PHYTOCHROME MEDIATED NITRATE REDUCTASE REGULATION IN *ZEA MAYS*

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CONTENTS

PREFACE

The research work embodied in this dissertation has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. The work is original and has not been submitted so far, in part or full for any other degree or diploma of any university.

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ABBREVIATIONS

ACh	-	Acetylcholine
Act D	-	Actinomycin D
CHI	-	Cycloheximide
HIR	-	High irradiance response
M	-	Molarity
NADH	-	Nicotinamide adenine dinucleotide, reduced form
P_{fr}	-	Far-red absorbing form of phytochrome, physiologically active form
P_{fr}^e	-	Excited state of P_{fr} form
PP	-	Photophosphorylation
Pr	-	Red absorbing form of phytochrome
PS I	-	Photosystem I
P_{tot}	-	Total phytochrome ($P_r + P_{fr}$)
TCA	-	Trichloroacetic acid

INTRODUCTION

Light is the energy source on which plants and ultimately all living organisms depend. In addition to its utilization in the process of photosynthesis, light has an important regulatory role in plant growth and development. Virtually every phase of plant development, and many other physiological phenomena, not intimately involved in development, are subjected to photocontrol independent of photosynthesis. One of the photoreceptors to receive light signals from the environment to translate it for the developmental processes, is termed as phytochrome. A vast number of light-controlled plant responses are now believed to be phytochrome mediated. However, the actual mechanism by which P_{fr} (active form of phytochrome) transmits its sensory message to the cell remains obscure.

Nitrate reductase (E.C. 1.6.6.1; NAD(P)H : nitrate oxidoreductase) is the first enzyme involved in a series of reactions leading to inorganic nitrate assimilation. Although it is substrate induced, the induction of nitrate reductase has been shown to be dependent on environmental factors like light. In 1972, Jones and Sheard reported altogether a new effect of light on nitrate reductase. According to them, this enzyme is controlled by phytochrome.

The terminal buds of pea when exposed to a.short duration of red light showed an increase in the nitrate reductase activity. This effect could be reversed by the far-red light.

Recently, Duke and Duke (1978) reported that modulation of the nitrate reductase activity is not directly influenced by photosynthetic photoreceptor and that phytochrome is involved in the photocontrol of nitrate reductase activity. It was reported from this laboratory, induction of nitrate reductase was mediated through phytochrome in etiolated wheat leaves (Janaki, 1978).
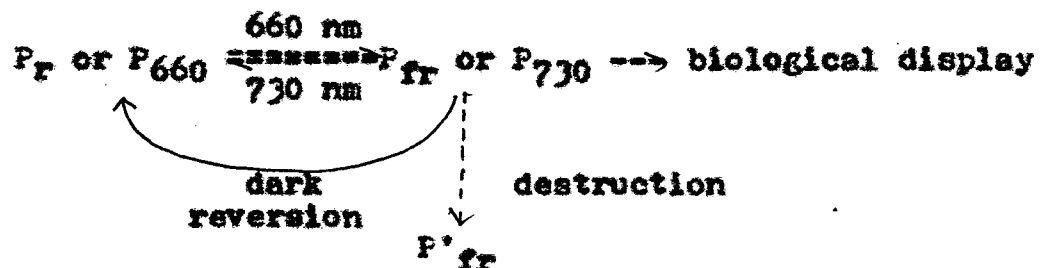
In the present investigation, an effort has been made to study whether the nitrate reductase activity in maize is under the control of phytochrome and if so, what is the mechanism of action of this pigment on the induction of this enzyme.

PREVIOUS WORK

Phytochrome - General Characteristics

Phytochrome is a reversible biological switch activated by light. It has been isolated and purified from a number of species by several groups (see Briggs and Rice, 1972). It is a blue-green chromoprotein, and is present in small quantities in almost all potentially autotropic plants. Two principal forms of the molecule- P_r (red absorbing, $\lambda max = 660$ nm) and P_{fr} (far-red absorbing, $\lambda max = 730$ nm) are distinguishable on the basis of their absorption spectra and biological response. P_r is considered to be the biologically inactive form, whereas P_{fr} is the biologically active form, i.e. capable of inducing a measurable biological response.

The two forms are reversibly interconvertible by red and far-red light ("phototransformation"). In addition, whereas the P_r form is stable in the dark, P_{fr} can revert thermally to P_r ("dark reversion") or undergo an irreversible loss of photoactivity ("destruction").

These properties of the phytochrome system are traditionally summarized in the following scheme:

$$P_r \text{ or } P_{660} \underset{730 \text{ nm}}{\overset{660 \text{ nm}}{\rightleftharpoons}} P_{fr} \text{ or } P_{730} \longrightarrow \text{biological display}$$

dark reversion

destruction

$$P'_{fr}$$

Two types of phenomena have been attributed to phytochrome, the so called "induction-reversion" and "high irradiance response" (Mohr, 1972).

Induction and Reversion Response - These are displays induced by a brief pulse of low intensity red light and reversed by far-red pulse (Borthwick, 1972). The photoreversibility of the response is classically accepted criterion for establishing phytochrome involvement in a process. Quantitative increase in the magnitude of the induced response with increasing light dose is interpreted as being a function of the degree of photoconversion of P_r to P_{fr} i.e. the more quanta, the more P_r is converted into P_{fr} and, therefore, resulting in greater response.

High Irradiance Response - In action spectral studies of many photoresponses with prolonged irradiation period, the maximum action is, however, localized in the blue and far-red region of the spectrum, e.g. hypocotyl elongation in Lactuca sativa seedlings (Hartmann, 1966). Such a response has been termed as "high irradiance response" and characterized by the magnitude of function of irradiance, lack of photoreversibility and reciprocity failures.

Far-red mediated HIR has been explained by two hypotheses: (a) Phytochrome is the only photoreceptor involved (see Hartmann, 1966); (b) it arises by an interaction between P_{fr} and photosynthesis, particularly PS I (see Grill and Vince, 1970).

Hartmann (1966) proposed that HIR responses are brought about by phytochrome, by maintaining a low but relatively constant level of P_{fr}, the maximum effect achieved close to 720 nm when P_{fr}/P_{tot} equilibrium is about 3%. Since HIR show irradiance dependence (Lange et al., 1971), it has been interpreted as a consequence of cycling between P_{fr} and P_r (Hartmann, 1966) or alternately to formation of excited P_{fr} molecule-P_{fr} (Mohr, 1972; Schopfer and Mohr, 1972) which is more effective than P_{fr} (ground state). The equilibrium constant of P_{fr} is a function of the rate of photochemical turnover of P_{fr} and therefore of irradiance. Recently, Schafer (1976) and Mancinelli and Rabino (1975) have advanced a model of HIR based on capacity of P_{fr} form to bind to membranes (Quail et al., 1973a; Marme et al., 1974; Marme, 1977). Schafer's model essentially consists of a scheme which propose that HIR is controlled by P_{fr} receptor complex maintained under condition of pigment cycling. Mancinelli and Rabino (1975) observed that under cyclic far-red treatments,

magnitude of anthocyanin synthesis of cabbage and mustard seedlings depends on the total dose. Further, cyclic treatments were found to be as effective as continuous far-red light, if total radiation dose is equal and applied over equal period. The reciprocity relationship is valid under cyclic far-red treatment.

The possibility of a second photoreceptor, beside phytochrome, in HIR has been raised by Grill and Vince (1970). The evidences in favour of such participation are mostly based on inhibitor studies. Phytochrome-mediated anthocyanin biosynthesis is inhibited by NH_4^+, dinitro phenol and antimycin A which are known inhibitors of cyclic photophosphorylation (FP) (Margulies, 1962; Schneider and Stimson, 1971; 1972). Salicylaldoxime, inhibitor of PS I has been shown to inhibit light-induced anthocyanin formation (Giudici de Nicola et al., 1972a). Further support for photosynthetic participation in HIR is based on development of photosynthesis under continuous far-red light (Schneider and Stimson, 1971, 1972; Oelze-Karow and Butler, 1971; DeGreff et al., 1971).

Evidences for non-involvement of photosynthesis also came from the inhibitor studies. Greening inhibitors, like chloramphenicol and streptomycin, enhance anthocyanin formation (Mancinelli et al., 1974). Eddy and Mapson (1951)
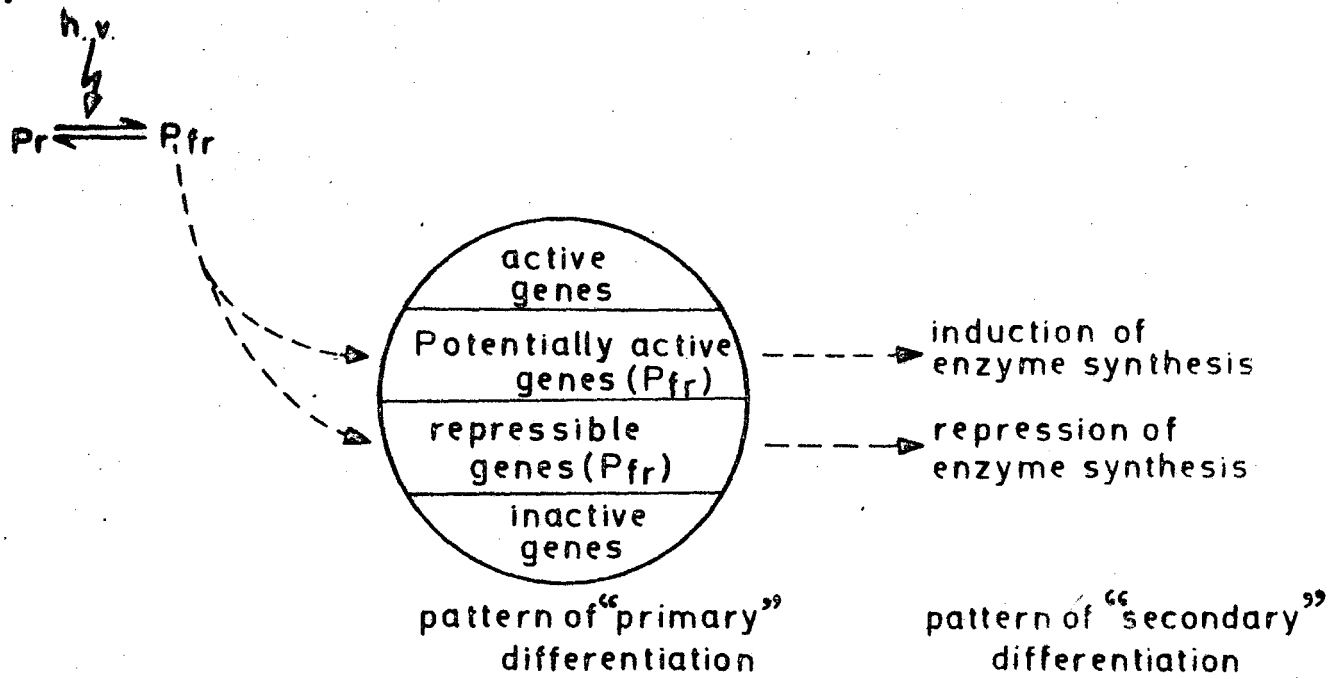
demonstrated that NH_4^+,-inhibitor of cyclic.PP- has no effect on photo-induced anthocyanin synthesis. Duke et al. (1976) reported a negative correlation between greening and anthocyanin biosynthesis in maize seedlings. Furthermore, under far-red light, chlorophyll synthesis is minimal (Mohr, 1972; Masoner et al., 1972), and anthocyanin biosynthesis can be correlated to phytochrome level (Drumm et al., 1975).

