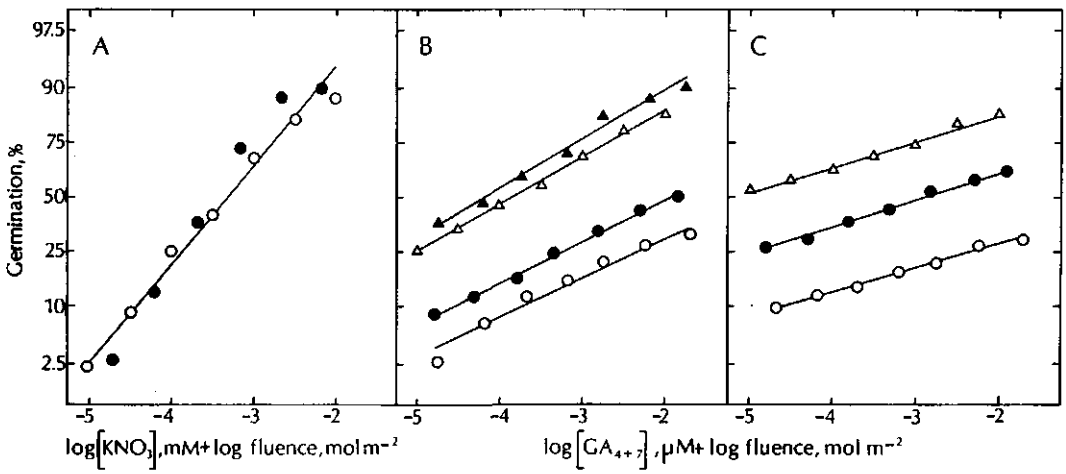

FIG. 4. Plot of germination against the sum of logarithm of nitrate (A) or GA_{4+7} (B, C) concentration and fluence of *S. officinale* (A, B, data obtained from Figs. 2 and 5) and the *ga-1* mutant of *A. thaliana* (C, data obtained from Fig. 6). A, 1, (○) and 2 (●) mM KNO_3; B, C, 2 (○), 5 (●), 10 (△), and 20 (▲) μM GA_{4+7}.

what increased by the addition of nitrate (Fig. 3). The LDP line for the nitrate stimulated germination was much steeper than the line for the germination in water (Table I). Addition of tetcyclacis caused a shift to higher fluence values (Fig. 3), which was parallel (Table I). Moreover, the dark germination was lowered to zero and maximal germination was reduced.

The results described here show remarkable similarities between the two species with respect to response range, threshold values of fluences, slopes of LDP lines, and effects of tetcyclacis.

Fluence-Response Curves under Non-GA-Producing Conditions. Fluence-response curves for the GA_{4+7}-stimulated germination of *Sisymbrium* seeds (Fig. 5) shared all the characteristics of the curves of the *ga-1* mutant of *Arabidopsis* (Fig. 6). In both species the R-sensitive subpopulations germinated in the fluence range of 10^{-7} to 10^{-2} mol · m^{-2} and showed a shift to higher fluence values at nonsaturating GA_{4+7}-concentrations. This shift was parallel as can be deduced from the slopes of the LDP lines (Table I). In both species a GA_{4+7}-dependent germination response was shown in the VLF range ($<10^{-6}$ mol·m^{-2}). This response remained at a constant level until fluence values were reached in the LF range (10^{-6} to 10^{-2} mol·m^{-2}). In both species the germination response range (Table I, R^+) of the two highest GA_{4+7} concentrations was of the same magnitude. Maximum level of germination, therefore, depended on the initial

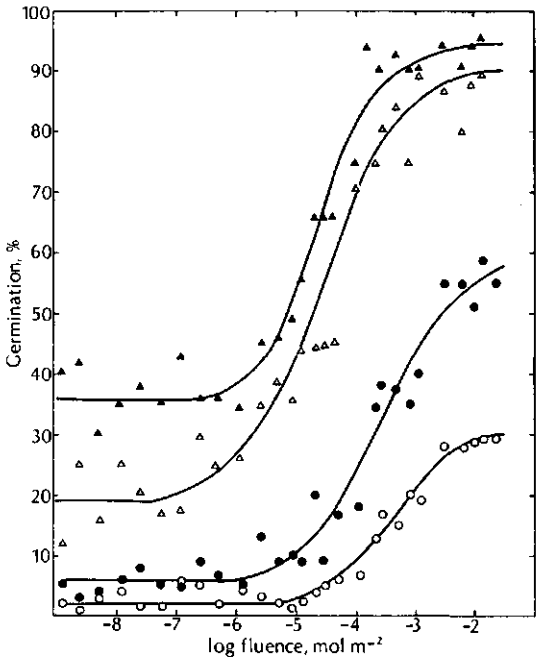

FIG. 5. Fluence-response curves for the germination at 24°C of *Sisymbrium* seeds in 2 (○), 5 (●), 10 (△), and 20 (▲) μM GA_{4+7}. Curves are calculated from population parameters.

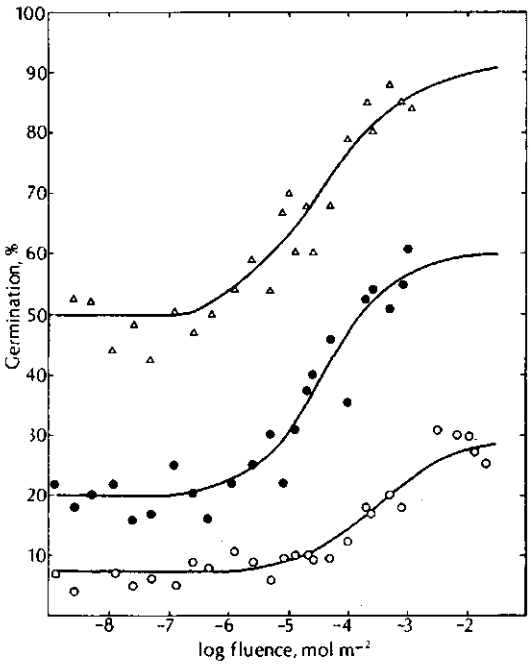

FIG. 6. Fluence-response curves for the germination at 24°C of the *ga-1* mutant of *A. thaliana* in 2 (○), 5 (●), and 10 (△) μM GA_{4+7}. Curves calculated from population parameters.

'dark' germination. In contrast with the fluence-response curves of the nitrate stimulated germination of *Sisymbrium* seeds (Fig. 4A) the correlation lines of the product of fluence and GA_{4+7} concentration and the germination response did not coincide (Fig. 4, B and C), suggesting an additive type of co-action of Pfr and GA_{4+7}.

DISCUSSION

From the present results it can be argued that the effect of light on seed germination is indeed regulated by two different light-reactions. The use of two related species with their specific requirements for germination reinforces the arguments since the results were remarkably similar. Light-effect I was expressed under conditions where GAs could be synthesized and germination did not depend on application of GA_{4+7}. These conditions were met in nitrate-incubated *Sisymbrium* seeds and in wild type *Arabidopsis* seeds. Light-effect II was demonstrated under conditions at which GA-synthesis was blocked and germination was absolutely dependent on exogenous GA_{4+7}. These conditions were realized by depriving *Sisymbrium* seeds of nitrate and by using the *ga-1* mutant of *Arabidopsis*. Both light-effects and their interrelationship will be discussed in detail.

Light-Effect I. The promotive action of nitrate on seed germination has been the subject of a number of studies (22, 28). Besides several hypotheses on its possible role in dormancy mechanisms (1, 22) there have been speculations on the action of nitrate on the Pfr-stimulated germination (5). However, there is no agreement as to its general mechanism of action. The present results strengthen our previous hypothesis that nitrate- and Pfr-action are closely connected (11). The nitrate concentration modulated the fluences required for half-maximal germination of both *Arabidopsis* and *Sisymbrium* seeds (Figs. 2, 3; Table I). A plot of the logarithmic sum of fluence value and nitrate concentration against the germination response of *Sisymbrium* seeds (Fig. 4A) shows that a certain response can be generated by more than one combination of nitrate and Pfr concentrations. The response is a function of the product of both factors and by definition this represents a multiplicative interaction between both stimulators. This type of interaction expresses the action of both factors at different points of the same pathway of reactions (23). In both species light-effect I was expressed essentially in the LF range. Nitrate had no effect on the threshold value of approximately 10^{-5} mol m^{-2}. Similar values were reported for *Kalanchöe blossfeldiana* (5) and *Rumex obtusifolius* (14). The results indicate that light-effect I may reflect a general mechanism for the light-stimulated germination. In addition, other factors, like nitrate, may be essential or nonessential to this stimulation. For both species it can be argued that light-effect I plays an essential role in GA-biosynthesis. Direct evidence for this hypothesis is difficult to obtain, but we have shown that in both species germination in the dark was absolutely dependent on the presence of exogenous GA_{4+7}. Moreover, the *ga-1* mutant of *Arabidopsis*, which lacks the ability to synthesize GAs, germinated after R only when exogenous GA_{4+7} was present (Fig. 1A). In a previous paper (11) we demonstrated that the R induced germination of *Sisymbrium* seeds could be inhibited by tetcyclacis. The effect of tetcyclacis could be fully reversed by exogenous GA_{4+7}. In the present study both tetcyclacis under GA-producing conditions (Figs. 2, 3) and exogenous GA_{4+7} under non-GA-producing conditions (Figs. 5, 6) shifted the fluence-response curves to higher fluence values and reduced maximal germination. Tetcyclacis may therefore indeed modulate the lev-

els of endogenous GAs. Tetcyclacis also opposed the effect of nitrate (Fig. 2; Table I) possibly because, in this case, nitrate is not the limiting factor but the synthesis of GAs. The low number of wild type *Arabidopsis* seeds that germinated in water in the VLF range (Fig. 3) was also reduced by tetcyclacis, indicating that a small fraction of these seeds is capable of synthesizing GAs with very low levels of Pfr.

Light-Effect II. Gibberellins have been found to substitute for light in the seed germination of several species (25). However, it is difficult to determine whether this substitution is complete. Very low levels of (preexisting) Pfr may still play a role. Seeds of *Sisymbrium* and the *ga-1* mutant of *Arabidopsis* showed dark germination which depended on the concentration of exogenous GA_{4+7} (Fig. 1, A and B). The level of dark germination had no effect on the slopes of the LDP lines (compare data on R^- and B in response to GA_{4+7} in Table I). Dramatic changing of the slopes of fluence response curves caused by preexisting Pfr has been reported for wild type *Arabidopsis* seeds (4). Since the germination level in the VLF range remained fairly constant with varying fluence in our case (Figs. 5, 6) we conclude that the dark response to exogenous GA_{4+7} is not limited by Pfr. In both species, limiting GA_{4+7} concentrations shifted *m* to higher fluence values (Table I). These results indicate that the expression of light-effect II depends on the GA_{4+7} concentration. An interaction between GA_{4+7} and Pfr does not occur. This can be concluded from the plot of the logarithmic sum of GA_{4+7} concentration and fluence against germination response (Fig. 4, B and C) which results in parallel lines for different GA_{4+7} concentrations. This is a good argument for an independent co-action of the two factors (23).

Light-Effects I and II. The present results make clear that the two light-effects are of a different nature with respect to their respective interactions with nitrate and GAs. Previous observations on *S. officinale* have shown that both light-effects differ in more respects (11). Upon incubation at temperatures that favor secondary dormancy light-effect I gradually disappeared while seeds remained responsive to light-effect II. Moreover, the escape time for far red reversion of light-effect I was approximately 8 h and of light-effect II approximately 1 h (HWM Hilhorst, unpublished results). Thus it can be argued that light-effect I is the limiting factor for the light-stimulated germination. If it is assumed that the germination response is proportional to the number of active GA-receptor complexes and that the formation of such complexes primarily depends on the availability of GAs and active receptor sites, we may hypothesize that light-effect I acts through the production of GAs and light-effect II through the production of active receptor sites. It should be noted that the concept of sensitivity not only comprises the formation of active GA-receptor complexes but also includes phenomena like affinity, response capacity, and uptake efficiency (9). The physiological relevance of light-effect I may be its role in linking the reception of favorable light conditions with germination through GA-biosynthesis. The expression of this light-effect may be regulated by other environmental factors such as nitrate, probably depending on the specific environmental requirements of the plant that grows from the seed. The physiological importance of light-effect II is less clear. It may occur before light-effect I but will not come to expression in the absence of the first light-effect. As both light-effects are initiated in the same fluence-range it may be questioned why the receptor activation is under Pfr control. As yet we have not been able to find natural conditions under which this process is limiting for germination. The present study makes clear that seed germination of *S. officinale* and *A. thaliana* is under control of both the level of endogenous GAs and the sensitivity to GAs. Both controls are regulated by Pfr. We believe that our results may add arguments to the recent discussion on the hormonal regulation of growth and development (27). Instead of a choice between control by changing hormone levels and changing sensitivity to hormones a dual control by light of both mechanisms would be favored.

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CHAPTER 4

Nitrate reductase independent stimulation of seed germination in *Sisymbrium officinale* L. (hedge mustard) by light and nitrate

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Nitrate Reductase Independent Stimulation of Seed Germination in *Sisymbrium officinale* L. (Hedge Mustard) by Light and Nitrate

HENK W. M. HILHORST and CEES M. KARSSEN

Department of Plant Physiology, Agricultural University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands

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ABSTRACT

After a 72 h preincubation in darkness at 15 °C seed germination of *Sisymbrium officinale* L. (hedge mustard) at 24 °C was stimulated by a combination of red light and nitrate. In the presence of nitrate the seeds escaped from the inhibiting effect of far-red irradiation with an escape time of approx. 8 h. After red light, the exposure of seeds to nitrate could be delayed for 3 h without affecting maximal germination. Prolonged delay resulted in a decrease of the germination response. The possibility that nitrate reduction was involved in the stimulation of germination was studied by pre-incubating seeds for 72 h in nitrate and subsequently transferring them to water and irradiating with red light. During the first 8 h period after the red irradiation in which induction of germination occurred, total nitrate levels (endogenous + leachate) remained constant, indicating an absence of nitrate reductase activity. During the next 8 h visible germination started and total nitrate levels declined, suggesting induction of nitrate reduction. It is concluded that nitrate reduction does not play a role in the induction of germination. The conclusion was supported by the lack of inhibition of seed germination by sodium chlorate and sodium tungstate in spite of an inhibition of nitrate reduction of 80 and 100 %, respectively. The contrasts between our results and hypotheses concerning the mechanism of action of nitrate in seed germination are discussed.

Key words: *Sisymbrium officinale* L., hedge mustard, germination, light, nitrate, nitrate reductase.

INTRODUCTION

Seed germination in many common weed species is stimulated by nitrate ions (Roberts and Smith, 1977). For a number of species a positive interaction between light and nitrate has been observed (Vincent and Roberts, 1977). More detailed studies have shown that the effect of nitrate is dependent on Pfr (Hilton, 1983; Probert, Gajjar and Haslam, 1987; Karssen and de Vries, 1983; Hilhorst, Smitt and Karssen, 1986). From studies on seed germination in *Kalanchoë blossfeldiana* it was concluded that nitrate increased the sensitivity of the seed to Pfr (DePetter *et al.*, 1985).

In *Sisymbrium officinale*, the germination response is determined by the product of the concentrations of Pfr and nitrate, indicating a multiplicative interaction (Hilhorst and Karssen, 1988). Moreover, it was indicated that the effects of Pfr and nitrate are closely connected in time during the process of induction of germination (Hilhorst *et al.*, 1986).

In general, hypotheses concerning the mechanism of action of nitrate in seed germination include the reduction of nitrate to nitrite by nitrate reductase (NR). These hypotheses link the effect of nitrate with respiratory processes by its tentative action as an alternative electron acceptor (Roberts and Smith, 1977; Hendricks and Taylorson, 1972; Hilton and Thomas, 1986). If so, could light then be the indispensible factor for induction of nitrate reductase activity (NRA) and thus explain the observed interaction between light and nitrate? Light has indeed a strong effect on the appearance of NR in higher plants (see for reviews Duke and Duke, 1984; Rajasekkar and Oelmüller, 1987).