Attempts to rationalize the vast diversity of observed photoresponses in terms of a molecular mechanism of phytochrome action have resulted in recent years in two major hypotheses - differential gene activation hypothesis advanced by Mohr (1966) and the membrane permeability hypothesis of Hendricks and Borthwick (1967).

Phytochrome as a Regulator of Gene Expression - This hypothesis seeks to explain phytochrome action through the activation, or repression, of specific genes. The subsequent changes in enzyme levels are held to be responsible for the amplification, and the specificity is determined by the differential status of the genome of the particular cell.

Most of the evidences in support of this hypothesis is based on the inhibition of P_{fr}-mediated response by substances known to inhibit nucleic acid (Carr and Reid, 1966; Mohr and Bienger, 1967; Dittes et al., 1971; Drumm et al., 1971) and protein synthesis (Drumm et al., 1971,1972).

Fig. 1 : A general model illustrating the hypothesis of
differential gene activation and repression as
the mode of action in differential induction and
repression of enzyme synthesis of P_{fr} (After
Schopfer, 1977).

h.v.

Pr ⇌ Pfr

active
genes

Potentially active
genes (P_{fr})

repressible
genes (P_{fr})

inactive
genes

pattern of "primary"
differentiation

induction of
enzyme synthesis

repression of
enzyme synthesis

pattern of "secondary"
differentiation

Beside the use of inhibitors, by density labelling method
also it has been established that phytochrome controlled
de novo synthesis of some enzymes (Acton and Schopfer, 1974;
Attridge, 1974; Acton et al., 1974).

Criticism of this approach is broadly two fold. The
data so far obtained provide no direct evidence that genetic
activity is, inf act, altered, let alone that P_{fr} interacts
with genome. This hypothesis does not explain the rapid
action/rapid experssion phenomena. Not only are these
responses are more rapid than would be expected from
genetic regulation, but in some cases at least have proved
insensitive to transcriptional and translational inhibitors
(Weintraub and Lawson, 1972; Satter and Galston, 1973).
Phytochrome induced changes in membrane, on the other hand,
account for even the most rapid phytochrome mediated phenomena.

Membrane-A Possible Site of Primary Action of Phytochrome-

Some of the phytochrome-controlled responses have a
lag period of only 1-5 minutes, such as nyctinastic move-
ment of Mimosa leaflets (Fondviolle et al., 1966), or
attachment of excised mung bean root tips to negatively
charged glass surface (Tanada, 1968). To explain such
responses, Hendricks and Borthwick (1967) suggested that
the primary action of phytochrome may take place via
some changes in functional properties of membranes. Perhaps,

Fig. 2 : The transport factor hypothesis for phytochrome
action. Active phytochrome is considered to be
located in membranes in which it acts to control
transport of a second messenger X, from one side
to other; X is then able to interact with many
processes (After Smith, 1975).
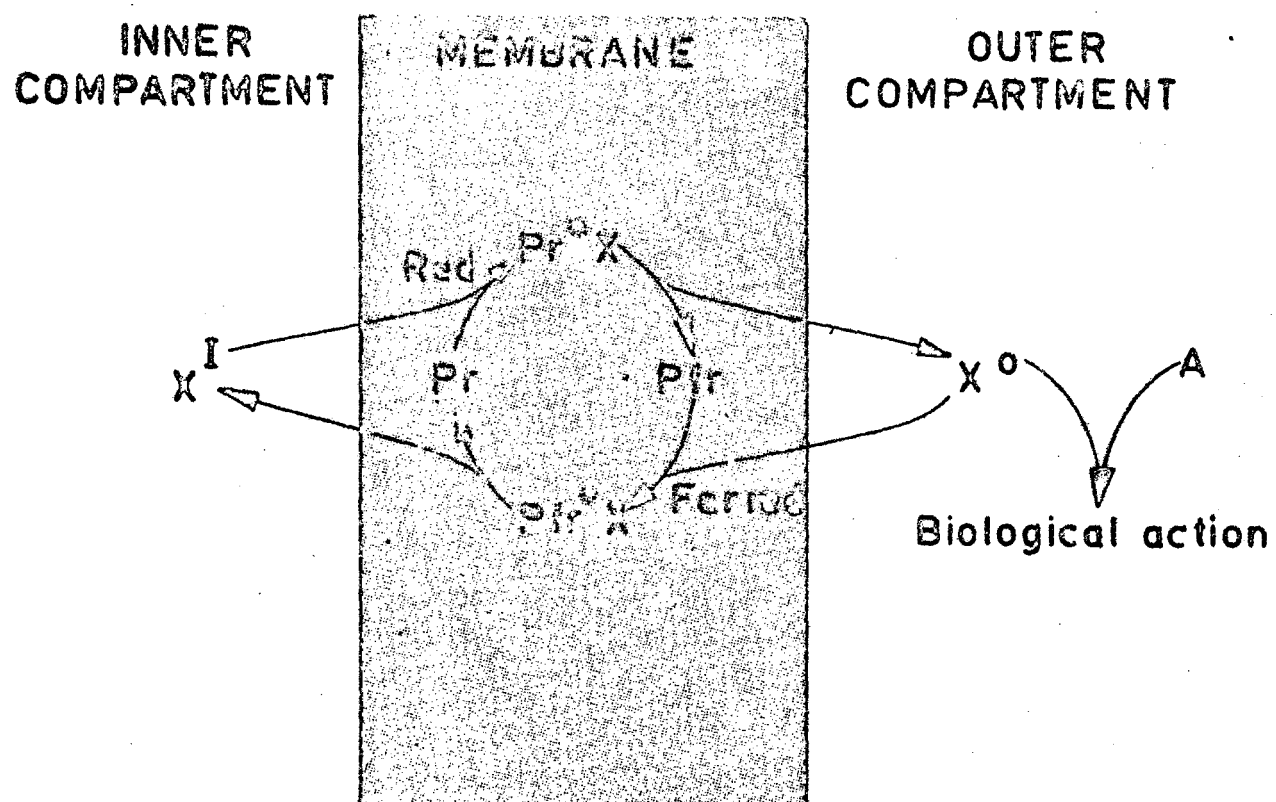
in a variety of cellular membranes, emanating from a single, fundamental membrane alteration could afford the opportunity for changes in ion flux, activation of membrane bound enzymes, altered compartmentalization, release of bound hormones, and so on. Furthermore, a phytochrome membrane association affords the possibility of a photocoupling function for phytochrome in the HIR. Any or all of these might lead ultimately to altered gene expression.

Smith (1970, 1975 and 1976a) has proposed a model which enviages that phytochrome is localized in membranes, and acts by regulating transport of some important metabolites, perhaps a second messenger, which may subsequently elicit photoresponse. Haupt and Weisenseel (1976) speculated P_{fr} may function as Ca^{2+} carrier in membranes.

P_{fr}-Mediation Through Acetylcholine - The occurrence of acetylcholine (ACh) in plants was discovered as early as 1953 by Fielder in Crataegus. Since then, several reports have appeared confirming this finding. Jaffe (1970) demonstrated phytochrome-mediated concentration changes of acetylcholine in mung bean root tips and such changes have also been detected in moss callus (Hartmann and Killbinger, 1974). ACh mimiced the phenomenon of red light mediated root tip attachment to negatively charged surface,

and the red light effect could be effectively blocked by
acetylcholine inhibitors (Jaffe, 1970). Jaffe proposed
that acetylcholine may act as the local hormone regulating
phytochrome-mediated phenomena at the membrane level.

Many other workers could not find any involvement
of ACh in other photoresponses e.g. nyctinastic leaflet
movement in Albizzia (Satter et al., 1972), induction of
phenylalanineammonia lyase (Kasemir and Lohr, 1972),
flavanoid level (Saunders and McClure, 1972) and ATP level
(Krishner et al., 1975). Tanada (1972) suggested that ACh
may function by its cation interfering capacities.
Kasemir and Mohr (1972) proposed that ACh effect may be
restricted to plants or organs in touch with water.

Though there are several proposals to explain phyto-
chrome action, any single hypothesis alone can not explain
all phytochrome mediated responses. So the primary molecular
mechanism of phytochrome action remains an unresolved
question.

Nitrate Reductase and Its Regulation by Phytochrome

The enzyme nitrate reductase is involved in the
reduction of nitrate to nitrite. Nitrate reductase has
been shown to be molybdo-protein and has a molecular weight
around 500 K - 600 K (Beevers and Hageman, 1969). Nitrate

reductase is substrate inducible enzyme. In addition to substrate i.e. nitrate, the development of nitrate reductase activity depends on several genetic, ontogenetic and envoronmental factors, including light, hormones and supply of other forms of nitrogen. The exact mechanism by which light controls the level of nitrate reductase still is not clear. Phytochrome has been implicated in light induction of nitrate reductase activity by Jones and Sheard (1972, 1974) and Johnson (1976).

The first report on phytochrome-mediation of nitrate reductase induction came from Jones and Sheard (1972). They demonstrated enhancement of nitrate reductase activity by 5 minutes of red light treatment and which was readily reversed by immediate exposure to far-red light. They also reported increase in levels of nitrate reductase after exposure to blue light for a brief period. The effectiveness of blue light was intermediate between those of red and far-red light. These light treatments exclude the possibility of photosynthesis as a process contributing to induction. Under these conditions, induction is shown to be reversibly controlled by red and far-red light, an effect ascribable to the pigment phytochrome.

Johnson (1976) also reported that 5 minutes of red light irradiation leads instaneously to 45% increase in

nitrate reductase enzyme activity in Sinapis alba. This
increase was insensitive to cyclohexinide whereas in the
same species, cycloheximide inhibits induction of nitrate
reductase by nitrate. Thus, it appeared that light and
nitrate acted through different mechanisms in controlling
nitrate reductase activity.

Jones, and Sheard (1975) demonstrated that although
phytochrome mediates increased uptake of nitrate in pea
terminal buds as well as causing an increase in nitrate
reductase activity, increased nitrate itself did not
lead to further increase in nitrate reductace. Dimethyl
sulfoxide and sugars did not enhance the induction in dark
when nitrate uptake was greatly increased by these
chemicals and light enhancement of nitrate uptake did
not occur before the induction of nitrate reductase. So
they concluded that increased nitrate availability is not
the cause of phytochrome-mediated enzyme increase. An
alternate theory suggested that light controls the
polyribosome level and the capacity for amino acid
incorporation in protein synthesis (Jordan and Huffaker,
1972; Travis et al., 1970; Travis and Key, 1977).

Travis et al (1970), Travis and Key (1971) suggested
that light-induced increase in polyribosome level produce
a non-specific effect on the rate of nitrate reductase

synthesis. Pine and Klein (1972) and Smith (1976) have
shown that polyribosome level is indeed controlled by
phytochrome in bean leaves. So the reversibility control
of nitrate reductase activity by red and far-red lights
may, therefore, be both a result and a partial cause of
effects on protein synthesis in general.

A further suggestion proposed by Sawhney and Naik
(1972) is that redox changes taking place in greening
tissues as a result of Hill reaction create conditions
for the enhanced rate of synthesis of the enzyme. Such
a mechanism clearly need not involve phytochrome since
phytochrome mediated responses can be promoted under
conditions in which photosynthesis does not occur (brief
light exposures).

Jordan and Huffaker (1972) inferred that reduction
of nitrate reductase by light in etiolated barley leaves
depend upon the development of photosynthetic CO_2 fixation.
In contrast, Jones and Sheard (1973) showed that the
induction in etiolated pea buds, after 5 minutes exposure
to white light was a high proportion of the total induction
after 2 hours of continuous white light.

Johnson (1976) has shown that cycloheximide did not
inhibit enzyme activity which was mediated by phytochrome
significantly in Sinapis alba. If the enzyme is assayed

in the cotyledons immediately after 5 minutes of red light treatment there is a 45% increase in the extractable activity, without any lag phase. The rapidity of these responses, and no lag period strongly suggest, but does not prove, that the transcriptional control is not involved in the induction of nitrate reductase. And it also seems inconceivable that the instaneous response to a short dose of red light can be due to an increased rate of enzyme synthesis. In fact, neither the nitrate nor phytochrome mediated responses are inhibited by the addition of tungsten at the onset of the treatment. It suggests that either nitrate induction may not involve synthesis of the whole of the protein moeity of the enzyme or else of protein synthesis required for the response is not the synthesis of nitrate reductase. However, increase in nitrate reductase activity by continuous irradiation might be due to the increase in the protein synthesis.

Analogous red and far-red reversible effects have been shown for a number of other enzymatic activities in etiolated peas. Bottomley (1970) showed that the activity of nuclear RNA polymerase in peas was increased by red light, and the effect was reversed by far-red light. The development of polyribosomes and the amino acid incorporating

activity of isolated ribosome, was shown by Williams and
Novelli (1968) to be enhanced more by red than blue light
in etiolated seedlings.

Jones and Sheard (1977) also reported enhancement
of nitrate reductase activity by blue light in etiolated
pea terminal buds. After 5 minutes light exposures
(white light, red, far-red, blue), there was no significant
differences in the effectiveness of the different wave
bands, indicating that only a small quantity of P_{fr} was
required to saturate the control exhibited via phytochrome.
However, in 2 hours of continuous irradiation about 40%
more enzyme activity was induced in extracts of terminal
buds exposed to white and blue lights when compared to
continuous irradiation with red and far-red light. The
greater effectiveness of blue light during prolonged
exposure points to the operation of so called "High
Energy Reaction", which probably involves the action of
pigments other than phytochrome.

Although the mechanism of blue light action remains
unknown, the effect of blue light may have implications
concerning overall mechanism of light regulation of nitrate
reductase. The effect of blue light induction of nitrate
reductase is not only confined to etiolated peas, but also

occurs in green corn grown under photosynthesizing conditions.

Recently, Duke and Duke (1978) reported that modulation of nitrate reductase activity is not directly influenced by photosynthetic photoreceptors and that phytochrome is involved in the photocontrol of nitrate reductase activity in maize. Their results also indicate that light quality at the end of day influences both nitrate reductase activity during night as well as time required to reach maximal nitrate reductase activity during the next photoperiod.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Fresh seeds of maize (Zea mays var shakti) were obtained from the Indian Agricultural Research Institute, New Delhi, and stored at 4^oC in dessicator.

Seeds of uniform size were selected and kept under running tap water for about 18 hours. Imbibed seeds were thoroughly washed with distilled water. These were then grown in petridishes for 6 days in complete darkness on moist absorbent paper in a seed germinator maintained at 27 ± 1^oC. Seedlings were supplied once a day with nitrate free Hoagland's modified nutrient solution, the composition of which is given below:

Composition of Hoagland's solution:

Solution 1:

Stock solution 1M

	ml/L
KH_2PO_4	1
$MgSO_4$	2

Solution 2:

Trace elements

	ppm
B	0.5
Mn	0.5
Zn	0.05

Cu	0.02
Co	0.01

Solution 3:

Fe tartarate (stock solution 0.5%) used 1 ml/L.

A daily screening of petridishes was done and ungerminated and infected seeds, if any, were removed. All operations except experimental treatments were conducted under dim green safe light.

Apical leaves excised from 6 day-old plants were chosen as the experimental material .

Incubation of Excised Apical Leaves

Samples of excised etiolated apical leaves were floated in 35 ml of incubation medium (60 mM KNO_3) either in dark or in light as indicated in Tables and Figures. For each treatment, approximately 250 mg apical leaves were employed. All treatments were replicated at least twice. All incubations were carried out at a constant temperature of $25 \pm 1^\circ C$.

Light Sources

The green safe light was obtained through 8 layers of green cellophane paper from a cool white fluorescent tube light. The intensity of light at the plant level was never more than 1 µW cm^{-2}. Red light was obtained from two tungsten lamps (100 and 60 watts) wrapped with

two layers of red cellophane paper. The intensity of red-light at the height of seedlings was 500 $\mu W\ cm^{-2}$. The light from a 300 watt reflector lamp was filtered through 8 cm of continuously flowing tap water and a CBS-750 filter to obtain far-red light. The intensity of far-red light was 140 $\mu W\ cm^{-2}$ at the plant level. White light was obtained by three cool white fluorescent tube lights. The intensity of white light at the plant level was 1200 $\mu W\ cm^{-2}$. Temperature during all these treatments were maintained at 25 ± 1°C.

Enzyme Extraction

Apical leaves were removed from the incubation medium at different time intervals, washed and dried on a blotting paper and ice-chilled. The chilled tissues were homogenized in 2 ml of 50 mM Tris-HCl buffer, pH 8.5, containing 3 mM EDTA and 3 mM cysteine. The homogenate was centrifuged at 20,000 x g for 20 minutes at 0°C. The supernatant was employed for the determination of enzyme activity.
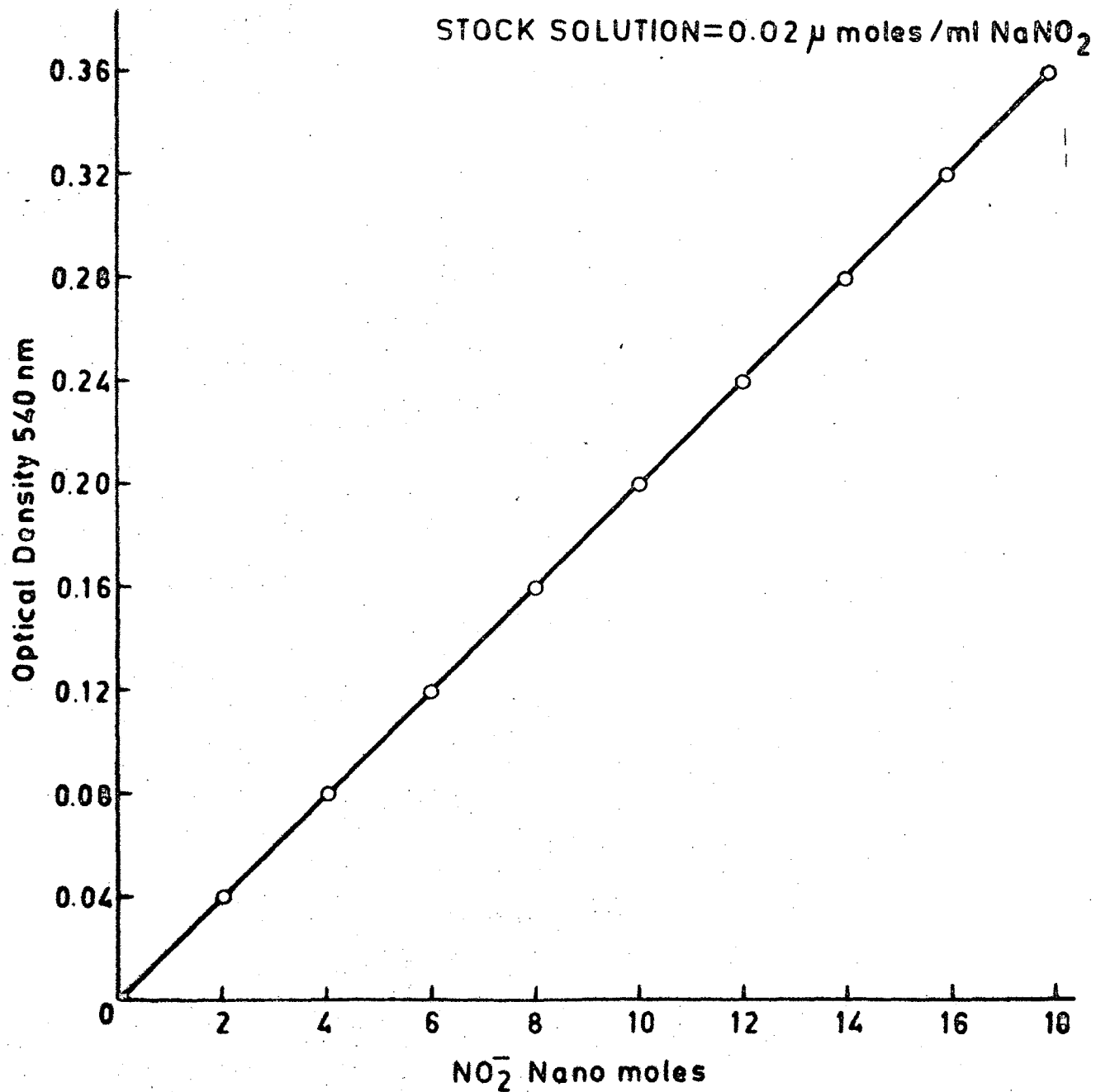
Enzyme Assay

Assay of nitrate reductase was performed essentially according to the procedure of Hageman and Hucklesby (1971). The assay mixture contained the following: 1 ml of 0.1 M K-phosphate buffer, pH 7.5, 0.2 ml of 0.1 M KNO$_3$; 0.2 ml

Fig. 3 : Standard curve for KO_2^- .