The objective of the present study was to determine if and when NRA was induced during the induction of germination in seeds of *Sisymbrium officinale* by red light and nitrate. For this purpose the effective periods for the action of both

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red light and nitrate were determined in germination tests and NRA was measured by monitoring endogenous and exogenous nitrate levels during the induction of germination. Nitrate levels were determined in the absence and presence of sodium chlorate, a competitive inhibitor of nitrate reduction, and sodium tungstate, an inhibitor of *de novo* synthesis of active NR.

MATERIAL AND METHODS

Seeds

Ripe seeds were collected from wild plants growing in the vicinity of Wageningen. A batch collected in 1985 was used. Seeds were cleaned and stored dry at 2 °C. Under these conditions no changes in dormancy occurred during the experimental period.

Germination conditions

Triplicates of 50 seeds were sown in 5 cm Petri dishes on one layer of filter paper (Schleicher and Schüll no. 595) and moistened with 1·5 ml water or the test solution. Broadband red light (620–700 nm) was obtained from six red fluorescent tubes (Philips TL 20W/15) filtered by 3 mm red Plexiglas (Red 501 Röhm and Haas, Darmstadt, FRG). Fluence at seed level was $1{\cdot}5 \times 10^{-3}$ mol m^{-2} and irradiation time was 10 mins. Far red irradiation (< 690 nm) came from six incandescent tubes filtered by one layer Red 501 and two layers Blue 627 Plexiglas (Röhm & Haas). Fluence at seed level was $1{\cdot}7 \times 10^{-3}$ mol m^{-2} and irradiation time was 15 mins. Sowing and all further handling of the seeds was performed in dim green light obtained by filtering one green fluorescent tube (Philips TL 40W/17) with two layers of yellow (no. 46) and two layers of blue (no. 62) Cinemoid filters (Strand Electric, London, UK). Where test solutions had to be removed or changed during incubation, the seeds were rinsed with two portions of 100 ml of distilled water on a Büchner funnel, sucked dry, transferred to a clean Petri dish, whereafter 1·5 ml of the new solution was added. Germination was counted after 3 d at 24 °C.

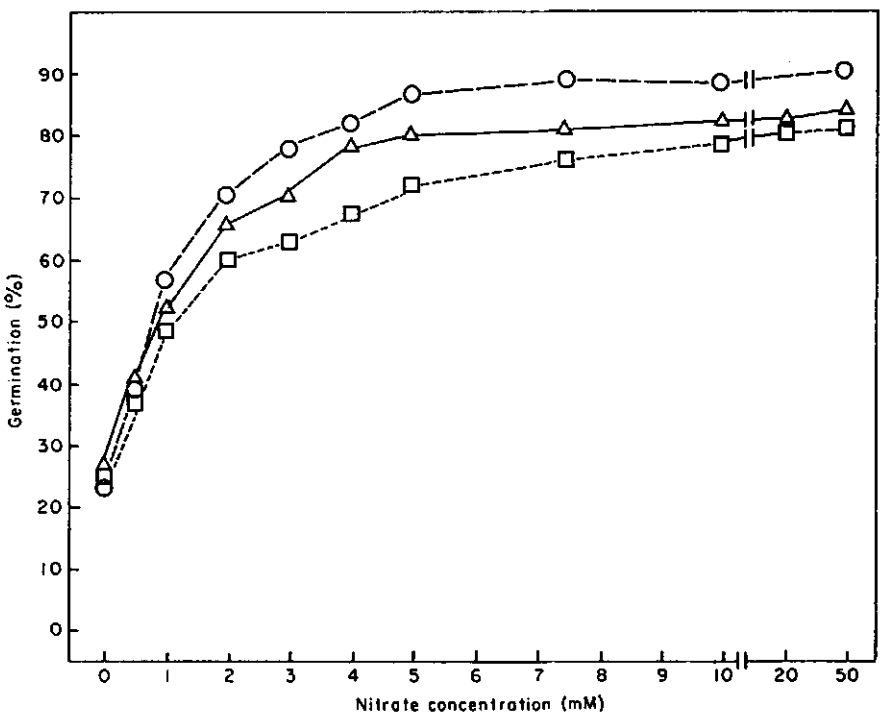

FIG. 1 Germination at 24 °C of seeds preincubated for 72 h in darkness at 15 °C in the indicated concentrations of KNO_3 alone (○), or in combination with 50 mM $NaClO_3$ (□) or 50 mM Na_2WO_4 (△). At the end of the preincubation seeds were rinsed, transferred to water (○), 50 mM $NaClO_3$ (□) or 50 mM Na_2WO_4 (△) and irradiated with R. Germination was counted after 3 d at 24 °C.

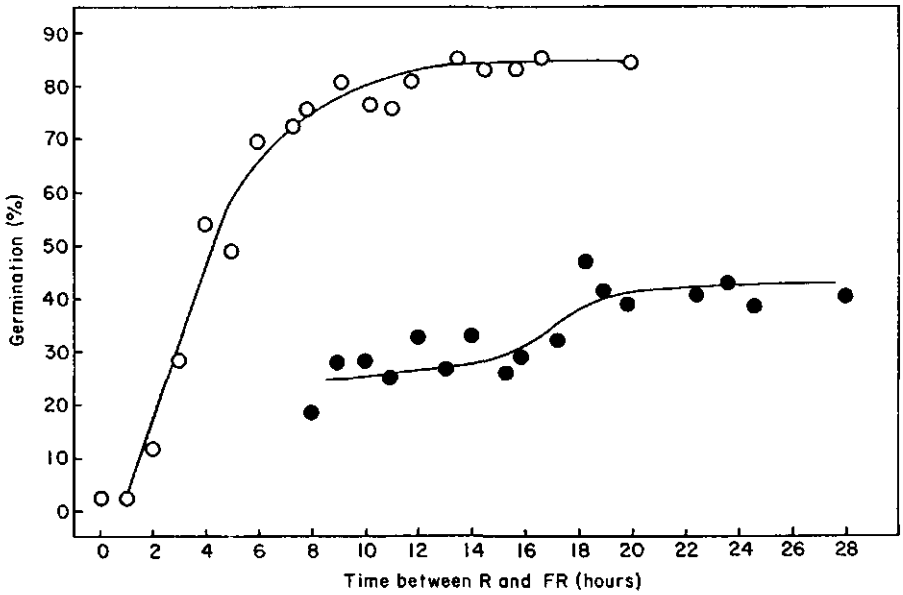

FIG. 2 Escape from FR inhibition of seeds pre-inucubated in 10 mM KNO_3 (○) or water (●) at the conditions of Fig. 1 and irradiated with R. The seeds that were pre-incubated in water were transferred to 10 mM KNO_3 at 8 h after R. FR was given at the indicated times. Germination was counted after 3 d at 24 °C.

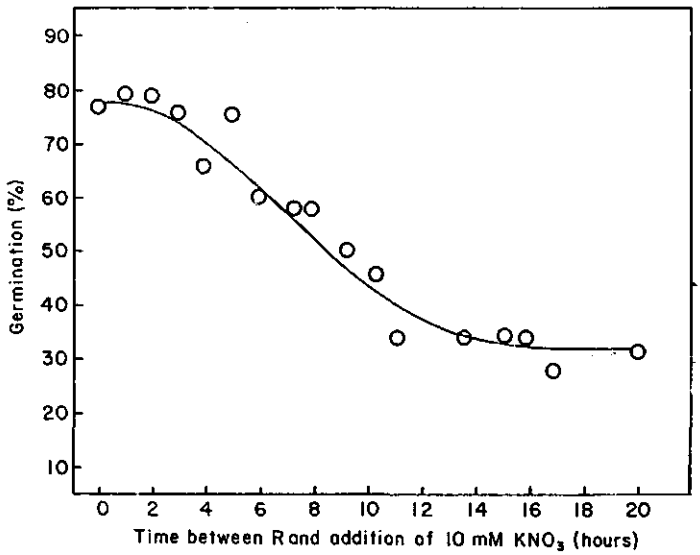

FIG. 3 The effect of a delay of the application of 10 mM KNO_3 on the germination of seeds pre-incubated in water at the conditions of Fig. 1 and irradiated with R at the end of the pre-incubation. Germination was counted after 3 d at 24 °C.

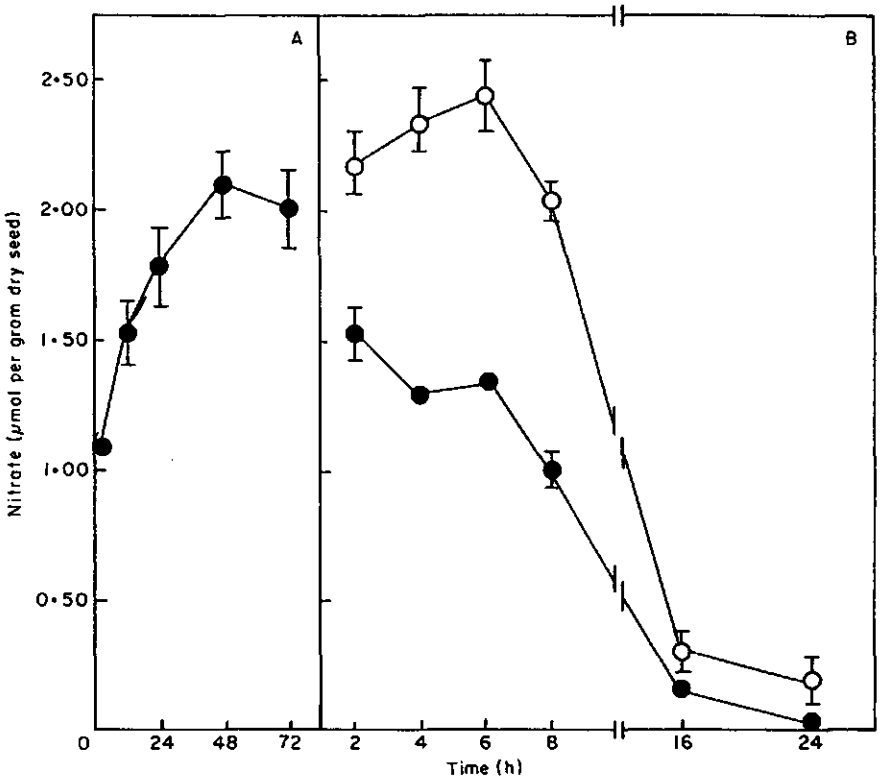

FIG. 4. (A) Nitrate levels in seeds during a 72 h pre-incubation in the dark at 15 °C in 10 mM KNO_3 (●). (B) Nitrate levels in seeds (●) and total nitrate levels (○) calculated from the levels of endogenous nitrate plus nitrate in the leachate, during a 24 h incubation at 24 °C after transfer of the seeds from 10 mM KNO_3 to water and a R pulse at the end of the 72 h pre-incubation. Vertical bars indicate s.d.

Nitrate measurements

50 mg (±0·5 mg) of seeds (d. wt) were rinsed and sucked dry on a Büchner funnel after incubation, and transferred to a plastic vial on ice. After freezing and thawing, the seeds were homogenized in 1·5 ml of distilled water with a stainless steel rod for 1 min. The homogenate was left standing on ice for 1 h. After centrifugation for 15 mins at 16000 *g*, 1 ml of the supernatant was transferred to another vial containing 5 mg of activated charcoal CN-1 (Norit Clydesdale Co. Ltd, Glasgow, UK). The vial was shaken in the cold for 15 mins, centrifuged for 15 mins at 16000 *g* and the supernatant passed through a MA 25 prefilter (Millipore, Etten-Leur, The Netherlands).

The sample (20 μl) was injected into a Model 3500B HPLC system (Spectra Physics, Santa Clara, California, USA) equipped with a Model 770 spectrophotometric detector (Spectra Physics) set at 210 nm, and a model C-R1B integrator (Shimadzu, Kyoto, Japan)). The column was a Lichrosorb 10NH2 column (Chrompack, Middelburg, The Netherlands). The mobile phase consisted of 25 mM KH_2PO_4 pH 3·7 or 10 mM KH_2PO_4 pH 3·0 when nitrate was measured in the presence of chlorate. Nitrate levels were calculated on the basis of a linear relationship between concentration and peak height of pure nitrate standards and expressed in μmoles of nitrate per gram dry seed.

RESULTS

A preincubation of 72 h in the dark at 15 °C resulted after a R pulse in a germination of 25% in water and of maximally 90% in potassium nitrate, saturating between 4 and 7 mM (Fig. 1). Addition of 50 mM sodium chlorate or 50 mM sodium

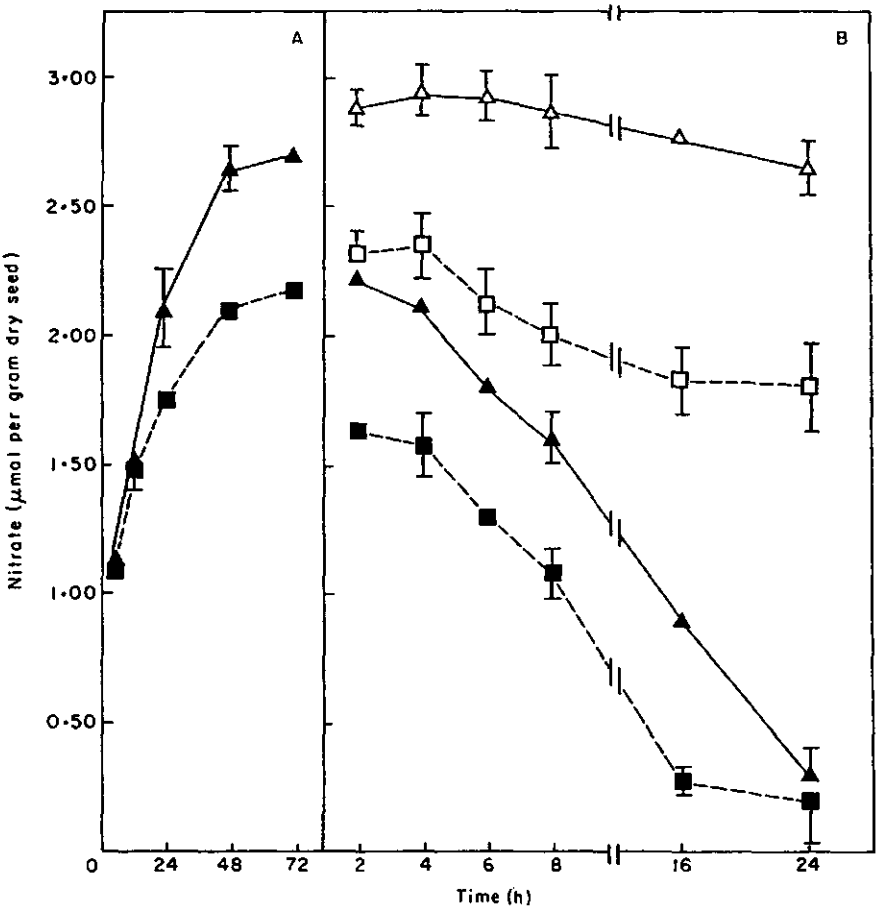

FIG. 5. (A) Nitrate levels of seeds during a 72 h pre-incubation at 15 °C in darkness in 10 mM KNO_3 + 50 mM $NaClO_3$ (■) or 10 mM KNO_3 + 50 mM Na_2WO_4 (▲). (B) Nitrate levels in seeds (■, ▲) and total nitrate levels (□, △) calculated from the levels of endogenous nitrate and nitrate in the leachate, during a 24 h incubation at 24 °C after a R pulse and transfer to 50 mM Na_2WO_4 (△, ▲) or 50 mM $NaClO_3$ (□, ■) at the end of the pre-incubation.

tungstate to the incubation medium from the start of imbibition had no significant influence on the dose–response relationship (Fig. 1). However, during the experiments it was seen that growth of the seedling rootlet was somewhat inhibited in the presence of chlorate. Tungstate severely inhibited growth of both the rootlet and the hypocotyl.