STANDARD CURVE FOR NO_2^- ESTIMATION

STOCK SOLUTION=0.02 μ moles/ml $NaNO_2$

of NADH (1 mg/ml). In control tubes NADH was omitted.
The reaction was started by the addition of 0.2 ml of
the enzyme extract. After incubating for 15 minutes
at $30^{\circ}C$ the reaction was stopped by the addition of
0.1 ml of 1 M zinc acetate solution. The resulting
precipitate was removed by centrifugation. One ml
aliquot was taken from the supernatant for color
development. To this 1 ml of sulfanamide (1% in 1.5 N
HCl) and 1 ml of 0.02% N-1-napthyl-ethylene-diamine
dihydrocholride were added and allowed to stand for
15 minutes. Optical density at 540 nm was determined
in Bausch and Lamb Spectronic 20. The concentration
of nitrite formed was calculated from the standard
curve (Fig. 3). Nitrate reductase activity is expressed
as nanomoles nitrite formed for 1 mg protein for 1 hour.

Protein Estimation

For determination of protein 0.2 ml of aliquots of
cell free extracts were precipitated by equal amounts of
10% TCA. The precipitate was collected by centrifugation.
The pellet was dissolved in 0.4 ml of 0.1 M NaOH and
protein was estimated by the method of Lowry et al. (1951).
The absorbancy at 500 nm was measured with Spectronic-20.
The E_{500} values were then converted to amount of protein
by means of a standard curve obtained by using bovine
serum albumin (Fig. 4).

Fig. 4 :   Standard curve for protein estimation.

STANDARD CURVE FOR PROTEIN (BSA)
STOCK SOLUTION 200 μg / ml
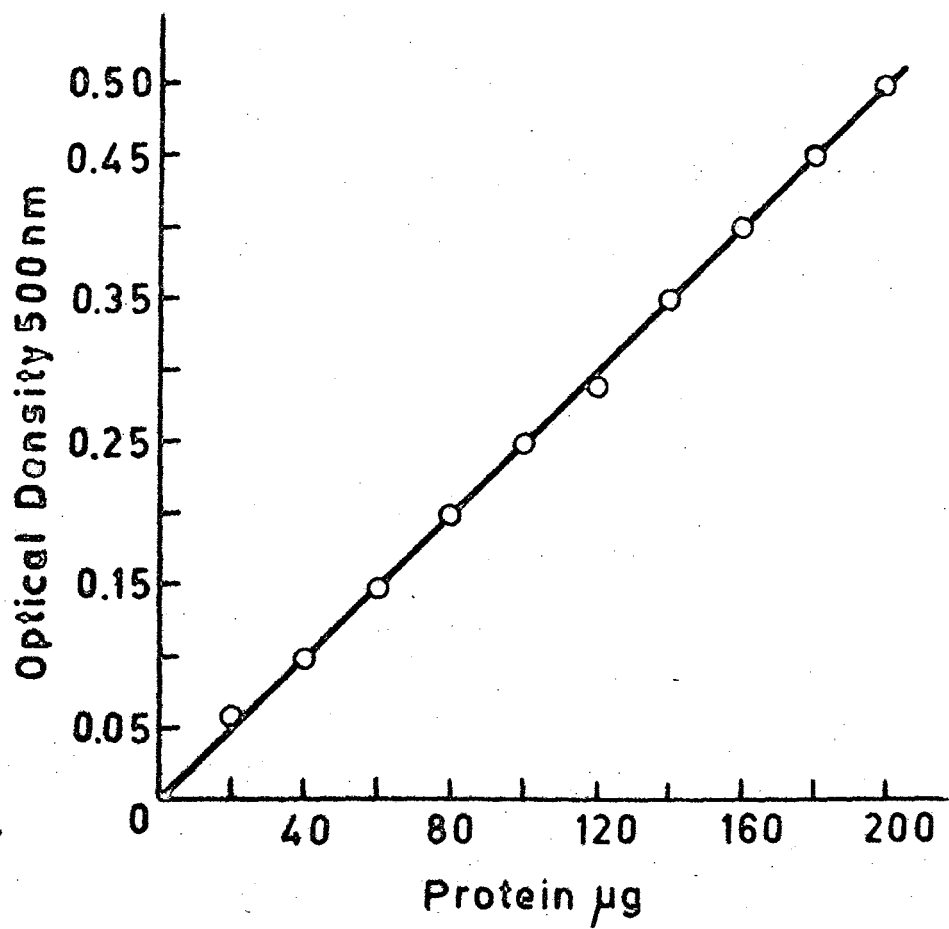
Chemicals

All inorganic chemicals were of reagent grade and were obtained from British Drug House (India) or E. Merck, Darmstadt, Germany. NADH and cycloheximide were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; actinomycin D was obtained from Calbiochem, Los Angeles, Calif, U.S.A.

RESULTS

Experiments were undertaken to determine the role of phytochrome in induction of nitrate reductase in etioloted maize seedlings. Apical leaves were taken as the experimental material. As stated earlier (Materials and Methods), six-day-old seedlings have been employed for all treatments.

Effect of Brief Treatments of Red Light on Nitrate Reductase Activity

Etiolated, excised leaves were incubated in induction medium. These were exposed to red light for 5 minutes and returned to darkness. After 4 hours nitrate reductase activity was measured. As can be seen in Table 1, irradiation of excised leaves with red light for 5 minutes increased the nitrate reductase activity by 342%. In different sets of experiments the increase in nitrate reductase activity varied from 250% ato 400%.

Effect of Brief Treatments of Far-Red Light on Nitrate Reductase Activity

To find out the effect of far-red light, excised leaves in induction medium were irradiated with far-red light for 5 minutes. After irradiation, leaves were transferred to dark for 4 hours before the assay of the

TABLE 1

EFFECT OF BRIEF IRRADIATION OF RED LIGHT ON NITRATE
REDUCTASE ACTIVITY

Excised leaves from 6 day-old dark grown maize
seedlings were floated on induction medium (60 mM
KNO_3) and exposed to red light for 5 minutes. After
treatment, leaves were transferred to dark. Nitrate
reductase activity was assayed after 4 hours. Nitrate
reductase activity is expressed as nanomoles nitrite
formed/mg protein^{-1}/hr^{-1}.

| Treatments	Specific activity	Relative activity (%)
Dark 4 hrs	69	100
Red light 5 min + Dark 4 hours	246	342

TABLE 2

EFFECT OF BRIEF TREATMENT OF FAR-RED LIGHT ON NITRATE
REDUCTASE ACTIVITY

Leaves excised from 6-day-old dark grown maize
seedlings were floated on induction medium. Brief
treatment of far-red light was given to these leaves
before returned to darkness. Nitrate reductase
activity was measured after 4 hours. Nitrate reductase
activity is expressed as nanomoles nitrite formed/
mg protein^{-1}/hr^{-1}.

Treatments	Specific activity	Relative activity (%)
Dark 4 hrs	98.07	100
Far-red 5 min + dark 4 hrs	99.04	101

Fig. 5 : Effect of brief irradiation of far-red light
on nitrate reductase activity.
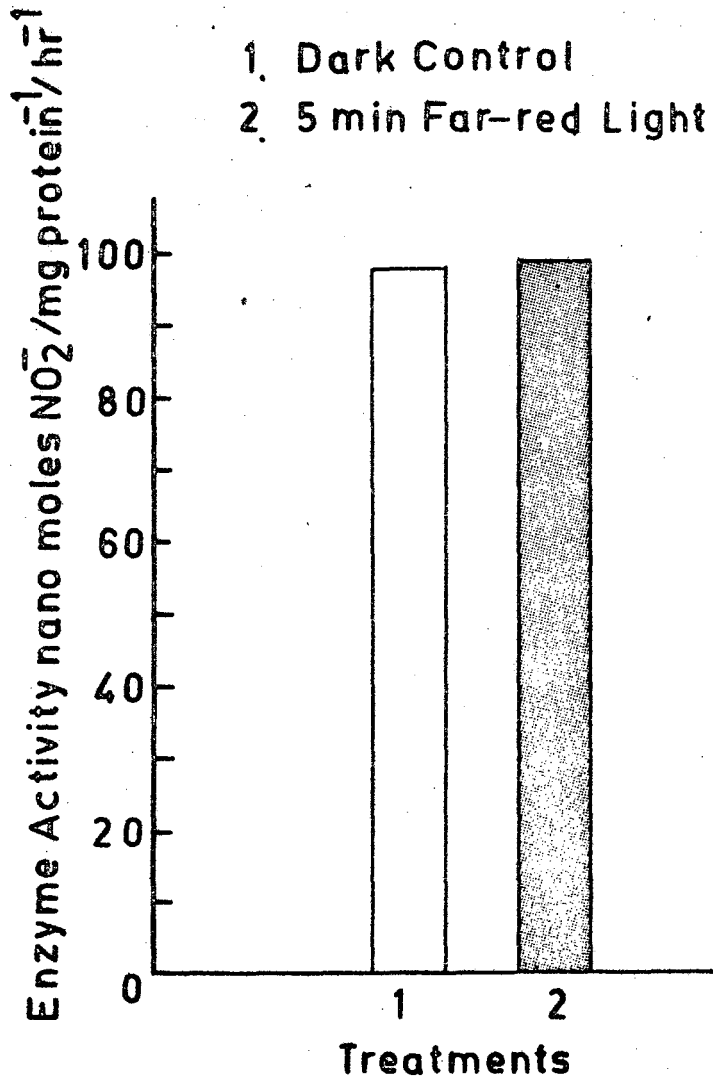
enzyme activity. The data in Table 2 (Fig. 5) show that
5 minutes far-red light had no effect on the nitrate
reductase activity.

Effect of Different Duration of Red Light on Nitrate Reductase Activity

As is evident from Table 1, five minutes of red light
irradiation increased the nitrate reductase activity. To
find out the time required for maximum induction of the
enzyme, excised leaves were irradiated with red light for
5, 10 and 15 minutes and kept back in dark for 4 hours
prior to assay of the enzyme activity. The data presented
in Table 3 (Fig. 6) indicated that red light irradiation,
for a period of more than 5 minutes, did not enhance the
nitrate reductase activity. Prolonged red light irradiations
(i.e. 10 and 15 minutes) led to a fall in the enzyme
activity as compared to 5 minutes irradiation.

Effect of Red and Far-Red Light of Nitrate Reductase Activity

Since the brief treatments with red light (Table 1)
showed that there is a greater increase in the nitrate
reductase activity as compared to dark, experiments were
undertaken to see if the effect of red light could be
reversed by far-red light and vice versa to ascertain
the involvement of phytochrome in the induction of nitrate
reductase. Dark and white light treatments were kept as
controls. The data presented in Table 4 indicate that

TABLE 3

EFFECT OF DIFFERENT DURATION OF RED LIGHT IRRADIATION ON NITRATE REDUCTASE ACTIVITY

Leaves, excised from 6-day-old dark grown maize seedlings, were floated on induction medium. Treatments were given to these leaves and transferred to dark. The enzyme activity was assayed after 4 hrs. Nitrate reductase activity is expressed as nanomoles nitrite formed/mg protein^{-1}/hr^{-1}.