In order to establish the time course of Pfr action after the R pulse the escape time for FR irradiation was determined (Fig. 2). Most of the seeds escaped from FR inhibition within 8 h from R and some seeds between 8 and 16 h. If the addition of nitrate was delayed for 8 h the escape of the nitrate-dependent subpopulation did not start. It can be seen that the nitrate-independent subpopulation of approx. 25% did escape from FR inhibition. Upon addition of nitrate a lag time before escape took place was observed, probably due to the time required for nitrate to reach its active site. The fact that only a very small part of the seed population escaped from FR inhibition is probably the result of induction of secondary dormancy and/or dark reversion of Pfr to Pr (Hilhorst *et al.*, 1986). Such changes may also be responsible for the decreased germination response after a delay of nitrate application to red irradiated seeds (Fig. 3). For a maximum response, 10 mM KNO_3 nitrate had to be applied within 3 h after R. The data of Figs 2 and 3 suggest that induction of germination by Pfr and 10 mM KNO_3 took place

during the first 8 h after R. If essential for the induction of germination nitrate reduction would be expected to occur during this 8 h period. We decided to monitor the nitrate levels during this period to see whether nitrate reduction took place.

After a doubling of the endogenous nitrate level during the 72 h pre-incubation in 10 mM KNO_3 at 15 °C (Fig. 4A), endogenous levels slowly decreased during the first 8 h after R at 24 °C (Fig. 4B). Since total levels of nitrate (endogenous + leachate) remained constant, leaching of nitrate from the seeds was fully responsible for this decrease, indicating that nitrate reduction did not occur during the first 8 h. Between 8 and 16 h after R the decline in the endogenous levels was no longer compensated for by leaching. Between 8 and 16 h visible germination started and it can be concluded that apart from reduction of endogenous nitrate also exogenous nitrate was taken up and reduced by the germinating seeds.

In order to eliminate the possible effect of a small but significant reduction of nitrate, the experiment was repeated in the presence of 50 mM $NaClO_3$ or 50 mM Na_2WO_4. The uptake of nitrate from 10 mM KNO_3 during the 72 h pre-incubation in the presence of chlorate (Fig. 5A) was similar to the uptake in its absence (Fig. 4A). However, in the presence of 50 mM tungstate more nitrate was taken up by the seeds. The reason for this higher uptake remains obscure but is not relevant for our objective. Also in the presence of chlorate or tungstate endogenous levels of nitrate decreased during the first 8 h after R but total levels remained constant (Fig. 5B). After the first 8 h total nitrate levels decreased to approx. 80% of the initial level in the presence of chlorate, indicating a nitrate reduction proportional to the nitrate/chlorate ratio. In the presence of tungstate, however, total levels of nitrate did not significantly decrease during the 24 h after R indicating a complete inhibition of nitrate reduction before and after visible germination. Germination in the presence of chlorate or tungstate was not affected (Fig. 1).

DISCUSSION

The present results clearly show that induction of seed germination of *Sisymbrium officinale* by R and nitrate took place during the first 8 h after R. This was concluded from the data of Fig. 2 which shows that the escape time for the inhibiting effect of FR irradiation was approx. 8 h. The presence of nitrate was essential for the escape reaction since no escape of the nitrate-dependent subpopulation occurred in the absence of nitrate. For a maximal germination response in 10 mM KNO_3, nitrate application had to take place within 3 h after R (Fig. 3). Thereafter the interaction of Pfr and nitrate was affected by dark reversion of Pfr to Pr, resulting in a lower germination response. Thus, during the 8 h period after R, Pfr was effective in establishing with nitrate a FR-insensitive intermediate that was essential to the induction of germination. Measurements of nitrate levels during the 24 h period after R revealed that in the first 8 h nitrate reduction did not occur. During the next 8 h visible germination started and nitrate levels declined, which could be attributed to nitrate reduction (Fig. 4A). Despite an inhibitor/nitrate ratio varying from 100 (at 0·5 mM KNO_3) to 5 (at 10 mM KNO_3), chlorate and tungstate did not affect germination (Fig. 1) although nitrate reduction was inhibited by 80 and 100%, respectively, during the period of visible germination (Fig. 4B). The observed anomalies of seedling growth in the presence of the inhibitors may be due to the inhibited nitrate assimilation, besides possible toxic effects.

We now conclude that reduction of nitrate is not a prerequisite for the nitrate-stimulated germination of *Sisymbrium officinale* seeds.

In white mustard (*Sinapis alba* L.) seedlings inducibility of NRA by nitrate and Pfr depended on the stage of development of the seedling, which appared to be well after the start of visible germination (Schuster, Oelmüller and Mohr, 1987; Rajasekkar and Oelmüller, 1987). Moreover, induction of NRA by Pfr in this species proceeds via a stored light signal with a lifetime of approx. 12 h which remains active even in the absence of Pfr. In contrast, the light effect for induction of germination that we observed depended on Pfr during the first 8 h after establishing maximal Pfr levels. This leads to the conclusion that Pfr and nitrate-induced germination is a different process than the Pfr and nitrate induced NRA. It may be argued now that nitrate has a dual role: (1) induction of germination in the unreduced state and (2) induction of NRA after visible germination. From studies on cereals it was concluded that as long as the endosperm is still actively supplying reduced nitrogen to the seedling, exogenous nitrogen is a relatively minor source of plant nitrogen (Oaks, 1983). Therefore nitrate as nitrogen source, and consequently NR, are relevant to the growth of the seedling and not to the induction of germination.

However, the mechanism of nitrate-induced germination remains obscure. Earlier hypotheses regarding nitrate as an alternative electron-acceptor to molecular oxygen (Roberts and Smith, 1977; Hendricks and Taylorson, 1972) are invalid for *Sisymbrium officinale*. A promising suggestion

for direct nitrate action seems the induction of membrane potential changes, which have been described for nitrate uptake by mesophyl cells of *Lemna gibba* (Ullrich and Novacky, 1981) and in cortical cells of 4-d-old maize roots (Thibaud and Grignon, 1981). In relation to this phenomenon the effect of Pfr on several membrane related processes may be of importance (Roux, 1986).

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CHAPTER 5

Dose-response analysis of factors involved in germination and secondary dormancy of seeds of *Sisymbrium officinale*.

I. Phytochrome

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ABSTRACT

The germination of seeds of Sisymbrium officinale is light- and nitrate dependent. A close interaction between the effects of light and nitrate on germination has been reported previously (HWM Hilhorst, CM Karssen [1988] Plant Physiol 86: 591-597). In this study a detailed dose-response analysis of the light-induced germination during induction of secondary dormancy is presented. Germination in water dropped from 90 to 0 percent after a dark incubation at 15°C of approximately 160 hours. In the presence of 25 millimolar KNO_3 the decrease of the germination level was delayed. At 24-hour intervals fluence-response curves were obtained in the presence of 25 millimolar KNO_3. With increasing length of the pre-incubation period fluence-response curves shifted along the abscissa to the right. After 120 hours the maximal germination level started to decline. The fluence-response curves were simulated by using formulations from the receptor occupancy theory for a simple bimolecular reaction in which the reaction partners were Pfr and its tentative receptor X. A good simulation was obtained when cooperativity of the binding of Pfr to X was assumed. The experimental curve parameters could then be interpreted as binding parameters. The role of phytochrome receptors, their possible location in membranes, and the action of nitrate with respect to dormancy are discussed.

INTRODUCTION

The involvement of light in the seed germination of many wild species has been known for a long time. Since the majority of the species studied shows reversibility of the light-induction by far-red irradiation, there is little doubt that phytochrome is involved. The active form of phytochrome, Pfr, is assumed to be the trigger for germination of light-requiring seeds and possibly also of light-independent seeds. The levels of pre-existing Pfr in these seeds may be higher than the threshold for germination (Cone and Kendrick, 1985).

Induction of secondary dormancy is characterized by a loss of sensitivity to environmental factors (Karssen, 1980/81). The declining response to red light (R) after prolonged dark incubation of seeds of *Rumex crispus* and *Portulaca oleracea* was compatible with a model in which germination is initiated after attainment of a certain level of interaction between Pfr and a phytochrome receptor, X, (Duke et al., 1977). According to this model, the decreasing germination response during induction of dormancy was the result of declining levels of active receptors X, while the total level of phytochrome (P_{tot}) and the Pfr decay rate remained constant. However, in a subsequent paper it was concluded that the declining response to R of *Rumex crispus* seeds upon dark incubation was the result of changes in the level of PfrX required for 50 % germination. This level depended on several unknown factors but not on the level of X or P_{tot} (Duke, 1978).

A model based on indications that the phytochrome molecule is a dimer (VanDerWoude, 1985) could explain biphasic fluence-response curves which were observed after sensitization treatments of lettuce seeds (Blaauw-Jansen and Blaauw, 1975, 1976). This model assumed the existence of two different active phytochrome-receptor complexes, Pr:Pfr-X and Pfr:Pfr-X, which were responsible for the very low fluence response (VLFR) and the low fluence response (LFR), respectively. Implications were that the level of X was about 10^{-3} $P:P_{tot}$. Moreover, occupation of less than 40 % of X was sufficient to maximize the LFR.

Germination of *Sisymbrium officinale* seeds has an absolute requirement for light and nitrate (Karssen and de Vries, 1983; Hilhorst *et al.*, 1986, Chapter 2). Detailed studies have shown a strong interaction between light

and nitrate (Hilhorst and Karssen, 1988, Chapter 3). These studies were performed on seeds that possessed a certain degree of dormancy. The aim of the present study was to monitor the response to R during induction of secondary dormancy and to give changes in the response a physico-chemical meaning, with respect to the interaction between phytochrome and its receptor.

MATERIALS AND METHODS

Seeds. A seed lot of *Sisymbrium officinale*, harvested in the vicinity of Wageningen in 1985 was used. The seeds were cleaned and stored dry at 4°C.

Germination experiments. Duplicates of 75 seeds were sown in 50 mm Petri dishes on one layer of filter paper (Schleicher and Schull no. 595) and moistened with 1.5 ml water or 25 mM KNO_3. Seeds were pre-incubated at 15°C in the dark, irradiated, and transferred to 24°C. Germination was counted after 3 days in the dark. Broad-band red light (610-700 nm) was obtained from six red fluorescent tubes (Philips TL 20W/15) filtered by 3 mm red Plexiglas (Red 501, Rohm and Haas, Darmstadt, FRG). Fluence at seed level was $1.5*10^{-3}$ mol m^{-2}. Irradiation time was 10 minutes. Fluence-response experiments were performed essentially as described earlier (Hilhorst and Karssen, 1988, Chapter 3). Sowing and all further handling of the seeds were done in dim green light from one green fluorescent tube (Philips TL 40W/17) that was filtered by two layers of yellow (no. 46) and two layers of blue (no. 62) Cinemoid filters (Strand Electric, London, UK). All experiments were repeated at least once with qualitatively similar results.

Calculations. Fluence-response curves were calculated from the data points by weighted linear regression analysis in a log-dose probit diagram as described before (DePetter *et al.*, 1985; Hilhorst and Karssen, 1988, Chapter 3).

RESULTS

Time course of induction of secondary dormancy

In order to determine the pattern of dormancy induction with respect to light, seeds were pre-incubated in water or 25 mM KNO_3 at 15°C for various periods and germinated at 24°C (Fig. 1). It can be seen that seeds from this batch did not possess primary dormancy. After a pre-incubation as short as 2 h germination was already at its maximum. It is clear that the light-requirement of this batch is absolute, seeds did not germinate in nitrate in darkness.

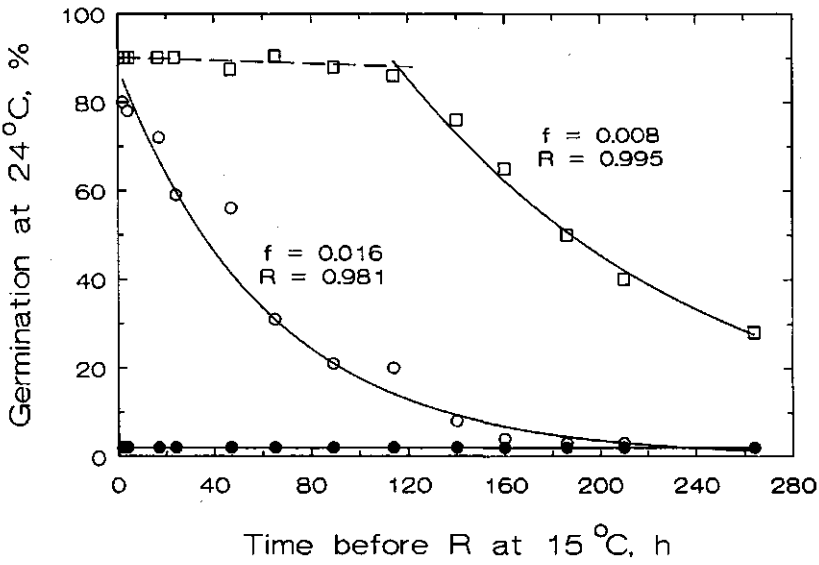

Fig. 1: Germination at 24°C of seeds pre-incubated for variable periods at 15°C in water (○) or 25 mM KNO_3 (●,□) and irradiated with red light (○,□) or kept in the dark (●). Decreasing parts of the curves were exponentially fitted to $y = (1-f)^t$, with y = response, t = time in h, f = rate of decrease (% h^{-1}). R = correlation coefficient.

The germination level of seeds that were pre-incubated in water declined to zero in approximately 120 h. Induction of dormancy of seeds in 25 mM KNO_3 was delayed. The maximal response was maintained until about 120 h, whereafter a decrease occurred. Both responses decreased exponentially. In the absence of nitrate the decrease was faster than in its presence (compare f values in Fig. 1).

Fluence-response curves.

At 24 h intervals seeds were exposed to a range of red light fluences and were germinated in 25 mM KNO_3. A set of 4 representative fluence-response curves is shown in Fig. 2. The parameters of the best fitting curves for all pre-incubation times are shown in Table 1. Up to a pre-incubation time of approximately 120 h, the major change was a shift of the curve to higher fluence values, expressed by an increasing m (log-fluence value for half-maximal germination, Table 1).

Table 1: Parameters of observed fluence-response curves after several pre-incubation periods.

h at 15°C	R^-(%)	s(R^-)	R_{max}(%)	s(R_{max})	m	s(m)	B	s(B)
24	4.96	0.94	97.76	1.62	-4.48	0.03	1.88	0.11
48	2.57	0.61	97.80	1.17	-4.33	0.02	1.63	0.07
72	4.64	0.50	90.10	1.20	-4.13	0.02	1.75	0.07
96	2.89	0.72	94.35	1.58	-4.16	0.03	1.70	0.09
120	1.59	0.67	96.65	1.36	-4.16	0.03	1.84	0.10
144	1.93	0.35	92.98	1.05	-3.96	0.02	1.98	0.09
192	1.04	0.32	84.15	1.31	-3.97	0.02	2.50	0.18
264	0.38	0.15	30.83	1.68	-3.58	0.14	2.72	0.31

Parameters were calculated by means of weighted linear regression analysis in a log-dose probit diagram. R^-: minimal response; R_{max}: maximal response; m: log fluence for half-maximal response; B: slope of log-dose probit line; s: standard deviation; %: percentage germination.

The shift was parallel, with the exception of the two longest pre-incubations, which were slightly steeper as seen from increasing values of B (slope of log-dose probit line). Differences in B values of the other pre-incubation periods were not significant. After 120 h the curves did not only shift to higher fluence values but maximum germination levels started to decline.

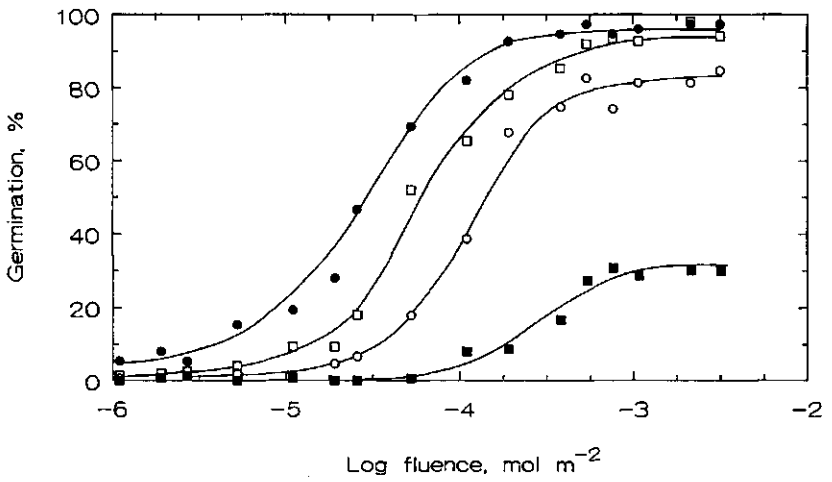

Fig. 2: Fluence-response curves obtained after 24 (●), 120 (□), 192 (○) and 264 (■) h pre-incubation at 15°C in 25 mM KNO_3. Seeds were irradiated and germinated at 24°C. Curves were calculated from population parameters.

Simulations

To relate the observed changes to the quantitative behavior of a receptor-mediated process, we tested whether models from general receptor theory were appliable. The simplest meaningful model was developed by Clark in 1933 (see Boeynaems and Dumont, 1980). To use this model a simple monomeric interaction between Pfr and its receptor X has to be assumed:

$$Pfr + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PfrX \xrightarrow{k_e} Response \qquad (1)$$

where k_1 and k_{-1} are kinetic association and dissociation constants, respectively, and k_e is a proportionality constant between the response and the receptor occupancy. It is assumed that all receptors are equivalent and independent; the response is proportional to the number of occupied receptors; the response is measured after the interaction between Pfr and X has reached a state of equilibrium; and Pfr can only exist in two states: free or bound to X. The model can be described by the following equation:

$$[PfrX] = [X]_T[Pfr]/(K_D + [PfrX]) \qquad (2)$$

with $[X]_T$ = total level of [X] = [X] + [PfrX]

K_D = dissociation constant = k_{-1}/k_1

If response $R = k_e[PfrX]$ and R_{max} = maximal response = $k_e[X]_T$, then

$$R/R_{max} = [Pfr]/(K_D + [Pfr]) \qquad (3)$$

For $R = 0.5R_{max}$: $K_D = [Pfr]_{0.5}$ (4)

The level of [Pfr] for each applied fluence was calculated with the following equation (Hartmann and Cohnen-Unser, 1972):

$$[Pfr] = \{1-\exp(-(S1 + S2).a.F)\}.\phi.[P_{tot}] \qquad (5)$$

with S1 and S2: photoconversion constants, mol m^{-2}

a: attenuation factor, depending on seed coat transmission characteristics, dimensionless; value between 0 and 1.

F: photon fluence, mol m^{-2}

ϕ: photo-equilibrium = S1/(S1 + S2)

With the aid of equations 3 and 5 fluence-response curves were calculated.

The values for K_D were calculated by substituting the values for m (Table 1) in eq. 5. The values for R_{max} were also taken from the observed curves (Table 1). Fig. 3 shows the simulations for the curves of Fig. 2. Although Clark's equation generated curves that showed qualitatively similar changes as the observed curves (increase of m, decrease of R_{max}), the simulations showed considerable discrepancies between observed and calculated individual curves (Table 2). R_{max} values of the calculated curves were generally too low, while the slopes of the curves, expressed by B, showed no variation and were considerably less steep than those of the observed curves.

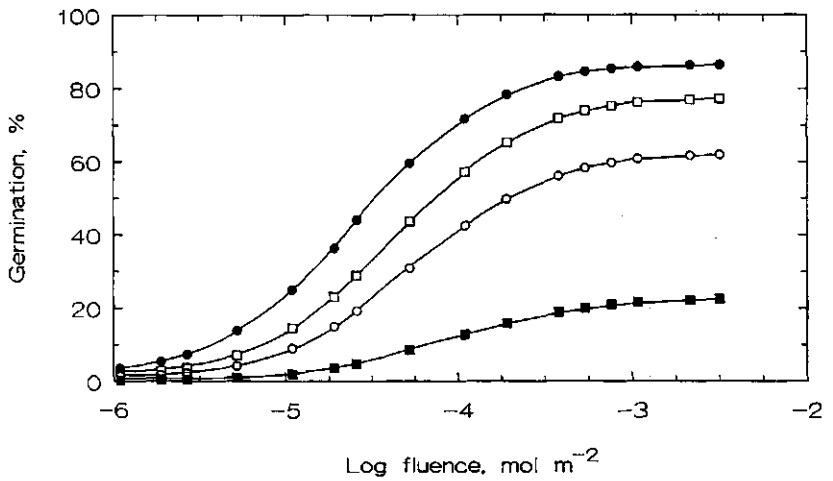

Fig. 3: Fluence-response curves of Fig. 2 simulated with eq. 3 assuming that a = 1. Equation parameters were derived from Table 1. Curves were calculated from population parameters.

By definition, a slope at $0.5R_{max}$ greater than 0.576 in a semi-logarithmic plot is indicative for positive cooperativity of the receptor (Boeynaems and Dumont, 1980). Therefore, Clark's equation was modified by entering the parameter n, often called interaction-, cooperativity- or Hill-coefficient (eq. 6). This coefficient was first used by Hill in 1910 to describe the sigmoid-shaped saturation curves for the binding of oxygen to hemoglobin

$$R = R_{max}[Pfr]^n/([Pfr]^n + K_D) \quad (6)$$

Furthermore, the low but significant dark germination could be explained by assuming the existence of 2.5 % pre-existing Pfr. (Fig. 2). Eq. 5 was therefore modified according to Cone and Kendrick (1985). Since n is defined as the slope of the sigmoidal curve at the inflection point, *i.e.* at half-maximal response, n could be substituted by the parameter B from the observed curves (Table 1). B is the slope of the log-dose probit line, which is similar to the slope of the curve at half-maximal response. K_D was calculated from $[Pfr]^n_{0.5}$.

Table 2: Curve parameters and standard deviations (s) of fluence-response curves of Fig. 2 simulated with eq. 2.

h at 15°C	R^-(%)	s(R^-)	R_{max}(%)	s(R_{max})	m	(m)	B	s(B)
24	2.30	0.16	84.09	0.16	-4.57	0.004	1.55	0.01
120	1.58	0.17	75.72	0.31	-4.36	0.08	1.57	0.02
192	1.14	0.12	60.92	0.35	-4.25	0.01	1.58	0.03
264	0.26	0.04	22.25	0.26	-4.06	0.02	1.57	0.04

Values for R_{max} were taken from Table 1. [Pfr] for each fluence was calculated from eq. 5. Values for $[Pfr]_{0.5}$ were calculated from observed m values (Table 1) and eq. 5. Photochemical constants were taken from Kelly and Lagarias (1985). Symbols as in Fig. 2. Curves were calculated from population parameters.

It is clear that insertion of the cooperativity coefficient into eq. 3 greatly improved the simulations (Fig. 4). There was no significant difference between observed and calculated values of m (compare Tabs. 3 and 1). With the exception of the 24 h value, the observed values for R_{max} were consistently 5-10% (absolute value) higher. The differences between observed and calculated B values were small but significant for pre-incubation times up to 144 h, while after 192 and 264 h values were statistically similar.

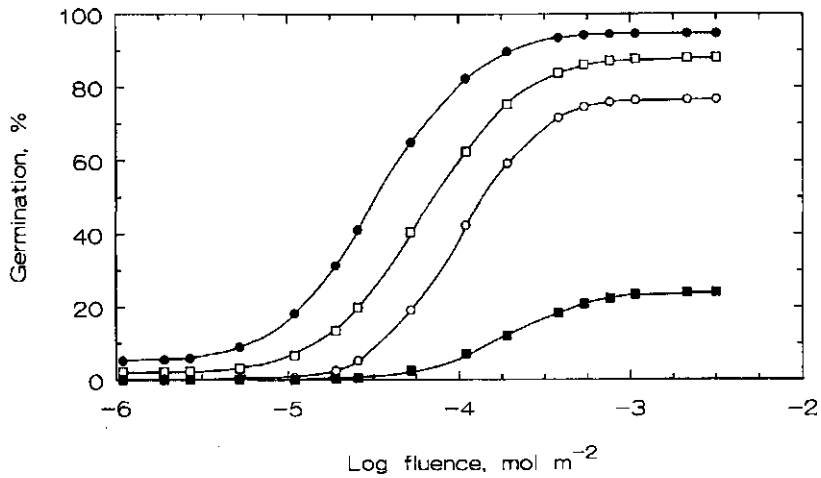

Fig. 4: Fluence-response curves of Fig. 2 simulated with eq. 6. Equation parameters were taken or derived from Table 1. Symbols as in Fig. 2. Curves were calculated from population parameters.

Table 3: Curve parameters and standard deviations of fluence-response curves of Fig. 2 simulated with eq. 6.

h at 15°C	R^-(%)	s(R^-)	R_{max}(%)	s(R_{max})	m	s(m)	B	s(B)
24	5.15	0.15	94.60	0.28	-4.46	0.04	2.14	0.02
48	5.05	0.21	90.75	0.43	-4.35	0.06	2.02	0.03
72	2.15	0.10	80.42	0.33	-4.19	0.06	2.08	0.03
96	2.71	0.11	84.49	0.32	-4.22	0.05	2.05	0.02
120	2.17	0.10	87.96	0.30	-4.21	0.05	2.15	0.02
144	0.85	0.08	79.97	0.40	-4.04	0.07	2.24	0.04
192	0.28	0.04	76.56	0.30	-4.01	0.06	2.61	0.04
264	0.08	0.02	24.04	0.35	-3.73	0.13	2.40	0.08

Equation parameters were taken or derived from Table 1. Curves were calculated from population parameters.

DISCUSSION

The present study shows that fluence-response curves in the LFR range can be simulated by an equation for a simple bimolecular reaction from general receptor occupancy theory. This equation is formally similar to the mathematical formulation that describes any sigmoidal curve. However, in the present study the curve parameters R_{max}, K_D and n were not given theoretical values but were derived from observed curves (R_{max}, m and B, respectively). This interaction model does not necessarily reflect the actual interaction between Pfr and X and the subsequent response. However, Clark's equation and its modified version accounting for cooperativity have proven to be good descriptions of many hormone-receptor interactions, both *in vitro* and *in vivo*, in animal physiology (Hollenberg, 1985). Moreover, although not yet supported by analysis of actual hormone-receptor binding properties, several responses induced by plant hormones fit the equation, *e.g.* the ABA-induced stomatal closure in epidermal strips of *Commelina communis* (Weyers *et al.*, 1987).

Evidence is accumulating that the interaction between Pfr and X is not simply bimolecular. The phytochrome molecule is a dimer *in vitro* (Jones and Quail, 1986) and probably also *in vivo* (Brockmann *et al.*, 1987) and consists of two identical subunits each containing one chromophore. In his dimeric model VanDerWoude (1985) has clearly shown that the formation of Pfr/P_{tot} by fluences in the LFR range closely resembled the formation of $Pfr:Pfr/P_{tot}$, because the level of $Pr:Pfr/P_{tot}$ could be neglected in this fluence range. Thus, the responses in our studies may be regarded as the LFR component of the dimer model.

Furthermore, it should be noted that we are dealing with seed populations. Since we defined the germination response to be proportional to the amount of PfrX formed, the translation into 'population terms' is that tolerances or thresholds for PfrX of individual seeds are normally distributed around a PfrX level for half-maximal germination (DePetter *et al.* 1985).

The response to light at 24°C declined exponentially upon pre-incubation in the dark at 15°C (Fig. 1). In the presence of nitrate the decline started approximately 120 h later. The decay rate, expressed by f, was two

times faster in the absence of nitrate than in its presence. It may be assumed that a certain level of PfrX generates the maximal germination response of about 90 %. Seeds that were germinated in water only showed a maximal response after a few hours of pre-incubation. Thus it may be argued that for an increasing number of seeds the established number of occupied receptors became too low for germination. If so, nitrate may generate more active Pfr-receptors, sufficient to maintain PfrX levels above threshold levels for at least 120 h. Extrapolation of the decay curve for seeds in nitrate to time zero gives a value of 220 %, against 88 % in the absence of nitrate. Since $R_{max} = k_e R_T$, this indicates that in the presence of nitrate more active receptors are present. Hence, nitrate may create a receptor reserve. In other words, approximately 40 % of the total amount of active receptors may be sufficient for a maximal response. A similar value was calculated by VanDerWoude (1985) for the binding of Pfr:Pfr to X to maximize the LFR of non-sensitized lettuce seeds. Obviously, changes in receptor levels above the level required for a maximal response cannot be observed by determining germination responses at saturating fluences. Therefore, fluence-response experiments were performed (Fig. 2, Table 1). Since these curves fitted equation 6, we used the properties of this equation to describe the observed changes in the curves upon induction of dormancy. From equation 2 it is evident that a decreasing level of $[X_T]$ can only result in a maximum response when the concentration of [Pfr] is increased to achieve the same concentration of [PfrX], providing n remains unchanged (eq. 6). Hence the fluence-response curve shifts to the right. This shift can only occur when 'spare' receptors are present. When the receptor number is reduced below the level required for a maximal response, R_{max} will be reduced. However, since $R_{max} = k_e[X_T]$, reduction of R_{max} may also be the result of postreceptor defects. The cooperativity coefficient n increased at the two longest incubation periods (192 and 264 h). It is not likely that true cooperativity of the receptor is enhanced during induction of dormancy. If the cooperativity coefficient is not an integer, indicating the number of receptor protomers involved in binding the promoter, it is a purely phenomenological parameter (Boeynaems and Dumont, 1980). However, when $n > 1$ (or $B > 0.576$) the binding to the receptor is a cooperative process. The increase of B observed here is probably the result of the fact that

the 192 and 264 h curves saturate close to the fluence value that establishes the maximum Pfr/P_{tot} ratio of 0.883. The thresholds of the seeds that are induced at the left-hand part of the curve shift to the right while the fluence value for saturation of the curve does not change. The remaining sub-population that germinates is still log-normally distributed around a fluence value for half-maximal germination; the symmetry index $F_{0.9}*F_{0.1}/F_{0.5}^2$ with F = fluence for 0.9, 0.1 or 0.5 of the maximum response, is very close to 1, indicating symmetry of the semi-logarithmic plot around the mid-saturation point.

From the present study a model can be proposed in which induction of dormancy of light-requiring seeds is the result of a decline of the number of active phytochrome receptors. The receptor is proposed to be a membrane bound component since anesthetics and other agents that influence membrane properties influence phytochrome control of seed germination and dormancy (Taylorson and Hendricks, 1980/81; Taylorson, 1988). In lettuce seeds it was found that sensitization of the fluence-response by chilling was very similar to sensitization by ethanol (VanDerWoude, 1985). Based on these observations and the present results we speculate that induction of secondary dormancy in light-requiring seeds is the result of a change in membrane properties which gradually decrease the number of accessible Pfr-binding sites and/or make the Pfr-receptor complex less active. The role of nitrate seems to be an enhancement of the number of active Pfr-binding sites or an inhibition of their inactivation. In earlier studies it was shown that nitrate increased the slope of the fluence-response curve (Hilhorst and Karssen, 1988, Chapter 3). Thus, nitrate may have a positive influence on the cooperativity of the binding of Pfr to its receptor. An increase of cooperativity implies that binding of one Pfr molecule would enhance the binding of a second molecule to a larger extend. However, the receptor-occupancy theory provides an alternative explanation for an apparent increase in cooperativity. One of the assumptions that have to be justified before applying the occupancy theory is that the fraction of bound agonist (here Pfr:Pfr-X) can be neglected with respect to the total amount of agonist (Pfr:Pfr + Pfr:Pfr-X). If nitrate increases the number of Pfr-receptors, more Pfr:Pfr will be bound while the amount of total Pfr:Pfr remains unchanged. This will increase the ratio of bound to free Pfr:Pfr. At fluences at the low fluence side of the LFR range very little

Pfr:Pfr is formed. Hence, the assumption that the bound phytochrome fraction may be neglected may not be valid here. The consequences for the saturation curve will be two-fold: First, the fluence range over which the response occurs will be decreased; second, the saturation curve will no longer be symmetrical in semi-logarithmic coordinates: the right-hand part of the curve will be steeper than the left-hand part (Boeynaems and Dumont, 1980). Both consequences can be seen in our fluence-response curves (Chapter 3, Figs. 2 and 3). It is, however, not possible to determine whether the curves are non-symmetrical. The curves calculated from the data points are best fits to sigmoidal curves which are symmetrical. Because of the small fluence ranges it is impossible to assess the degree of symmetry of the original series of data-points.