Treatments	Specific activity	Relative activity (%)
Dark 4 hrs	97.14	100
Red light 5 min + dark 4 hrs	341.28	351
Red light 10 min + dark 4 hrs	264.18	272
Red light 15 min + dark 4 hrs	228.04	235

Fig. 6 : Effect of different duration of red light
irradiation on nitrate reductase activity.

1. Dark Control
2. 5 min Red Light
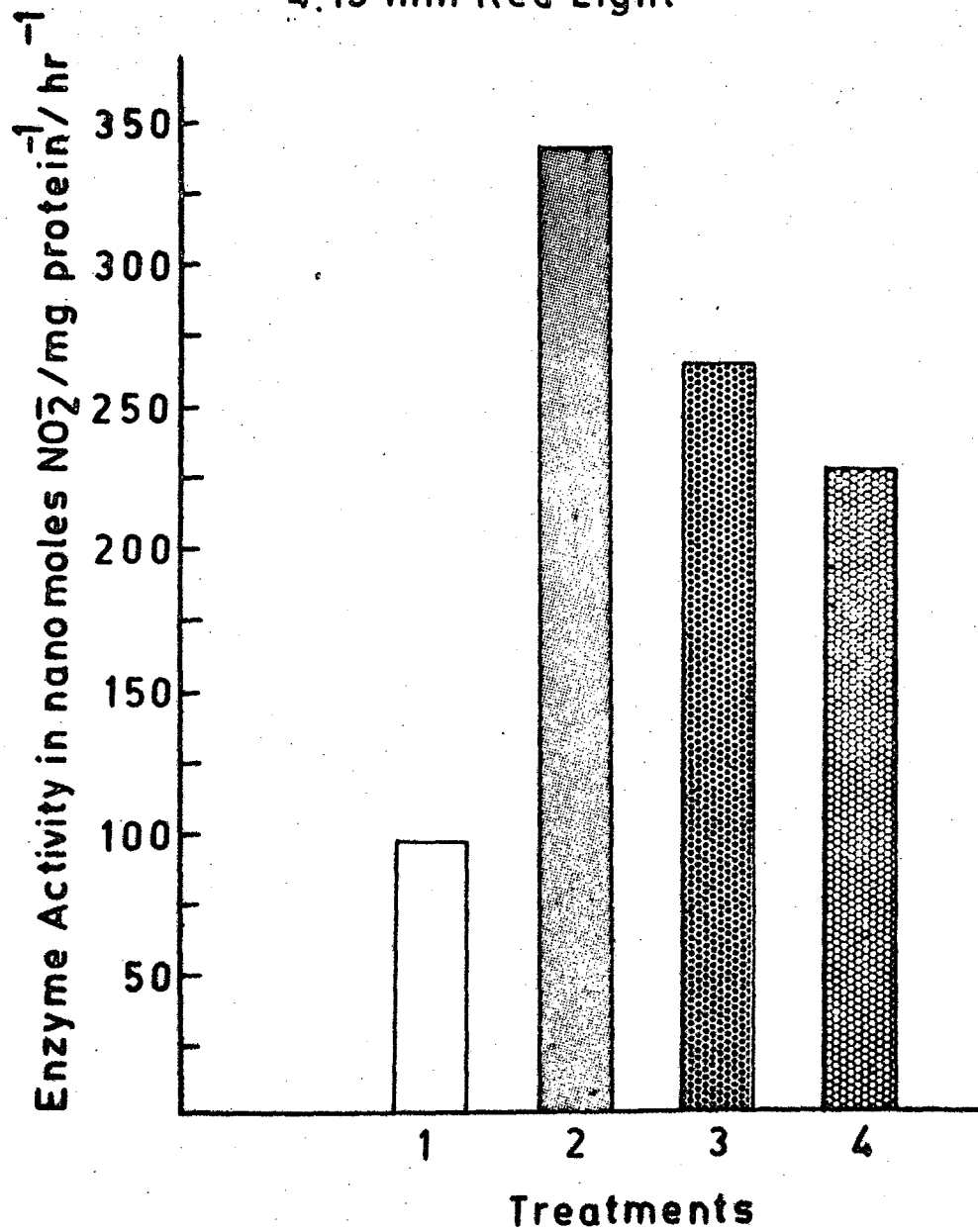3. 10 min Red Light
4. 15 min Red Light

TABLE 4

REVERSIBLE EFFECT OF RED AND FAR-RED LIGHTS ON NITRATE
REDUCTASE ACTIVITY

Excised leaves from 6-day-old dark grown maize
seedlings were floated ion induction medium. Treatments were given to these excised leaves. After
irradiation, these were transferred to dark. Nitrate
reductase activity was measured after 4 hours. Nitrate
reductase activity is expressed as nanomoles nitrite
formed/mg protein^{-1}/hr^{-1}.

Treatments	Specific activity	Relative activity (%)
Dark 4 hrs	82	100
RL + dark 4 hrs	252	306
RL + FRL + dark 4 hrs	38	46
RL + FRL + RL + dark 4 hrs	354	432
Continuous white light 4 hrs	728	885

RL - Red light 5 min; FRL - Far-Red Light 5 min.

Fig. 7 : Reversible effect of red and far-red lights
on nitrate reductase activity.
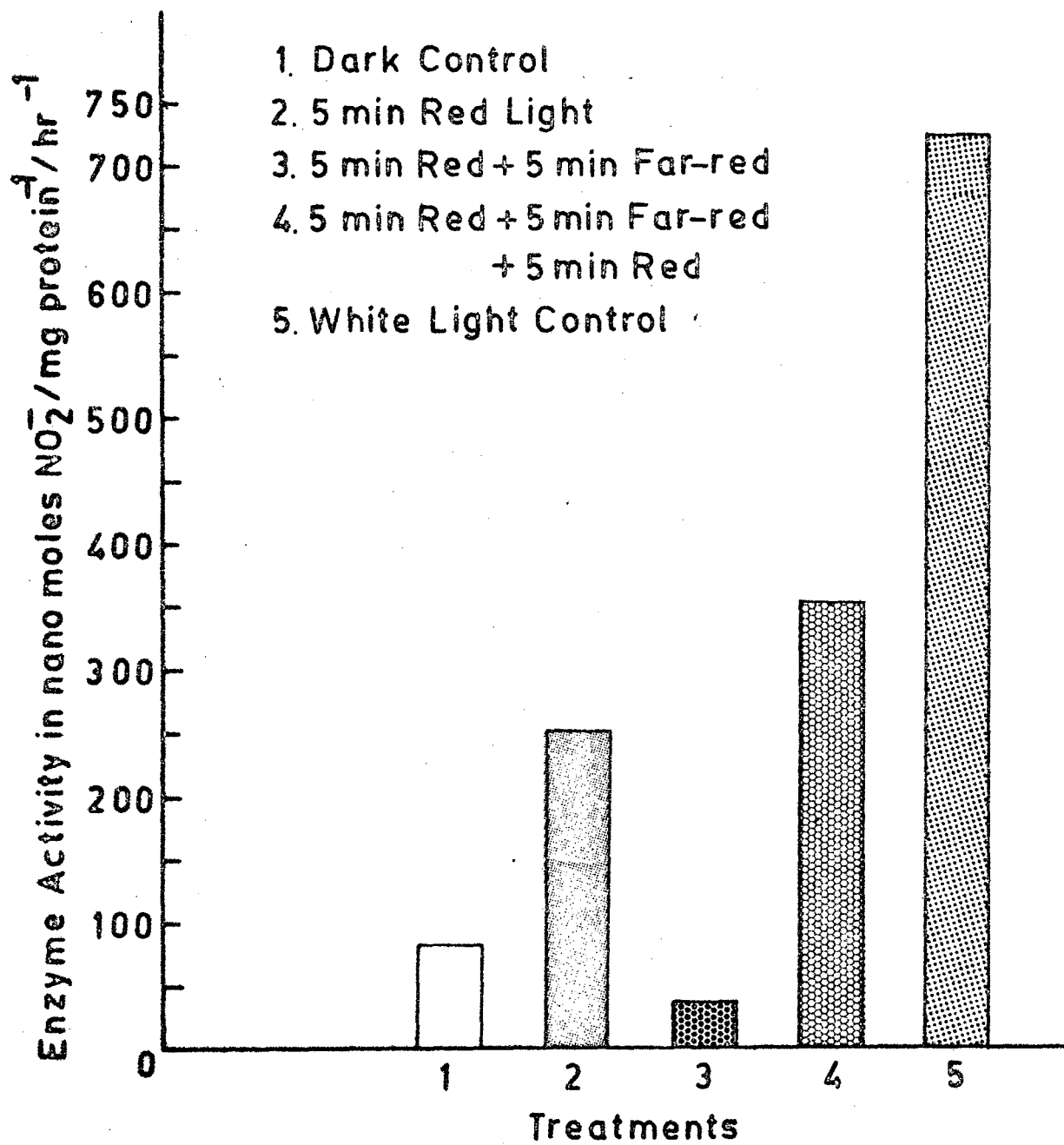
increase in the nitrate reductase activity over the dark
control is a function of quality of light to which excised
leaves were exposed eventually. Nitrate reductase induction
in white light (continuous) was, however, found to be
greater than red light treatments (Fig. 7).