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CHAPTER 6

Dose-response analysis of factors involved in germination and secondary dormancy of seeds of *Sisymbrium officinale*.

II. Nitrate

Dose-response analysis of factors involved in germination and secondary dormancy of seeds of *Sisymbrium officinale*
II. Nitrate.

ABSTRACT

The role of nitrate as a promoter of germination of Sisymbrium officinale seeds was examined in optimal light-conditions. It was shown that the requirement for nitrate was absolute. This was true for all seed lots used. The probit of germination in water was log-linearly related to the level of endogenous nitrate. Pre-incubation at 15 °C in water resulted in an immediate decrease in germination level, while in 25 millimolar KNO_3 the decrease was delayed. The decline of the germination in water was strongly correlated with the rate nitrate leached from the seeds. The germination response to a broad range of KNO_3 concentrations was followed during pre-incubation, at 24-hour-intervals. During 264 hours of pre-incubation the nitrate-response curves showed a right-hand shift parallel to the X-axis. After 120 hours the high maximum germination level started to decline. The curves could be simulated by an equation from the receptor-occupancy theory. It was concluded that induction of secondary dormancy is a result of a decrease of the number of nitrate receptors. After 24 and 48 hours of pre-incubation the nitrate-response curves were biphasic. The biphasic character could be related to the level of endogenous nitrate and to a differential requirement for nitrate of two fractions of the seed population. Similarities with the behavior of fluence-response curves under similar conditions led to the hypothesis that phytochrome and nitrate share the same site of action.

INTRODUCTION

Nitrate stimulates the seed germination of a broad range of wild species (Roberts and Smith, 1977). For its role as a dormancy breaking agent several models have been proposed. In these models reduction of nitrate and nitrite is required to reoxidize $NADPH_2$ to NADP, either directly by the specific reductases (Roberts, 1973; Roberts and Smith, 1977) or indirectly by the inhibition of catalase action, thus initiating a hypothetical peroxidase-regulated chain of reactions leading to reoxidation of $NADPH_2$ (Hendricks and Taylorson, 1975). It was suggested that the stimulation of the pentose phosphate pathway by NADP was essential for the relief of dormancy. However, it has been shown that these models are not valid for seeds of *Sisymbrium officinale* (Hilhorst and Karssen, 1989, Chapter 4). Inhibitors of nitrate reductase activity did not influence the nitrate-stimulated germination. Moreover, nitrate levels in the seeds did not decrease during induction of germination by red light (R). Hence, a direct regulatory role for the nitrate ion was proposed.

In several species nitrate and light may act synergistically on germination (Vincent and Roberts, 1977; Karssen and de Vries, 1983; Hilton, 1985). Detailed studies of the light- and nitrate-stimulated germination of *S. officinale* seeds revealed a very close interaction between both factors. They were only active in the presence of the other. Nitrate steepened the fluence-response curve and it was concluded that nitrate might be regarded as a co-factor for phytochrome action (Hilhorst and Karssen, 1988, Chapter 3).

In a study of the fluence-response during prolonged dark incubation it was concluded that nitrate had an effect on the phytochrome-receptor X, in that nitrate enhanced the number of active receptors and/or inhibited the inactivation of the receptors (Chapter 5).

The aim of the present study was to monitor the response to nitrate at saturating fluences and to compare the nitrate-response curves with the fluence-responses obtained under similar conditions but at saturating nitrate concentrations.

MATERIALS AND METHODS

Seeds. Seeds of *Sisymbrium officinale* (hedge mustard) were collected from plants growing in the vicinity of the laboratory. For most experiments a seed lot from 1985 was used. This lot was the same as used before (Chapter 5). In some experiments seeds from 1985, 1986 and 1987 were used. Experiments were performed in 1988. Seeds were cleaned and stored dry at 4°C in plastic containers. No changes in dormancy or viability could be observed over the experimental period.

Germination experiments. Duplicates of 75 seeds were sown in 50 mm Petri-dishes on one layer of filterpaper (Schleicher and Schull, no. 595) and moistened with 1.5 ml of water or nitrate solution. After various pre-incubation periods at 15°C the seeds were irradiated with red light (R) and transferred to 24°C. Germination was counted after three days in the dark. Broad-band R (620-700 nm) came from six red fluorescent tubes (Philips TL 20W/15) filtered by 3 mm red Plexiglas (Red 501, Rohm and Haas, Darmstadt, FRG). Fluence at seed level was $1.5*10^{-3}$ mol m^{-2} and the irradiation time was 10 min. All seed handling was done in dim green light from one green fluorescent tube (Philips TL 40W/17) filtered by two layers of yellow (no. 46) and two layers of blue (no. 62) Cinemoid filters (Strand Electric, London, UK). Experiments were repeated at least once with qualitatively similar results.

Calculations. Nitrate-response curves were calculated by means of weighted regression analysis of the data-points in a log-dose probit diagram (DePetter *et al.*, 1985).

Nitrate measurements. Seedlots of 50 mg, either dry or pre-incubated and rinsed with 100 ml of distilled water, were transferred to a plastic vial on ice. After freezing and thawing the seeds were homogenized with a stainless steel rod in 1.5 ml of distilled water. In one experiment extractions were carried out with 0.2 % Triton X-100, 50 mM KH_2PO_4 pH 3.7 or 0.2 M NaCl. In addition, extraction was carried out at elevated temperature (10 min. 100°C). The homogenate was kept on ice for 1 h and centrifuged for 15 min at 16000 g. Of the supernatant 500 µl was put on

top of a 3 mm layer of Lichroprep RP-8 (particle size 25-40 μm, Merck, Darmstadt, FRG) in a 2 ml plastic column supported by a MA 25 pre-filter (Millipore, Etten-Leur, The Netherlands). The column had been pre-washed with 3 portions of 500 μl methanol followed by 200 μl of the supernatant. Of the filtrate 20 μl was injected into a Model 3500B HPLC system (Spectra Physics, Santa Clara, California, USA) equipped with a Model 770 spectrophotometric detector (Spectra Physics) set at a wavelength of 210 nm, and a Model C-R1B integrator (Shimadzu, Kyoto, Japan). The column was a stainless steel Lichrosorb 10 NH2 column (Chrompack, Middelburg, The Netherlands). The mobile phase was 25 mM KH_2PO_4, pH 3.7. Nitrate levels were calculated on basis of the linear relationship between concentration and peakheight of pure standards which received similar treatments as the test samples. Recovery of nitrate added to the samples was generally higher than 95%. Nitrate levels in the incubation medium were measured by direct injection into the HPLC system. Measurements were performed on at least 4 independent replications.

Nitrate measurements in dry seed parts. To determine nitrate in dry seed parts, dry seeds were treated with abrasive paper of a very fine grade. This treatment caused the seed coats to break. The cotyledons were separated from the rest of the embryo. All collected seed parts were weighed and extracted for nitrate determination as described above. About 50 mg of seeds was used.

RESULTS

Requirement for endogenous nitrate

In order to determine whether the requirement of germination for nitrate was absolute, the nitrate contents of seeds from 5 different seedlots were measured. The seedlots were from different years (1985, 1986, 1987) and from different natural habitats, with the exception of the seedlot with the highest nitrate content. This batch was obtained from plants that were grown on hydroculture with nitrate added to the growth medium (the seeds were a gift from H. J. Bouwmeester). Nitrate contents were mea-

sured in the dry seeds and after 48 h of pre-incubation in water at 15°C. Seeds were irradiated after 2 h and 48 h, respectively, and germinated at 24°C in the dark. A linear relationship was obtained between dry seed nitrate levels and the probit of germination after 2 h at 15°C (Fig. 1).

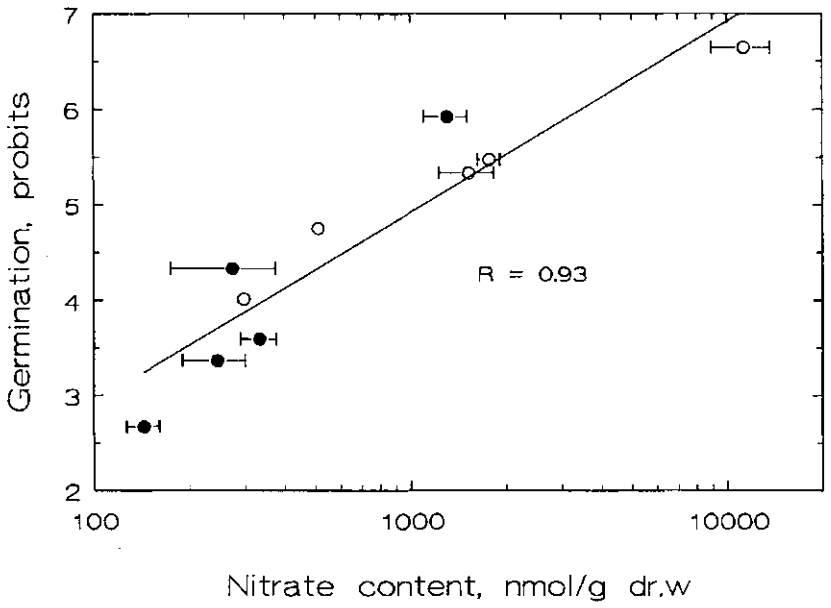

Fig. 1: Plot of nitrate content of 5 different seed lots against germination in probits. Seeds were incubated at 15°C for 2 (○) or 48 h (●), irradiated and germinated at 24°C. Nitrate contents were measured in dry seeds (○) or in seeds rinsed with distilled water after 48 h (●). R = correlation coefficient.

This suggests that nitrate levels are log-normally distributed over the collection of seedlots used here, around a value for half-maximal germination. After 48 h at 15°C nitrate levels were considerably lower, due to leaching of nitrate into the medium. However, a similar linear relation-

ship was maintained. At nitrate levels below 100 nmol g^{-1} germination percentage was close to zero while the germination response saturated at nitrate levels around 10,000 nmol g^{-1}.

Of a seed lot with a nitrate content of 1900 nmol g^{-1} the distribution of nitrate over different (dry) seed parts was determined (Table 1). The relative nitrate content was highest in the axes + radicle part. This part contributed only 10 % to the total weight of the seed. In an absolute sense, the seed coats, including the thin endosperm layer, contained most nitrate: approximately half of the total amount. The large amount of nitrate that leached out during the first few hours of imbibition (see below) probably mostly originated from the seed coat.

Table 1: Nitrate content of dry seed parts of a seed lot with a total nitrate content of 1900 nmol g^{-1}

	% weight of total	% nitrate of total	nitrate content, nmol g^{-1}
seed coats	30	49	3098
cotyledons	60	34	1078
axes + radicles	10	19	3653

Leaching of nitrate

During dark-incubation at 15°C in water the germination capacity declined to zero in approximately 120 h, while in 25 mM KNO_3 the germination response started to decrease after 120 h with a slower rate than in water (see Chapter 5). The decrease of the germination response in water was very well correlated with the decrease in nitrate content (Fig. 2). It should be noted that seeds lost most of their nitrate content during the first 24 h of imbibition. In the present case the nitrate level dropped within 24 h from approximately 1800 nmol g^{-1} in dry seeds to about

400 nmol g^{-1} after 24 h. This fast leaching process is probably due to nitrate that is very loosely bound to structures on or close to the surface of the tissues surrounding the seeds.

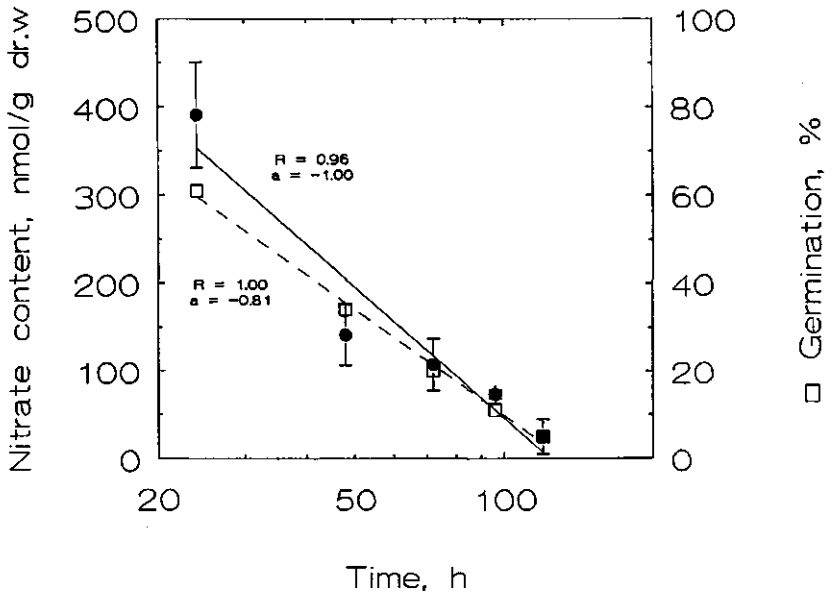

Fig. 2 Plot of nitrate content (●) and R-induced germination at 24°C in water (□) against the logarithm of pre-incubation time at 15°C. R = correlation coefficient; a = slope of regression line, relative to slope of decay of nitrate level (a = -1.00). Germination data are adapted from Chapter 5.

Nitrate-response curves

As suggested before (Chapter 5), the decreasing response to 25 mM KNO_3 after 120 h at 15°C might be the result of decreasing Pfr-receptor levels. If this is the case, this process should already be observable at periods shorter than 120 h when sub-saturating nitrate concentrations are used.

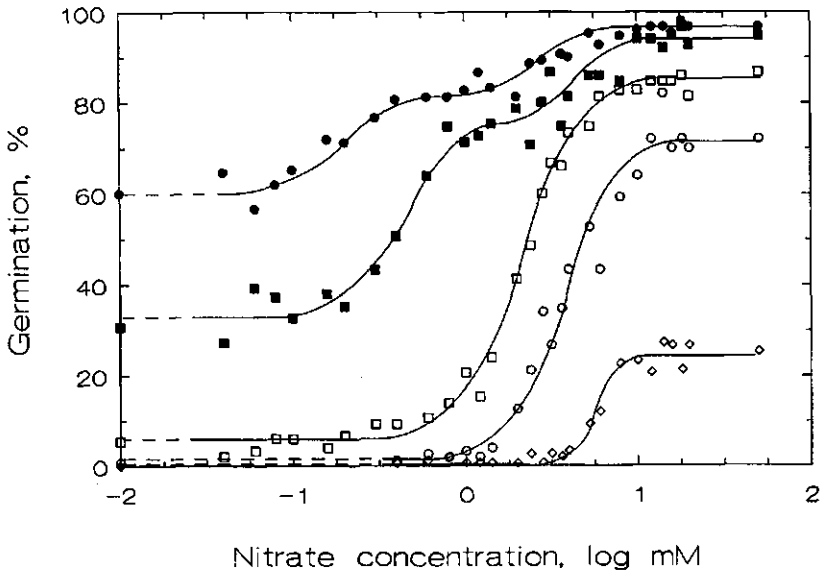

Fig. 3: Red light induced germination at 24°C of seeds pre-incubated at 15°C for 24 (●), 48 (■), 120 (□), 192 (○) and 264 h (◇) in a range of nitrate concentrations. Curves were calculated from population parameters. Values at 10^{-2} mM are similar to values for germination in water.

Nitrate-response curves were obtained at several intervals during 264 h of pre-incubation at 15°C (Fig. 3). The most remarkable aspect of the set of nitrate-response curves is the biphasic character of the 24 and 48 h curves. The very-low-nitrate-response (VLNR) occurred between 0.05 and 1 mM of exogenous nitrate, while the low-nitrate-response (LNR) occurred between 1 and 10 mM nitrate. From the curve parameters (Table 2) it can be concluded that both the VLNR and the LNR curves shifted to the right at increasing incubation periods since m (log nitrate concentration for half-maximal germination) increased significantly. The shift was parallel because, with exception of the 264 h curve, the slope of the curve, expressed by B, remained constant.

Table 2: Parameters of observed nitrate-response curves after several pre-incubation periods.

h at 15°C	R^-(%)	s(R^-)	R^+(%)	s(R^+)	m	s(m)	B	s(B)
24(1)	60.50	1.14	21.61	1.04	-0.75	0.04	3.63	0.62
48(1)	34.01	1.10	40.56	1.63	-0.37	0.03	5.04	0.96
24(2)	83.81	1.48	12.91	1.42	0.58	0.11	3.92	2.07
48(2)	77.06	0.74	17.82	1.44	0.79	0.08	4.12	0.99
120	5.77	0.46	79.14	0.75	0.33	0.01	3.30	0.17
192	1.15	0.48	71.96	1.65	0.58	0.02	3.33	0.23
264	0.73	0.13	24.05	1.64	0.77	0.02	6.98	0.72

Parameters were calculated by means of weighted linear regression analysis in a log-dose probit diagram. R^-: minimal response; R^+: response range; m: log nitrate concentration (mM) for half-maximal response; B: slope of log-dose probit line; s: standard deviation; (1): VLNR; (2): LNR.

In order to use the curve parameters as interaction parameters, we attempted to simulate the observed curves with an equation from the receptor-occupancy theory for a simple bimolecular cooperative interaction:

$$R = R_{max}[NO_3]^n/([NO_3]^n + [NO_3]^n_{0.5}) \quad (1)$$

with:
- R = response
- R_{max} = maximum response
- n = cooperativity coefficient
- $[NO_3]$ = nitrate concentration of the medium in mM
- $[NO3]_{0.5}$ = nitrate concentration for half-maximal response in mM

For the derivation and discussion of this equation see Chapter 5.

The parameters of eq. 1, R_{max}, $[NO_3]_{0.5}$ and n were substituted by the parameters of the observed curves, R^+, 10^m and B, respectively. In the case of the biphasic curves (24 and 48 h) the VLNR and LNR were treated as separate curves. Furthermore, contrary to the fluence-response curves (Chapter 5), the simulated curves were added to the minimum responses of the observed curves. This was done because the relationship between exogenous and endogenous nitrate levels was too complicated (see below) to be included in the calculations. Fig. 4 shows that equation 1, apart from the fluence-response curves, can also be applied to the nitrate-response curves.

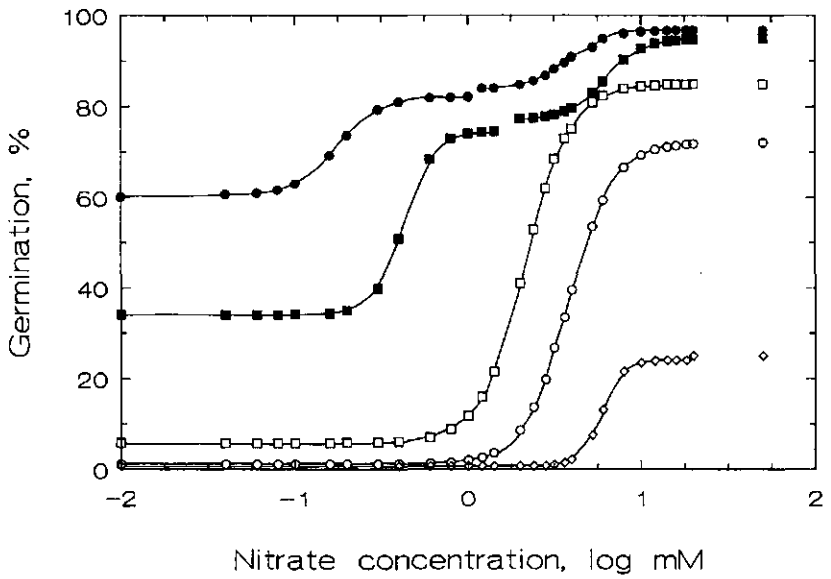

Fig. 4: Simulations of the curves of Fig. 3 by equation 1. Curves are calculated from population parameters. Symbols as in Fig. 3.

With the exception of the B values, all simulated curve parameters did not differ significantly from the observed ones (Table 3).

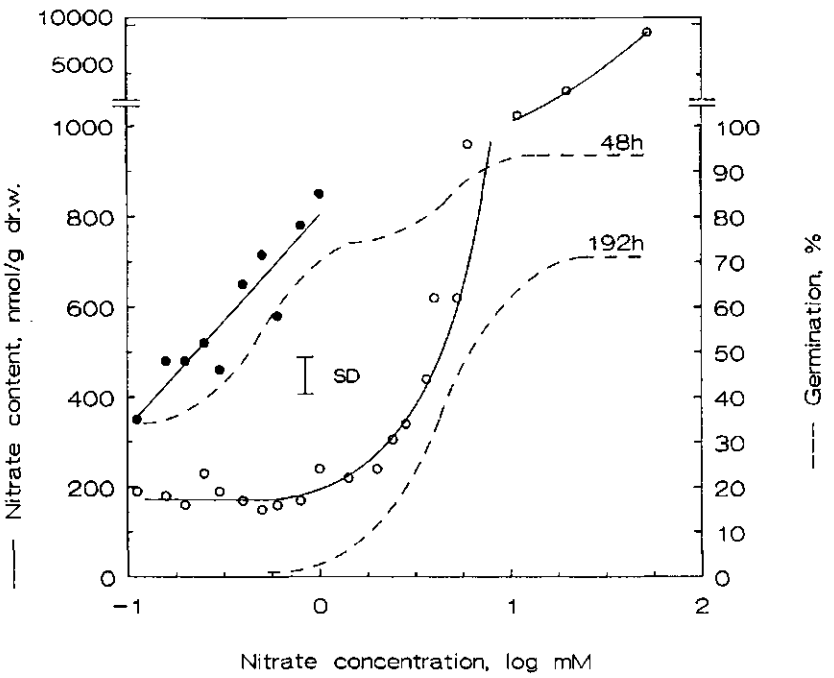

Fig. 5: Uptake of nitrate after 48 h incubation at 15°C. Nitrate uptake was calculated from levels of seed extracts (○) or from the decrease of nitrate levels in the medium (●). For comparison nitrate-response curves after 48 and 192 h pre-incubation from Fig. 3 are shown (dotted curves). SD = maximum standard deviation of the nitrate contents.

DISCUSSION

Role of endogenous nitrate

The present results clearly show that endogenous nitrate is the limiting factor in the light-induced germination in water of *S. officinale* seeds. Seeds that contained less than approximately 100 nmol g^{-1} nitrate

did not germinate, neither in light nor in darkness (Fig. 1). The log-dose probit relationship between nitrate levels and germination response is indicative for a log-normal distribution of endogenous nitrate contents around a level required for 50 % germination in water. This distribution was independent of the year of harvest and the length of the pre-incubation period at 15°C (Fig. 1).

The decrease of the germination in water after increasing pre-incubation periods was very well correlated with the decreasing endogenous nitrate level (Fig. 2). The decrease in nitrate content was due to leaching into the medium and not to nitrate reduction (Hilhorst and Karssen, 1989, Chapter 4). Similar slopes in the semi-logarithmic plot of the decrease of germination and nitrate levels suggest a direct relationship. It may now be argued that the loss of the dormancy-breaking agent, nitrate, induced secondary dormancy. However, in the 1985 seed lot pre-incubations longer than 120 h reduced germination in supra-optimal nitrate concentrations (Chapter 5). Since leaching of nitrate into a medium containing 25 mM KNO_3 is not very likely, this decline may be the result of a process other than leaching.

Following a similar line of argumentation as for the phytochrome-receptor interaction (Chapter 5) it may be concluded that the declining response in high nitrate concentrations is the result of the decreasing number of nitrate-receptors. This process may be super-imposed on a process in which nitrate (the ligand) is the limiting factor, providing that the number of receptors is not limiting.

The nitrate-response

That spare receptors are present may be concluded from the nitrate-responses after increasing pre-incubation periods (Fig. 3). These responses generally followed the same pattern as the fluence-responses (Chapter 5). The nitrate-response curves shifted along the X-axis to the right, while the maximal response started to decrease rapidly after about 120 h (Fig. 3, Table 2). Since the nitrate-responses could be simulated by equation 1, it is assumed that binding of nitrate to its receptor is a simple bimolecular interaction in which the receptor possesses a certain

degree of cooperativity (B > 0.576). If a receptor reserve is present, a similar maximum response may be generated at a reduced total number of receptors, but at higher levels of nitrate, resulting in a right-hand shift (Hollenberg, 1985). Therefore, it may be concluded that besides a reserve of Pfr-receptors also a reserve of nitrate-receptors exists.

From the present results and those of the fluence-response experiments (Chapter 5) it is difficult to make a clear distinction between receptors that are specific for Pfr or for nitrate. We may speculate that Pfr and nitrate bind to the same receptor. This is supported by the correlation between the values for half-maximal response of nitrate and Pfr/P_{tot} (Fig. 6), indicating similar decreases in receptor reserves.

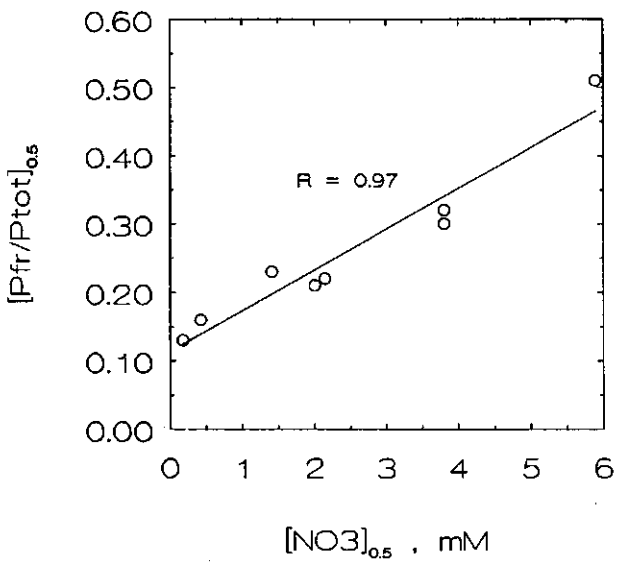

Fig. 6: Correlation diagram of values for half-maximal response for nitrate and Pfr/P_{tot}. Values of $[NO_3]_{0.5}$ were calculated from m (Table 1). Values for $[Pfr/P_{tot}]_{0.5}$ were calculated from fluence values for half-maximal response as described in Chapter 5. R = correlation coefficient.

The biphasic character of the nitrate-response

Surprisingly, the nitrate-response curves after 24 and 48 h of pre-incubation were biphasic. To our knowledge this is the first time a biphasic response is found for a germination response induced by nitrate or any other growth regulator other than light. This stresses the importance of applying sufficient detail to dose-response experiments. Since multiphasic patterns for the isothermic uptake of ions by root cells are well known (Nissen *et al.*, 1980) we studied the uptake of nitrate by the seeds (Fig. 4).

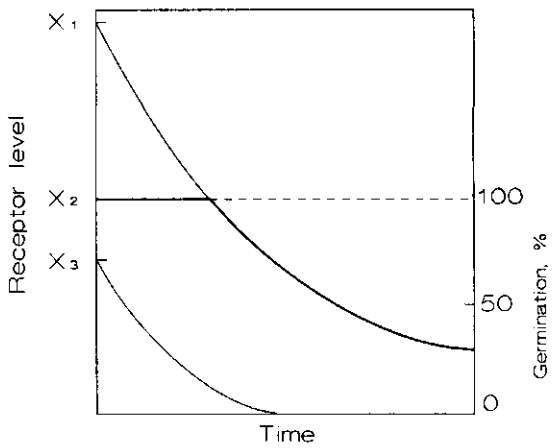

Fig. 7:. Diagrammatic representation of the change in receptor levels during induction of secondary dormancy. Receptor levels are related to germination response through the equation: Response = $k_e[X]$ (see Chapter 5). X_1 = receptor level in the presence of supra-optimal nitrate concentration. Decrease of X_1 is temperature-dependent. X_2 = receptor level required for maximal germination, hence X_1 - X_2 = receptor reserve.The bold line indicates the germination pattern in high nitrate concentration (see Chapter 5, Fig. 1). X_3 = receptor level present in seeds as a result of endogenous nitrate. In water the decrease of X_3 is directly related to leakage of nitrate (Fig. 2).

The VLNR response was correlated with the uptake of a nitrate fraction that was not extractable from the seeds, while the LNR coincided with a sharp rise in the amount of extractable nitrate. Clearly, the binding characteristics of the receptor are not limiting for the VLNR. For the LNR, however, higher amounts of 'free' endogenous nitrate seemed to be required to generate a response. One explanation could be that the LNR is generated by a different nitrate-receptor. However, the slopes of the VLNR and the LNR did not differ significantly. This is an indication for similar receptors. By analogy with the dimer model for phytochrome (VanDerWoude, 1985), it may as well be argued that for the LNR more nitrate molecules have to bind to the receptor than for the VLNR. The disappearance of the VLNR after prolonged incubation periods was not the result of an inhibition of uptake, neither was the uptake influenced by phytochrome (Table 4). Therefore, reduction of the VLNR may be the result of a loss of receptors. However, the level of endogenous nitrate also plays a role at these low exogenous nitrate concentrations. Lowering of the endogenous level will reduce the magnitude of the VLNR but will not generate a shift of the curve.

In conclusion, the present results favor a model in which induction of dormancy is a receptor-regulated process. Under conditions of nitrate depletion the process is nitrate-limited up to the point where the total number of receptors falls below the level required for the maximally attainable response at a certain nitrate level. In a nitrate-poor environment the loss of light-sensitivity is correlated with leaching of nitrate from the seeds (Fig. 7). It has been suggested before that loss of light-sensitivity upon dark-incubation in *Chenopodium album* (Karssen, 1970), *Rumex crispus* (Duke *et al*, 1977) and *Arabidopsis thaliana* (Cone and Spruit, 1983), was the result of declining levels of the Pfr reaction partner X. However, *R. crispus*, *C. album* and *A. thaliana* are also responsive to nitrate (Vincent and Roberts, 1977; Saini *et al*., 1985; Hilhorst and Karssen, 1988, Chapter 3). The present study shows that induction of secondary dormancy in seeds of *S. officinale* depends on two Pfr reaction partners, nitrate and X. Thus, loss of light-sensitivity during dark-incubation may be regulated by two processes: firstly, leaching of a germination stimulating component and, secondly, by a 'true' inactivation or loss of the phytochrome-receptor. Since numerous species

are known to be responsive to the combination of light and nitrate the results presented here may reflect a general mechanism.

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CHAPTER 7

General Discussion.

Secondary dormancy in light-requiring seeds: a model

GENERAL DISCUSSION

Secondary dormancy in light-requiring seeds: a model.

Introduction

During the past few decades several definitions and concepts for the phenomenon of seed dormancy have been proposed (Nikolaeva, 1977; Simpson, 1978; Bewley and Black, 1982; Lang *et al.*, 1988; see also Chapter 1). With a few exceptions (Simpson, 1978) no clear-cut distinction between breakage of dormancy and induction of germination is recognized. We believe that such a distinction is justified since there is considerable evidence that both processes have different requirements, for example temperature. Furthermore, as we hope to show, results from our studies on seeds of *Sisymbrium officinale* and results from those of others can be satisfactorily explained by presuming such a distinction. Simpson (1978) defined seed dormancy as: "**... that state in which a (living) seed fails to resume growth when it is rehydrated in an environment that will support normal germination and seedling growth of apparently identical, but non-dormant, seeds from the same species or even the same parent plant**". This definition specifically concerns primary dormancy, *i.e.* when the mature seed is shed from the motherplant in a dry state. However, when the word "rehydrated" in this definition is replaced by "in the imbibed state" the definition also applies to most observed cases of secondary dormancy. This definition clearly makes the distinction between dormancy and inhibition of germination by environmental factors. It will be a basic element of the following discussions.