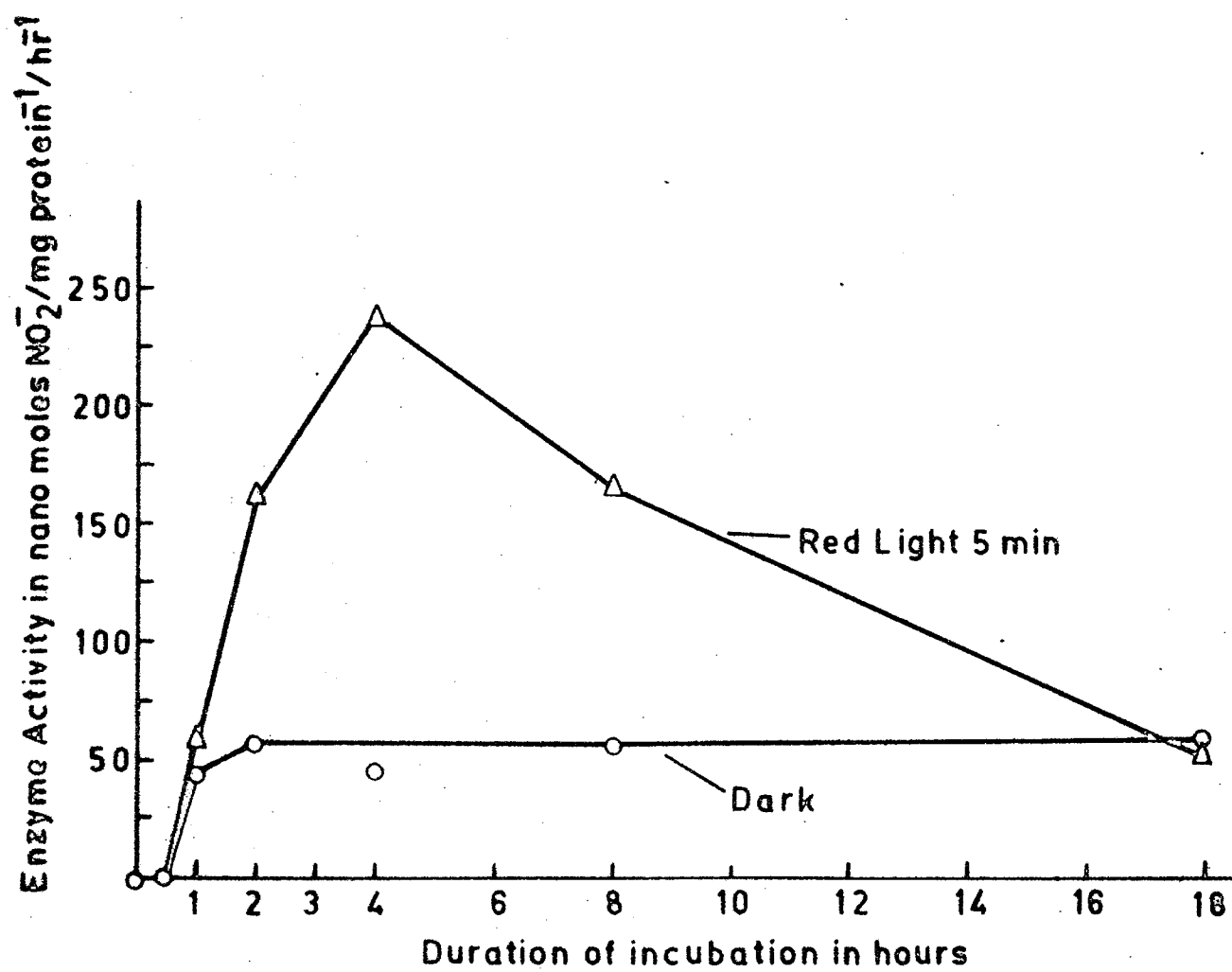## Kinetics of Nitrate Reductase Activity After 5 min Red Light Treatment

Earlier experiments showed that red light has an
enhancing effect on nitrate reductase activity (Table 1).
To find out the lag period and optimum duration for the
induction of nitrate reductase, after red light treatment
for 5 minutes materials were transferred to dark. After
different intervals, enzyme activity was measured. This
revealed that there was a lag phase of about 30 minutes,
and after that there was an instaneous increase in the
enzyme activity upto 4 hours, and then gradual decrease in
the enzyme activity. After 18 hours the red light effect
was completely diminished (Fig. 8).

Kientics of Nitrate Reductase Activity under Continuous Far-Red Light Treatment

Earlier experiments showed that exposure to far-red
light for 5 minutes did not enhance the enzyme activity.
To find out if the HIR response is operative in the nitrate
reductase induction, kinetics of nitrate reductase activity

Fig. 8 :   Kinetics of nitrate reductase activity after
            5 minutes red light treatment.

Fig. 9 : Kinetics of nitrate reductase activity under continuous far-red light treatment.
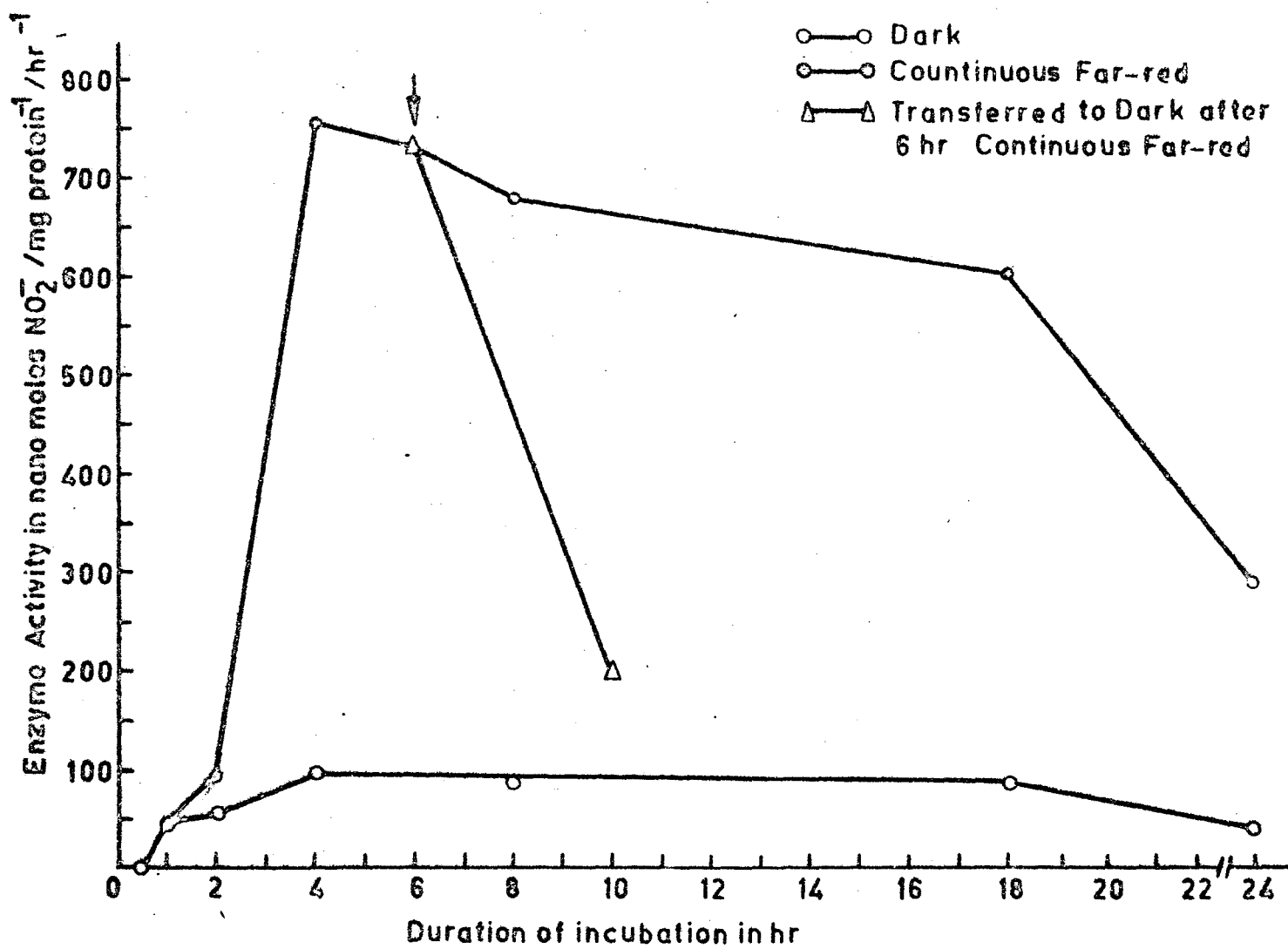
under continuous far-red light was studied. This study
revealed that there was a lag phase of about 30 minutes
and after that instaneous increase in the enzyme activity
upto 4 hours (similar to the kinetics of red light)
occurred but after 4 hours there was a gradual decrease
in the nitrate reductase activity. However, in contrast
to red light kinetics, after 24 hours, too, nitrate
reductase activity was greater than the dark controls.
On turning off the far-red light at the 6th hour, the
enzyme activity declined sharply but was still maintained
higher than the dark controls. This experiment indicated
that continuous far-red light was essential to maintain
higher levels of nitrate reductase activity (Fig. 9), or
P_{fr} was involved in the synthesis of nitrate reductase.

Effect of Acetylcholine on Nitrate Reductase Activity in Dark

Experiments were conducted with an aim to study
whether acetylcholine mimics the phytochrome in the
enhancement of nitrate reductase activity. The effect of
acetylcholine was seen in etiolated, excised leaves.
Exogenous acetylcholine (10^{-4}M) brought about an enhance-
ment in the nitrate reductase activity in excised leaves
(Table 5), but magnitude of increase in the nitrate
reductase activity by acetylcholine was comparatively less

TABLE 5

EFFECT OF ACETYLCHOLINE ON NITRATE REDUCTASE ACTIVITY
IN DARK

Excised leaves, from 6-day-old dark grown maize seedlings, were floated on induction medium. 10^{-4} M acetylcholine was supplied in the induction medium. Enzyme activity was measured after 4 hours of incubation in the dark. Nitrate reductase activity is expressed as nanomoles nitrite formed/mg protein^{-1}/hr^{-1}.

Treatments	Specific activity	Relative activity (%)
Dark 4 hrs	52.07	100
Red light 5 min + dark 4 hrs	299.8	525
Acetylcholine 5 min + dark 4 hrs	63.4	121
Acetylcholine for 4 hrs in dark	113.72	218

Fig. 10 : Effect of acetylcholine on nitrate reductase
activity in dark.