Temperature

Temperature is probably the most important regulating factor of dormancy and at least very important in germination. Many seeds require a chilling pre-treatment to break dormancy (Vincent and Roberts, 1979;

Bewley and Black, 1982). Often the optimum temperature for subsequent germination is considerably higher. It may be questioned whether chilling *per se* effects qualitative changes in metabolism or induces membrane transitions in seeds. Many examples can be found where temperature seems to affect the rate of breakage and induction of dormancy (*e.g.* Totterdell and Roberts, 1979; Cone and Spruit, 1983). In *Sisymbrium officinale* dormancy is broken by temperatures between 2 and 24°C (Chapter 2, Fig. 1). The rates for complete breakage of dormancy varied between 1 and 5 days. However, induction of secondary dormancy showed a much wider variation in rates. At 2°C induction of dormancy occurred over a period of weeks to months (results not shown) while at 24°C seeds were fully dormant after only 2 days. This phenomenon may be the reason why higher temperatures are often considered non-effective in breaking dormancy. If the intervals between observations at elevated temperatures are too long, faster processes such as breakage and subsequent induction of dormancy may be overlooked. Obviously, the requirement for a certain temperature-time combination is strongly related to natural conditions and the species, *e.g.* summer vs. winter annual (Karssen, 1982). In the light of our own observations (Chapters 5, 6) it may be argued that the breakdown rate of receptors for Pfr and nitrate is under temperature control.

Involvement of membranes.

The germination of many species is known to possess an optimum temperature (Lang, 1965; Thompson, 1973). Often the shift between favorable and unfavorable temperatures is very abrupt. Since this abruptness could be correlated with amino acid leakage from seeds of several species (Hendricks and Taylorson, 1976) and with changing fluorescence intensity from a membrane specific fluorescence probe in membrane preparations from *Setaria faberi* seeds (Hendricks and Taylorson, 1978), the involvement of membranes in Pfr-induced germination was strongly supported. Moreover, it has been shown that only the Pfr form of phytochrome binds to liposome membranes in a temperature-dependent way (Kim and Song, 1981). Treatments with agents that are known to affect cell membranes, such as anesthetics, greatly affected the Pfr-induced germination of lettuce seeds (VanDerWoude

and Toole, 1980). Hence, strong indications are present that the phytochrome receptor is located in a membrane. In lettuce seeds a very-low-fluence-response (VLFR) could be induced by chilling or by treatment with ethanol (VanDerWoude, 1985). It was hypothesized that these treatments changed the activity of the Pr:Pfr-X complex by means of changing its lateral mobility in the membrane. This process was called 'sensitization' of the complex, possibly through interaction with another membrane component. Whether this phenomenon may be compared to dormancy breakage remains unclear. Especially the short chilling period required to induce the VLFR stands in contrast with the long periods of chilling required to break dormancy in many other species.

In the present study we did not observe a VLFR in the Pfr-induced germination of *S. officinale* seeds (Chapters 3, 5). Possibly, the optimum germination temperature, around 24°C, induced a state of the membrane which caused exposure of the Pfr-receptor to Pfr molecules. Apparently, only binding of Pfr:Pfr dimers to the receptor resulted in a (low fluence) germination response.

It has been shown that nitrate is absolutely required in the Pfr-induced germination of *S. officinale* seeds (Chapter 6). Moreover, nitrate is only active in the presence of Pfr since escape from the antagonistic effect of far-red irradiation did not start until nitrate was added (Chapter 4). Also, delay of application of nitrate caused the dark-reversion of Pfr to Pr to become the rate-limiting process in germination. Together with the observation that the germination response was a function of the product of applied fluence and nitrate concentration (Chapter 3), it may be argued that the activity of nitrate is only expressed in the presence of Pfr or a Pfr-receptor complex. In addition, the multiplicativity of fluence and nitrate concentration points to action sites in the same chain of reactions (Schopfer, 1986). As nitrate was active in the unreduced state (Chapter 4) it may be hypothesized that the nitrate ion modifies the Pfr binding site, thereby allowing Pfr to bind. If the Pfr binding site is a membrane protein a possible role of nitrate could be the neutralization of positive charges on the surface of the protein, or nitrate may influence the membrane potential. Both possibilities may result in a change of affinity of the receptor for its agonist Pfr (Hollenberg, 1985). However, as stated before (Chapter 6), post-receptor

effects which are still under Pfr control could not be ruled out.

Applying the receptor-occupancy theory

Many responses of animal cells induced by effectors such as pharmaceutics can be described by formulations from the receptor-occupancy theory. There seem to be no formal objections to using these formulations in the analysis of plant cell responses, providing similar assumptions are made and justified (see Chapter 5). However, only a few examples in the field of plant physiology could be found in which these formulations were used. Examples are the ABA-induced closure of stomata of epidermal strips from *Commelina communis* (Weyers *et al.*, 1987) and the analysis of the width of dose-response curves for gibberellin- and auxin-induced responses (Nissen, 1985; Nissen, 1988). Recently, the auxin-induced proton-efflux from *Avena* coleoptile segments was studied by determination of sensitivity parameters (Fitzsimons, 1989). All these studies have in common that they made use of curve parameters such as R_{max}, n and K_D (for explanation see Chapter 5), without testing whether the curves generated from the calculated parameters matched the observed curves. As shown in Chapter 5, discrepancies between observed and calculated values may occur. The present studies (Chapters 5, 6) show that responses to Pfr and nitrate can be simulated by the equation for a simple bimolecular interaction of an agonist with its receptor, with a certain level of cooperativity. The good fits of simulated to observed curves do not necessarily mean that the model reflects the actual interaction. However, for a number of dose-response relations in animal cells it has been shown that the final response was closely related to the initial agonist-receptor binding properties.

Post-receptor events

Phytochrome and nitrate action form only the beginning of a transduction chain that ultimately results in germination. One step in this chain of processes is the synthesis of gibberellins (Chapters 2, 3). The role of gibberellins in seed germination is essential (Karssen *et al.*, 1989).

Gibberellin-deficient mutants of *Arabidopsis thaliana* and tomato did not germinate in the absence of exogenous gibberellins (Karssen and Lacka, 1986; Groot and Karssen, 1987). Tetcyclacis, an inhibitor of gibberellin biosynthesis, completely blocked the R- and nitrate-induced germination in *S. officinale* (Chapters 2, 3). Application of GA_{4+7} restored the original germination level, indicating that possible side-effects could be neglected. Evidently, direct evidence for R- and nitrate-induced biosynthesis of gibberellins can only be obtained by direct measurement of gibberellins during induction of germination. However, additional indirect evidence for *de novo* synthesis of gibberellins was obtained by comparing the gibberellin-induced germination of *S. officinale* in the absence of nitrate with the gibberellin-deficient mutant of *A. thaliana* (Chapter 3). Responses of both species to exogenous GA_{4+7} were remarkably similar, indicating that in *S. officinale* no endogenous gibberellins were present in the absence of nitrate. Applied gibberellins were also active in the dark, indicating a Pfr-independent action of GA. The possibility remains that in this case the gibberellins induced a VLFR, as reported for seeds of *Kalanchoë blossfeldiana* (DePetter *et al.*, 1985). However, Pfr-destructing treatments prior to R irradiation, such as high temperature (Cone, 1985), did not reduce the response to GA_{4+7} in the dark (results not shown).

In summary, two Pfr-mediated phenomena in the induction of germination can be recognized: (1) the induction of GA-biosynthesis (light-effect I) and (2) a co-action of Pfr and exogenous GAs. The co-action of exogenous GA_{4+7} and Pfr may be interpreted in two ways: Pfr exerts its effect on the gibberellin-receptor or the gibberellins do the same on the Pfr-receptor. For both possibilities evidence is presented (Chapter 3). We prefer the first possibility because the escape time for the inhibiting effect of tetcyclacis is considerably longer than the time needed for escape from the far-red reversal of the effect of R irradiation, 16 h and 8 h, respectively (Chapter 2). This indicates that the synthesis of gibberellins occurs well after the initiation of GA-biosynthesis by Pfr. Since reversion of Pfr to Pr occurred within 8 h after R irradiation (Chapter 4) it is not likely that under natural conditions gibberellins play a role in the Pfr-receptor interaction. Receptors for gibberellins remained present and active during induction of secondary dormancy since seeds could still germinate in the dark in the presence of exogenous GA_{4+7}. This response

could still be observed even when the seeds no longer responded to the combination of R and nitrate (Chapter 2). Thus, it may be argued that the Pfr effect on the gibberellin-receptor occurred well before the actual binding of gibberellins to the receptor.

A model

The results presented in this thesis and the discussions in this chapter may be summarized in a descriptive model (Fig. 1). In this model regulation of dormancy is located in a membrane. The initial state of the membrane in the figure is that of a fully imbibed seed at low temperature. The membrane is well-ordered and relatively immobile at this temperature (a). A mathematical model for the temperature dependency of dormancy breaking in plants by constant temperatures has been proposed (Fishman et al., 1987). The basis of this model is the temperature-dependent formation of a precursor of a dormancy-breaking compound. At a critical level of the precursor an irreversible cooperative transition occurs into the active compound. Some experimental support for such a model came from experiments in which dormancy of *Echinochloa crus-galli* seeds was broken by brief exposure to high temperature (Di Nola and Taylorson, 1989). It was found that this treatment affected the composition of membrane-bound proteins. Moreover, alterations in membrane structures were observed (L Di Nola, personal communication).

Therefore, in the present model it will be assumed that the dormancy-breaking compound is a membrane-located phytochrome receptor protein. These receptors are synthesized with a temperature-dependent rate (b). However, the membrane is still immobile and the receptor is inactive and unexposed (X_i). After changing the temperature to the optimal germination temperature the receptor becomes exposed as a result of increased membrane mobility which makes lateral movement of the receptor possible (c). This transition may be a transition from a gel-phase to the liquid-crystalline phase. Lateral movement of the receptor exposes nitrate binding sites, for example positive charges at the surface of the protein. Upon binding of nitrate the receptor undergoes a conformational change (d) which allows binding of Pfr:Pfr dimers to the activated receptors (X_a) (e). These

phytochrome dimers are only formed at fluences $F > 10^{-5}$ mol m^{-2}. It should be noted that this is only one of several possibilities how small ions may alter receptor properties (Hollenberg, 1985).

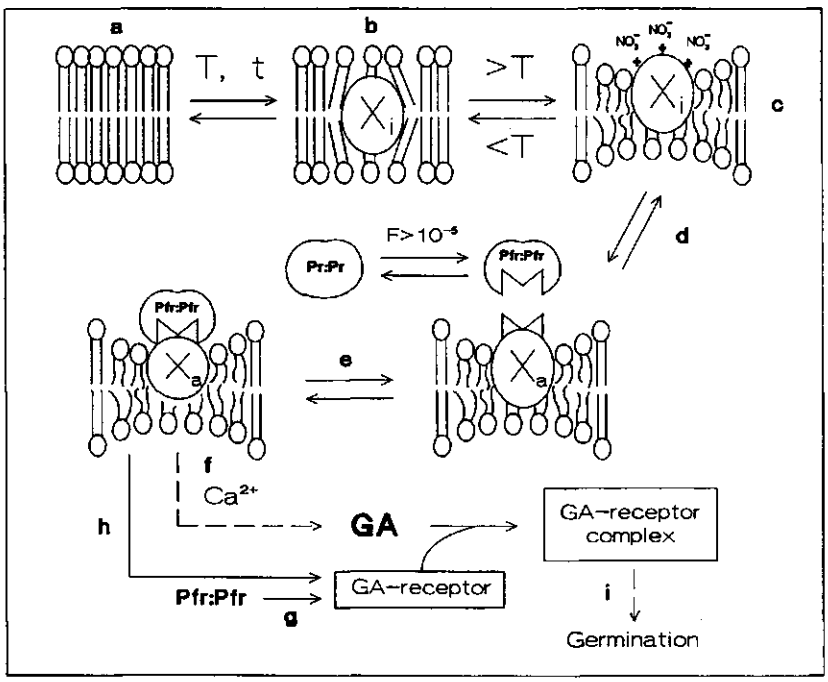

Fig. 1: Model for breakage and induction of dormancy, and stimulation of germination in light-requiring seeds. The phytochrome receptor, X, is assumed to be a membrane-protein. T = temperature, t = time, X_i = inactive receptor, X_a = activated receptor, F = fluence (mol m^{-2}), GA = gibberellin. Letters in the figure refer to description in the text.

The Pfr:Pfr-X complex generates a signal that leads to synthesis of gibberellins (f) (light-effect I). The nature of this signal is unknown. Evidence is now accumulating that the Ca^{2+} ion may function as a secondary

messenger of the Pfr-receptor-complex (Roux *et al.*, 1986). Binding of gibberellins to their receptors is enhanced by either Pfr:Pfr (g) or Pfr:Pfr-X (h) (light-effect II). If the latter is the case it opens the possibility that the GA-receptor is located close to the Pfr-receptor. Finally, the signal from the GA-receptor leads to germination (i), for example by inducing the synthesis of cell-wall degrading enzymes (Groot *et al.*, 1988).

This model is highly speculative. However, we believe that such models, based on analysis of dose-response relations of growth-regulating factors, may contribute to understanding the often observed interactions between these factors. Furthermore, this type of analysis may provide a context for receptor studies at the molecular level.

Future perspectives

Since the radio isotopes of nitrogen and oxygen have short life times and detection of the stable isotopes does not seem to be sensitive enough, study of the localization of nitrate at the (sub)cellular level is not possible. Therefore, future research should be directed at the receptors. Localization of immuno-labeled phytochrome has already been demonstrated. An interesting prospect would be to study the possible redistribution of phytochrome over the cell as a result of nitrate action. With respect to the suggested changes in membrane properties and membrane proteins during breakage and induction of dormancy, biochemical and morphological analysis of these changes seems to be promising.

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SUMMARY

SUMMARY

One of the most important aspects of the life cycle of seed plants is the formation and development of seeds on the motherplant and the subsequent dispersal. An equally important element of the survival strategy is the ability of seeds to prevent germination in unfavorable conditions, such as the wrong season, low light irradiance, or an unfavorable soil composition. Seeds of many species may remain in the soil, either in a dry state or fully imbibed, for hundreds of years without losing their viability. Earlier investigations have shown that this state of dormancy may be broken and induced in a seasonal cycle. Evidently, temperature is an important regulating factor in these cycles. When dormancy is broken, seeds may germinate, providing the conditions are adequate. Hence, seeds must be able to 'sense' their environment. Again, temperature is important, but also two naturally occurring environmental factors, light and nitrate, are known to stimulate the germination of many (wild) species.

Seeds of *Sisymbrium officinale* (hedge mustard) only germinate in the presence of light and nitrate (either endogenous or exogenous). Irradiation with far-red light (720 nm) antagonized the stimulating action of red light (660 nm). Both wavelengths are part of the daylight spectrum. This is proof that the light-induced germination is mediated by the plant pigment phytochrome. The induction of germination by light and nitrate could also be inhibited by application of tetcyclacis, an inhibitor of the biosynthesis of gibberellins. Application of gibberellins 4 and 7 antagonized the inhibition. It was concluded that synthesis of gibberellins is part of the transduction chain that leads to germination. Comparison of the escape times for the antagonizing action of far-red light and the inhibition of tetcyclacis led to the conclusion that induction of germination occurred during the first 8 hours after irradiation with red light and application of nitrate, while the synthesis of gibberellins was completed for all seeds after 16 hours.

Seeds only germinated in the dark when exogenous gibberellins were present. In the absence of nitrate, red light reduced the requirement for gibberellins. In other words, besides a promotive effect on gibberellin synthesis (light-effect I), light also enhanced the sensitivity to gibberellins (light-effect II). Light-effect I disappeared after prolonged

treatment at elevated temperatures while the seeds remained responsive to exogenous gibberellins and light-effect II (Chapter 2).

Both light-effects were studied in detail (Chapter 3). The influence of several concentrations of nitrate and gibberellins on the shape and position of fluence-response curves was examined. The results obtained with seeds of *Sisymbrium officinale* were compared with those of the gibberellin-deficient mutant of *Arabidopsis thaliana*, a related species. Remarkable similarities were observed. In both cases nitrate steepened the fluence-response curves. In other words, the fluence-range over which the total seed population responded was reduced. Application of gibberellins 4 and 7 to seeds, in the absence of exogenous nitrate, resulted for both species in a parallel shift to lower fluence values. Application of cofactor analysis led to the conclusion that the interaction between the effects of nitrate and phytochrome was multiplicative. The germination response was a function of the product of nitrate concentration and fluence value. This is an indication that the factors nitrate and phytochrome act on the same pathway. The interaction between the effects of phytochrome and gibberellins, however, was additive. Both factors acted independently in different pathways leading to the same response. Phytochrome might enhance the sensitivity of the gibberellin-receptors (light-effect II). However, this effect could only be brought to expression when sufficient gibberellins were present. The use of the gibberellin-deficient *Arabidopsis* mutant supported the hypothesis that in *Sisymbrium* no active gibberellins are present in the absence of nitrate.

The hypothesis that nitrate is active in the induction of germination because its reduction would lead to production of NADP, stimulator of the pentose phosphate pathway, was tested by studying the light- and nitrate-induced germination in the presence of inhibitors of nitrate reductase (Chapter 4). Furthermore, a method was developed to measure the nitrate content of seeds. The inhibitors of nitrate reductase, sodium chlorate and sodium tungstate had no influence on the light-induced germination in a range of nitrate concentrations. At several intervals after a pretreatment during which the seeds had taken up nitrate from the medium, the nitrate contents of the seeds and the medium were measured. It was found that during the induction of germination, the 8-hour period after irradiation, the nitrate content of the seeds decreased. However, this decrease could

be fully explained by leakage into the medium. Thus, no nitrate was reduced during this period. Nitrate reduction did not occur until actual growth (protrusion of the radicle) had started. Apparently, at that time the nitrate assimilation began. In the presence of the inhibitors this reduction was almost completely inhibited. The growth of the seedling was abnormally slow in the presence of the inhibitors. These results led to the conclusion that nitrate is active in the unreduced state at induction of germination.

To gain more insight in the process of dormancy induction and the decreasing sensitivity to light and nitrate during this process, dose-response experiments were carried out for these factors during induction of dormancy at constant temperature (Chapter 5). Fluence-response curves of seeds in supra-optimal nitrate concentrations shifted to higher fluence values upon increasing duration of the pretreatment. After approximately 120 hours at 15°C the maximum germination decreased. The slopes of the curves did not change. The observed fluence-response curves could be simulated by formulations from the general receptor-occupancy theory. This enabled us to interpret calculated curve parameters as interaction parameters of the binding of phytochrome to its receptor. The shifts of the curves with unchanged maximal response could in this way be explained by assuming that more phytochrome-receptors were present than required for maximal germination. The induction of secondary dormancy could then be the result of a temperature dependent decrease of the number of receptors. Once below a critical value this decrease could lead to reduction of the maximum response.

The role of nitrate was studied in a similar way (Chapter 6). The response to nitrate was monitored in optimal light conditions. The results of the nitrate-response experiments showed similarities with those of the fluence-response experiments. Upon increasing pretreatment duration a shift to higher nitrate concentrations was observed, followed by a decrease of the maximum germination. The nitrate-response curves could also be simulated with an equation for a simple bimolecular reaction. Furthermore, it was shown that the presence of nitrate was an absolute requirement for the light-induced germination. It was concluded that nitrate may act as an activator of the phytochrome-receptors. In the absence of exogenous nitrate, the response was limited by the amount of

endogenous nitrate. Since nitrate leached out during incubation in water the germination in water was correlated with the nitrate leaching. A remarkable aspect of the nitrate-responses was the occurrence of biphasic response curves. The very low nitrate response disappeared after 48-72 hours of pretreatment. The occurrence of these two phases was related to the uptake of nitrate from a range of nitrate concentrations. For the first phase the uptake appeared to be the limiting factor; all the nitrate that was taken up was bound. For the second phase relatively high endogenous nitrate levels were required. A considerable amount of this nitrate was not bound. The possibility of the existence of two different nitrate receptors was discarded. By analogy with an existing model for biphasic fluence-response curves it was suggested that the requirement of the nitrate-receptor for nitrate for a certain fraction of the seed population could differ from the rest of the population. Seemingly, induction of dormancy could influence the ratio of these fractions.

The results were summarized in a model in which the phytochrome-receptor is located in a membrane (Chapter 7). Breakage of dormancy, regulated by temperature, would induce synthesis of this receptor. Changing the temperature to an optimal germination temperature would induce a phase transition of the membrane, thus enabling the receptor to move laterally in the membrane. In this way the receptor may become accessible to nitrate. Nitrate would bind to the receptor and alter its conformation in such a way that phytochrome can bind. The transduction chain, leading to germination, may be initiated via a secondary messenger like calcium. Induction of dormancy makes this process impossible because the rate of degradation of phytochrome-receptors will exceed the synthesis.

SAMENVATTING

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Een van de belangrijkste aspecten van de levenscyclus van zaadplanten is de vorming en ontwikkeling van zaden aan de moederplant en de daaropvolgende verspreiding. Een minstens even belangrijk element van de overlevingsstrategie is het vermogen van zaden om niet te ontkiemen onder ongunstige omstandigheden, zoals het verkeerde seizoen, te lage lichtintensiteit, of een ongunstige bodemsamenstelling. Vele zaden kunnen wellicht honderden jaren in droge of geimbibeerde toestand in de grond verblijven zonder hun kiemkracht te verliezen. Eerder onderzoek heeft aangetoond dat deze kiemrust niet absoluut is. Voor een aantal soorten is aangetoond dat de kiemrust in een seizoenscyclus gebroken en geinduceerd wordt. Het ligt voor de hand dat de temperatuur een belangrijke regulerende factor is in deze wisselingen. Wanneer kiemrust wordt gebroken kunnen de zaden kiemen indien de omstandigheden gunstig zijn. Voorwaarde is derhalve dat zaden hun omgeving moeten kunnen "waarnemen". Van twee natuurlijk voorkomende factoren, licht en nitraat, is bekend dat zij de kieming van veel soorten kunnen stimuleren.

Zaden van *Sisymbrium officinale* (Gewone raket) kiemen alleen in licht en in aanwezigheid van nitraat. Bestraling met verrood licht (720 nm) antagoneert de stimulerende werking van rood licht (660 nm). Beide golflengten komen in het normale daglicht spectrum voor. Dit vormt een bewijs dat de bevorderende werking van licht verloopt via het plantepigment fytochroom. De kiemingsinductie door licht en nitraat kon ook geremd worden door toediening van tetcyclacis, een remmer van de gibberellinebiosynthese. Toediening van de gibberellinen 4+7 deed de remming teniet. Geconcludeerd werd dat synthese van gibberellinen onderdeel vormt van de reactieketen die tot kieming leidt. Vergelijking van de 'ontsnappingstijden' voor de antagonerende werking van verrood licht en de remmende werking van tetcyclacis leidde tot de conclusie dat de kieminductie plaatsvond in de eerste acht uren na bestraling met licht en toediening van nitraat, terwijl de gibberelline-synthese pas na 16 uur voor alle zaden voltooid was. Zaden kiemden alleen in het donker na toediening van gibberellinen. Rood licht verlaagde in afwezigheid van nitraat de behoefte aan gibberellinen. Met andere woorden, naast een bevordering van de gibberelline-biosynthese (lichteffect I) heeft licht ook invloed op de

gevoeligheid voor gibberellinen (lichteffect II). Lichteffect I verdween na langdurige voorbehandeling bij hogere temperaturen, terwijl de zaden gevoelig bleven voor gibberellinen en ook lichteffect II waarneembaar bleef (Hoofdstuk 2).

Beide lichteffecten werden in detail onderzocht (Hoofdstuk 3). De invloed van verschillende concentraties nitraat en gibberellinen op vorm en positie van fluence-respons curven werd onderzocht. De resultaten verkregen met zaden van *Sisymbrium officinale* werden vergeleken met die van de gibberelline-deficiente mutant van *Arabidopsis thaliana* (Zandraket), een verwante soort. Er werden opvallende overeenkomsten waargenomen. Nitraat maakte in beide gevallen de fluence-respons curve steiler, d.w.z. het fluence-bereik waarover de totale zaadpopulatie reageerde werd kleiner. Toediening van gibberellinen 4+7 aan zaden in afwezigheid van exogeen nitraat leverde bij beide soorten een paralelle verschuiving van de curve naar lagere fluence waarden op. Toepassing van co-factor analyse leidde tot de conclusie dat de interactie tussen fytochroom en nitraat multiplicatief was, d.w.z. de kiemingsrespons was een functie van het product van nitraatconcentratie en fluence waarde. Dit is een aanwijzing dat de factoren fytochroom en nitraat actief zijn in dezelfde reactie-keten. De interactie tussen fytochroom en gibberellinen daarentegen was additief. Beide factoren werkten onafhankelijk in verschillende reactie-ketens die echter wel naar dezelfde respons leidden. Fytochroom zou de gibberelline-receptoren gevoeliger kunnen maken (lichteffect II). Dit effect kan alleen dan tot expressie worden gebracht indien voldoende gibberellinen gesynthetiseerd worden (lichteffefct I). Het gebruik van de gibberelline-deficiente *Arabidopsis* mutant versterkte de hypothese dat in *Sisymbrium*, in afwezigheid van nitraat, geen actieve gibberellinen aanwezig zijn.

De hypothese dat nitraat werkzaam is doordat reductie leidt tot vorming van NADP, stimulator van de pentose-fosfaat route, werd getest door de licht- en nitraat- gestimuleerde kieming te bestuderen in aanwezigheid van remmers van het enzym nitraatreductase (Hoofdstuk 4). Tevens werd een methode ontwikkeld om nitraatgehalten in zaden te kunnen bepalen. De nitraatreductaseremmers natriumchloraat en natriumwolframaat hadden geen invloed op de kieming in een reeks van nitraatconcentraties. Op verschillende tijdstippen na een voorbehandeling waarin de zaden

nitraat hadden opgenomen uit het medium, werd het nitraatgehalte van de zaden en het medium gemeten. Het bleek dat gedurende de inductie van de kieming, tot 8 uur na bestraling met rood licht, het nitraatgehalte van de zaden wel afnam. Deze afname kon echter geheel verklaard worden uit de uitlek van nitraat in het medium. Pas tijdens het werkelijke kiemproces (groei van het doorgebroken worteltje) vond nitraatreductie plaats. Kennelijk kwam op dat tijdstip de nitraatassimilatie op gang. In aanwezigheid van voornoemde remmers werd deze reductie vrijwel geheel geremd. De groei van het gekiemde zaad was dan ook abnormaal traag in aanwezigheid van de remmers. Deze bevindingen leidden tot de conclusie dat nitraat in ongereduceerde vorm werkzaam was bij de feitelijke inductie van kieming.

Om meer inzicht te verkrijgen in het proces van kiemrustinductie en het ongevoelig worden voor licht en nitraat werden dosis-respons experimenten uitgevoerd voor de factoren licht en nitraat tijdens inductie van secundaire kiemrust bij constante temperatuur (Hoofdstuk 5). Fluence-respons curven van zaden in supra-optimale nitraatconcentraties verschoven naar hogere fluence waarden bij toenemende voorbehandelingsduur. Na circa 120 uur bij 15°C nam de maximale kieming af. De hellingen van de curven bleven nagenoeg gelijk. De waargenomen fluence-respons curven konden gesimuleerd worden met behulp van formuleringen uit de algemene receptorbezettingstheorie. Hierdoor konden curve-parameters geinterpreteerd worden als interactie-parameters van de binding van fytochroom aan een receptor. Hieruit volgde dat de verschuiving van de curven, met gelijkblijvende maximale respons, verklaard kon worden door aan te nemen dat er meer fytochroom-receptoren aanwezig waren dan noodzakelijk voor de maximale kiemingsrespons. De inductie van secundaire rust zou dan het gevolg zijn van een temperatuursafhankelijke afname van het aantal receptoren. Eenmaal onder een kritieke waarde gekomen zou deze afname leiden tot afname van de maximale kieming.

De rol van nitraat werd op gelijksoortige wijze onderzocht (Hoofdstuk 6). De respons op nitraat werd gevolgd onder optimale lichtcondities. De resultaten van de nitraat-respons experimenten vertoonden veel overeenkomst met die van de fluence-respons experimenten. Hier werd een verschuiving naar hogere nitraatconcentraties waargenomen, gevolgd door een afname van de maximale kieming. Ook de nitraat-respons curven konden gesimuleerd worden met behulp van een formulering voor een simpele bimoleculaire reac-

tie. Vastgesteld werd dat aanwezigheid van nitraat een absolute voorwaarde is voor de licht-geinduceerde kieming. Er werd geconcludeerd dat nitraat mogelijk werkzaam is als activator van de fytochroom-receptoren. Hieruit volgde tevens dat in afwezigheid van exogeen nitraat de maximale kiemrespons bepaald wordt door de hoeveelheid endogeen nitraat. Aangezien nitraat uitlekt tijdens incubatie in water zou de kieming gerelateerd moeten zijn aan deze uitlek. Er werd inderdaad een sterke correlatie gevonden tussen nitraatuitlek en afname van de kieming na incubatie in water. Een opmerkelijk aspect van de nitraat-respons was het voorkomen van twee-fasen respons curven. De eerste fase verdween na 48-72 uur voorbehandeling. Het voorkomen van deze twee fasen werd gerelateerd aan de opname van nitraat uit een reeks nitraatconcentraties. Voor de eerste fase bleek de opname de limiterende factor te zijn; al het opgenomen nitraat werd gebonden. Voor de tweede fase waren relatief hoge nitraatgehalten nodig in het zaad, waarvan een aanzienlijk deel niet gebonden werd. De mogelijkheid van het bestaan van twee verschillende nitraatreceptoren werd verworpen. Naar analogie met een bestaand model voor twee-fasen fluence-respons curven werd gesuggereerd dat de behoefte van de nitraatreceptor aan nitraat voor een bepaalde fractie van de zaadpopulatie kan verschillen van de rest van de populatie. Inductie van kiemrust kan de verhouding tussen de twee fracties kennelijk beinvloeden.

De resultaten werden samengevat in een model waarin de fytochroom-receptor gelocaliseerd is in een membraan (Hoofdstuk 7). Rustbreking, gereguleerd door de temperatuur, zou synthese van deze receptor bevorderen. Verandering van de voorbehandelingstemperatuur in een optimale kiemingstemperatuur zou laterale beweging van de receptor in het membraan mogelijk maken doordat het membraan een fase-overgang ondergaat. Hierdoor wordt de receptor toegankelijk voor nitraat. Nitraat zou vervolgens de conformatie van de receptor zodanig kunnen veranderen dat binding van fytochroom mogelijk wordt. Via een secundaire boodschapper zoals calcium, zou de transductieketen die leidt tot kieming geinitialiseerd worden. Inductie van rust maakt dit hele proces onmogelijk doordat de afbraak van fytochroom-receptoren sneller gaat verlopen dan de synthese.

NAWOORD

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CURRICULUM VITAE

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Henk Hilhorst is geboren op 5 oktober 1954 te Soest. Na behaling van het MULO-B diploma in 1971 werd begonnen aan de analistenopleiding van de Amersfoortse Laboratoriumschool. In 1974 werd het diploma HBO-A Klinische Chemie behaald. In datzelfde jaar begon hij aan de opleiding Hoger Natuurwetenschappelijk Onderwijs aan het Dr Struyckeninstituut te Breda. Het stagejaar van deze opleiding werd doorgebracht bij de vakgroep Plantkunde (nu Plantencytologie en Morfologie) van de Landbouwuniversiteit. Daar werd onder leiding van Dr. J. L. van Went onderzoek gedaan naar ultrastructurele veranderingen in tabaksbladeren als gevolg van infectie met tabaksmozaiekvirus, veroudering en toediening van ethyleen. Na voltooiing van deze opleiding in 1978 trad hij op 1 september van dat jaar als analist in dienst van de vakgroep Moleculaire Fysica. Daar assisteerde hij bij het onderzoek naar de assemblage van het tabaksmozaiekvirus. In 1980 begon hij aan de deeltijdopleiding MO-A Engels van de Centrale Opleidingscursussen voor Middelbare Akten te Utrecht. Deze opleiding werd in 1984 voltooid.
Op 1 oktober 1983 trad hij in dienst van de vakgroep Plantenfysiologie. Daar werd onder leiding van prof. dr. C. M. Karssen het onderzoek verricht dat tot dit proefschrift leidde.