1. Dark Control
2. Red Light (5 min)
3. Ach 10^{-4}M (5 min)
4. Ach 10^{-4}M (4 hr)
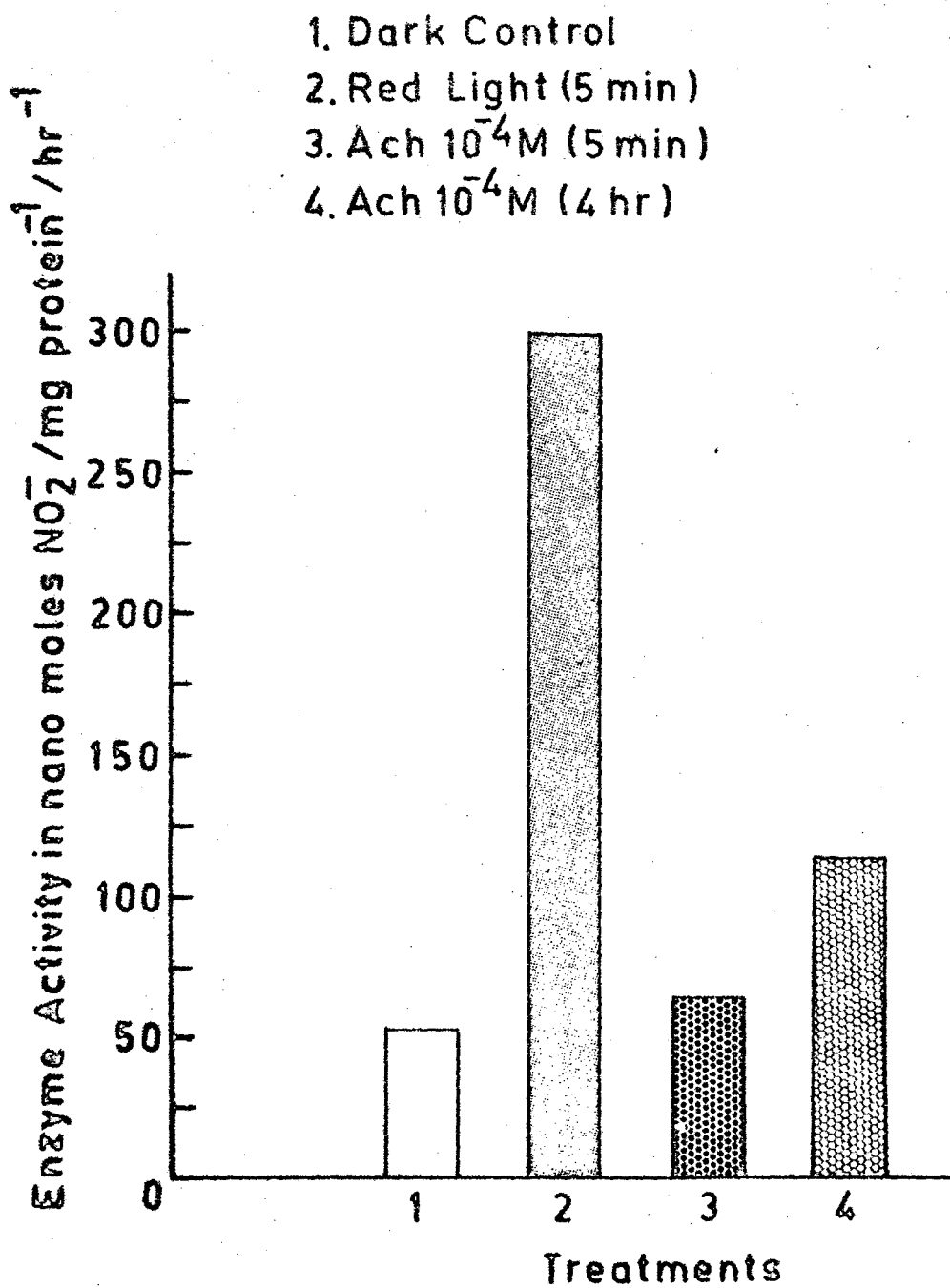
than the magnitude to phytochrome mediated nitrate reductase induction. However, if acetylcholine was supplied exogenously for a brief period it did not enhance the nitrate reductase activity (Fig. 10).

Effect of Transcription and Translation Inhibitors

To decipher the state of transcriptional and translational control during induction under continuous far-red light, inhibitors of RNA and protein synthesis were studied.

Effect of Actinomycin D - Excised leaves were supplied with actinomycin D in the induction medium. The enzyme activity was measured after 4 hours continuous far-red light irradiation. For one set, for the first two hours actinomycin D was supplied to the medium, and after that material was transferred to actinomycin D free medium and vice versa for another set. As shown in Table 6, actinomycin D was found to exert significant effect on the nitrate reductase activity if supplied in the first two hour-period, however, if it was supplied after on set of the irradiation for last 2 hours, it did not have any significant effect. If actinomycin D was supplied for the full 4 hour period, it almost totally inhibited the induction of the enzyme (Fig. 11).

TABLE 6

EFFECT OF ACTINOMYCIN D ON PHYTOCHROME-MEDIATED INCREASE IN NITRATE REDUCTASE ACTIVITY IN EXCISED LEAVES OF MAIZE

Leaves excised from 6-day-old dark grown maize seedlings were floated on induction medium and transferred to continuous far-red light. 15 μg/ml. actinomycin D was supplied with induction medium at the onset of far-red irradiation or as indicated in table. Enzyme activity was assayed after 4 hours. Nitrate reductase activity is expressed as nanomoles nitrite formed/mg protein^{-1}/hr^{-1}.

Treatments	Specific activity	Relative activity ($\%$)
Control (continuous) far-red without inhibitor 4 hrs)	561	100
2 hrs -Actinomycin D + 2 hrs actinomycin D	544	97
2 hrs Actinomycin D + 2 hrs -actinomycin D	333	59
Actinomycin D 4 hrs	41	7

Fig. 11 :   Effect of actinomycin D.

1 Continuous Far-red control (4 hrs)
2 First 2 hr (-Act D)+2 hr (Act D)
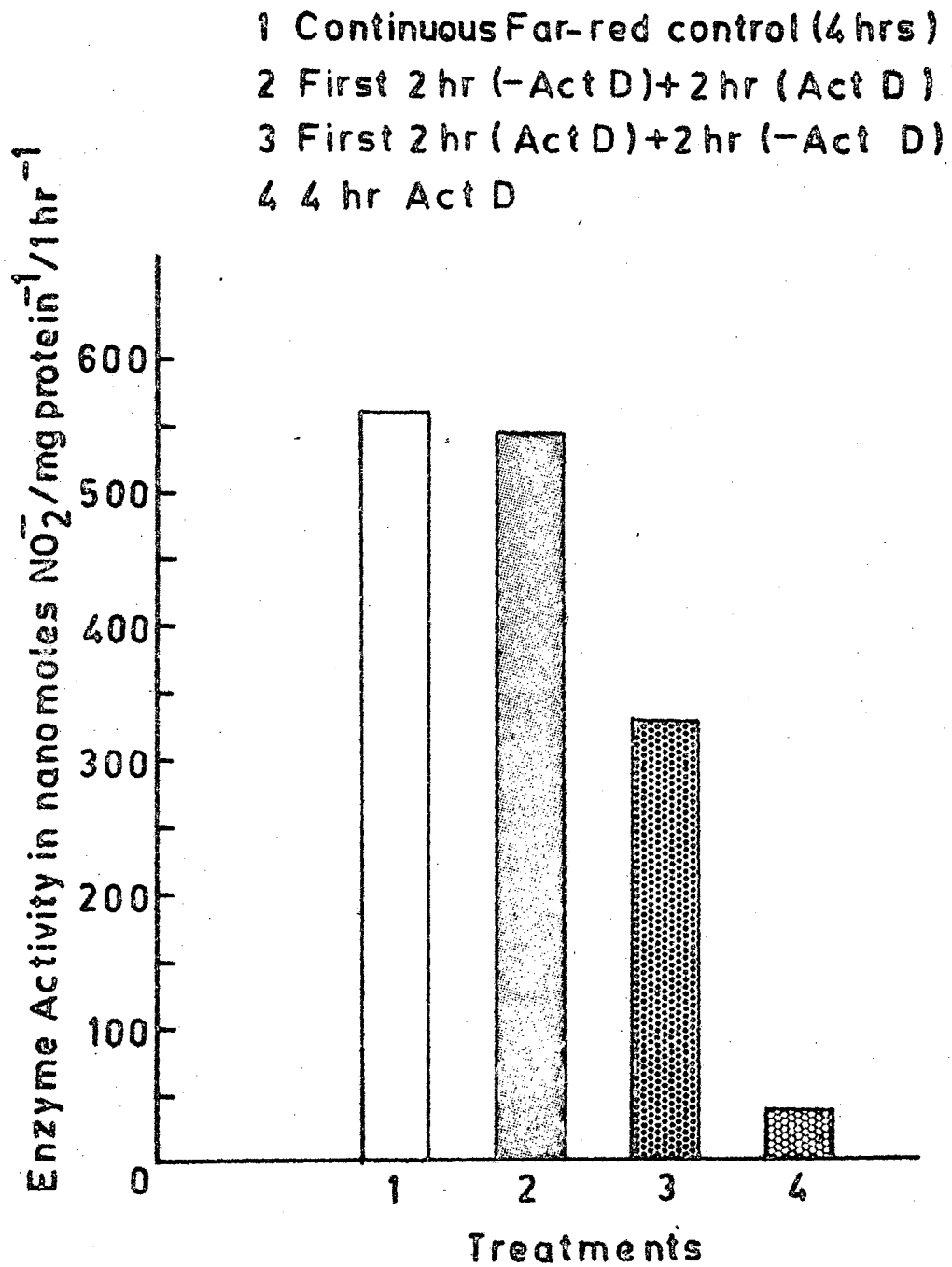3 First 2 hr (Act D)+2 hr (-Act D)
4 4 hr Act D

TABLE 2

EFFECT OF CYCLOHEXIMIDE ON PHYTOCHROME MEDIATED INCREASE
IN NITRATE REDUCTASE ACTIVITY IN EXCISED LEAVES OF MAIZE

Leaves, excised from 6-day-old dark grown seedlings were floated on induction medium and transferred to continuous far-red irradiation. 20 µg/ml cycloheximide was supplied with induction medium at the onset of far-red treatment or as indicated in table. Nitrate reductase activity was measured after 4 hrs. Nitrate reductase activity is expressed as nanomoles nitrite formed/mg protein^{-1}/hr^{-1}.

Treatments	Sepcific activity	Relative activity (%)
Control (continuous far-red light 4 hrs)	610.93	100
2 hrs Cycloheximide + 2 hrs —cycloheximide	121.42	20
2 hrs — cycloheximide + 2 hrs cycloheximide	102.00	17
4 hrs Cycloheximide	0.0	0

Fig. 12 : Effect of cycloheximide.

1. Continuous Far-red control (4 hr)
2. First 2 hr CHI + 2 hr (-CHI)
3. First 2 hr (-CHI) + 2 hr CHI
4. 4 hr CHI
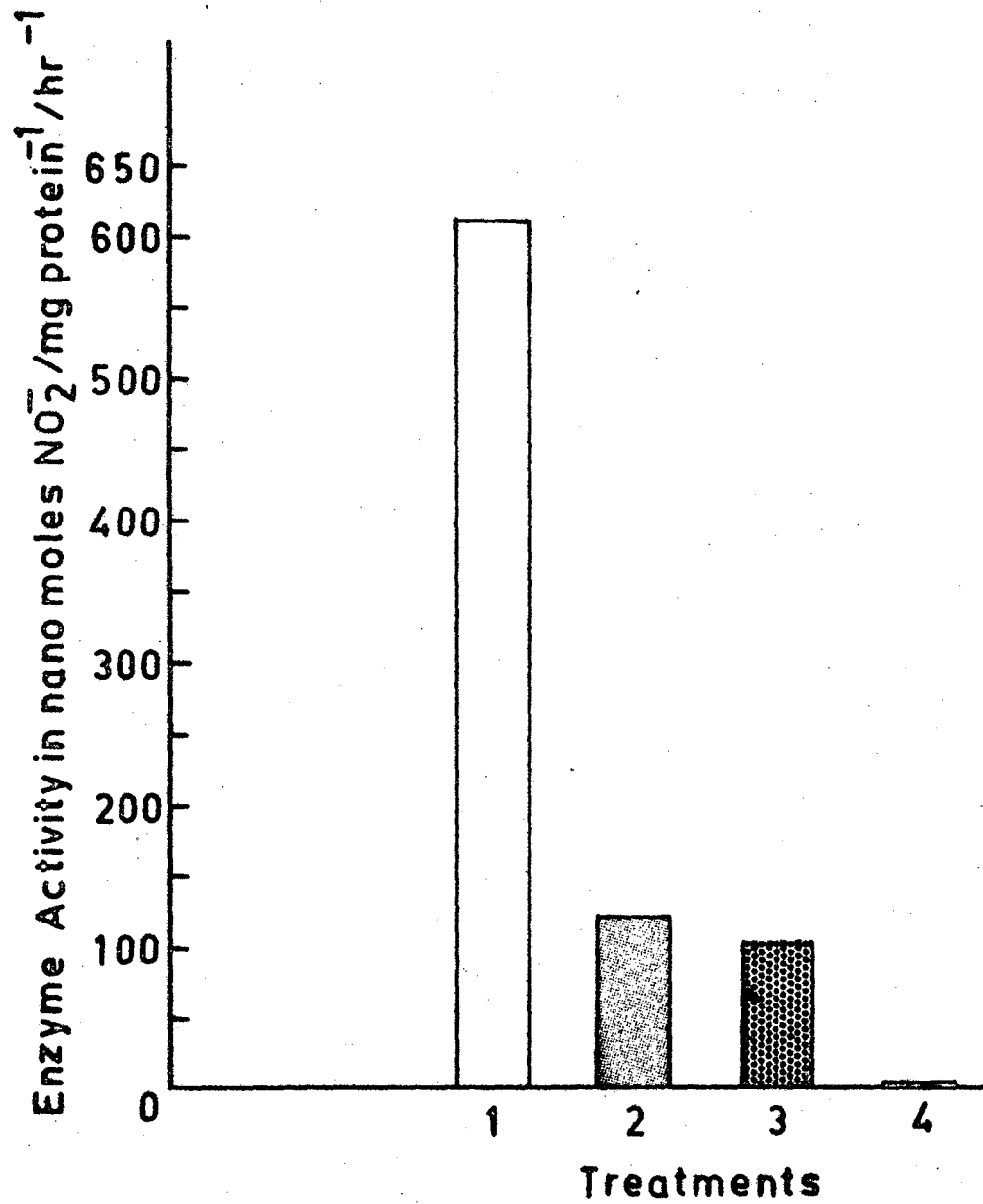
<u>Effect of Cycloheximide</u> - To find out the possible role of protein synthesis, etiolated, excised leaves were supplied with cycloheximide in the induction medium. The enzyme activity was measured after continuous far-red light irradiation for 4 hours. In one set cycloheximide was supplied for the first 2 hours and then transferred to inhibitor free induction medium for another 2 hours and vice versa for another set. Cycloheximide, a known inhibitor of protein synthesis, totally inhibited the nitrate reductase activity if it was supplied for the full 4 hour period (Fig. 12). However, even if it was supplied for any 2 hours in the 4 hour period, there was a significant decrease in the enzyme activity (Table 7).

DISCUSSION

Light plays an important regulatory role in the growth and development of plants. Since the development in biochemical terms is an orderly sequence of change in enzyme complement and modulation of activities of certain key enzymes, it becomes imperative to understand the mechanism of action of light at the enzyme level in order to decipher the relationship between light and development. The synthesis of a number of enzymes has been reported to be regulated by light, either directly or through the mediation of phytochrome (Schopfer, 1972). The mechanism by which phytochrome controls activity of enzyme is currently under intense debate (Mohr, 1972; Smith, 1975; Schopfer, 1977). In the present investigation, attempts have been made to find out whether the nitrate reductase activity in maize is phytochrome mediated and also to decipher the possible mechanism of action of phytochrome on the nitrate reductase activity.

When the excised, etiolated leaves of maize were exposed to a short treatment of red light, more than 350% enhancement in the nitrate reductase activity was observed (Table 1). However, far-red light irradiation of similar duration was found to be ineffective (Table 2). Red light has also been reported to enhance the nitrate reductase

activity in pea terminal buds (Jones and Sheard, 1972),
in mustard seedlings (Johnson, 1976) and in maize
seedlings (Duke and Duke, 1978). Besides, the nitrate
reductase, red light has also been reported to increase
activity of many other enzymes, for example, 33% increase
in activity was observed in ascorbate oxidase (Drumm et al.,
1972) after 24 hours of treatment, 162% of increase in
phenylalanine ammonia lyase after 4 hours of treatment
(Schopfer and Mohr, 1972) and 62% increase in peroxidase
activity after 24 hours of treatment (Sharma et al., 1976).

The established operational criterion for the
involvement of phytochrome in light mediated responses
required that an induction effected by red light can be
fully reversed by subsequent pulse of far-red light
(Mohr, 1972). In the present investigation, the data
obtained fulfilled this criterion for the involvement of
phytochrome in the modulation of nitrate reductase in
excised leaves of maize. Here, it was found that far-
red light negates the action of red light and vice versa,
an increase in nitrate reductase activity is a function
of quality of light to which the plants were last exposed
(Table 4). Analogous red and far-red reversible effect
has been shown for peroxidase enzyme in etiolated maize
shoots (Sharma et al., 1976).

In another set of experiments, where red light was given for 5, 10 and 15 minutes (Table 3), it was found that 5 minutes of irradiation was sufficient to saturate the photoresponse exerted by P_{fr}. By prolonging the duration to 15 minutes, the nitrate reductase activity declined. This drop in the nitrate reductase activity may be due to a stimulation of some secondary processes in the excised leaves which may have affected the nitrate reductase activity in one way or the other.

After 5 minutes exposure to red light, the nitrate reductase activity increased after a lag period of 30 minutes and reached optimum at 4 hours, following which the enzyme activity declined. The presence of lag phase before the increase in the enzyme activity points that the effect of phytochrome is not instaneous but requires building up of a potential to initiate the photoresponse. The existence of lag also rules out the possibility of direct photoactivation of enzyme as reported by Johnson (1976) in mustard seedlings. At 4 hours, the rate of enzyme synthesis could be greater than the rate of degradation, which can account for the maximum activity of nitrate reductase at 4 hours. After this period, the rate of degradation of enzyme could be greater than the rate of synthesis, due to which probably the enzyme activity declined.

Travis et al. (1970), Travis and Key (1971), suggested that light induced-increase in polyribosome level produce a non-specific effect on the rate of nitrate reductase synthesis. Pine and Klein (1972) have shown that polysome level is controlled by phytochrome in bean leaves. The increase in nitrate reductase activity by red, far-red light in maize leaves may be due to the increase in level of polysomes i.e. protein synthesis. Presence of lag phase also supports this idea in the present investigation.

An alternative way to confirm the involvement of phytochrome in a photoresponse is to demonstrate the 'High Irradiance Response' (HIR-Hartmann, 1966; Mohr, 1972; Schopfer and Mohr, 1972; Schafer, 1976; Rabino et al., 1977). The enhancement in the nitrate reductase activity in excised maize leaves irradiated with continuous far-red light, demonstrated the operation of HIR, confirming phytochrome involvement in the photoregulation of the nitrate reductase. Under continuous far-red light the nitrate reductase activity increased after a lag period of 30 minutes and reached some what steady state level at 4 hours (Fig. 9). Although the lag phase was similar in far-red light and dark treated materials, a marked difference in the enzyme activity was observed only after 2 hours. The presence of lag phase before the enzyme activity rules out the possibility of direct photoactivation of enzymes as has been reported for peroxidase in spinach (Panel and Greppin, 1973) and also in

membrane vesicles isolated from _Cucurbita_ pepo (Penel et al., 1976). Most of the phytochrome-mediated enhancement in enzyme activity is preceded by a lag phase e.g. 2 hours for inorganic pyrophosphatase in maize (Butler and Benett, 1969) and 3 hours for ascorbate oxidase (Drumm et al., 1972) and 3 hours for peroxidase (Sharma et al., 1976). Mohr (1972) assumed that the lag phase can be explained by the time necessary to make genes accessible to far-red irradiation, therefore, no lag phase can be detected in case of secondary irradiation. Huault (1974) has shown activation of inactive phenylalanine ammonia-lyase in cotyledons of _Raphanus sativus_, and suggests that this lag phase is related to the critical level of P_{tot}, which is about $27 \pm 3\%$ of maximum content of P_{tot} and the presence of lag phase is accounted only if P_{tot} content is below the critical level. The slow decrease in enzyme activity can be explained by two points. (i) In the later phase of far-red irradiation P_{fr} loses its effectiveness or (ii) this effect could be due to some inhibitory processes which are triggered by continuous far-red, which in turn affects the enzyme activity.

The termination of far-red light after 12 hours of irradiation brings about a decrease in the nitrate reductase activity although it is maintained at a higher level than the dark control. Since the termination of far-red leads to decline in P_{fr} level due to P_{fr} decay

(Butler et al., 1963), one can correlate enhancement in
the nitrate reductase activity and presence of P_{fr} in the
enzyme. In other words, enhancement of the nitrate
reductase activity requires continuous presence of P_{fr}
in the system.

The exogenous supply of acetylcholine enhanced the
nitrate reductase activity in dark when given for prolonged
period, whereas, if it was supplied for a short period,
it was ineffective (Table 5). The magnitude of enhancement
was, however, always less than that obtained by 5 minutes
of red light or continuous far-red light. The involvement
of ACh in phytochrome mediated photoresponse can be
unequivocally considered only if it satisfies the following
criteria:

1. Exogenously supplied ACh should mimic the action of
   phytochrome and kinetics of response induced by it
   should be similar to the kinetics of photoresponse.

2. The inhibitors which are known to affect the ACh level
   or counter their action should nullify the phytochrome
   response.

In the present investigation, we have not studied ACh
action elaborately. So although ACh enhanced the nitrate
reductase activity in dark, it cannot be considered as a
mediator in phytochrome response unless it follows the
above points.

One obvious prediction of differential gene activation hypothesis is de novo synthesis of enzyme molecules in positive photoresponse under the influence of P_{fr}. Evidence for this has come mainly from the use of the inhibitors of transcription (Carr and Reid, 1966; Mohr and Bienger, 1967; Dittes et al., 1971; Drumm et al., 1971) and translation (Drumm et al., 1971; 1972). These inhibitors either reduce or prevent the phytochrome effect. In the present investigation, actinomycin D and cycloheximide were used. If actinomycin D was supplied continuously for 4 hours the time required to get the maximum activity, it inhibited the nitrate reductase activity by 93%. However, if actinomycin D was supplied after 2 hours after the on set of far-red irradiance, there was no significant decrease in the enzyme activity. If it was supplied for the first 2 hours, the enzyme activity was inhibited by 40%. This showed that RNA synthesis is necessary for the induction of the enzyme essentially takes place in the first 2 hours in the 4 hour period of enzyme synthesis.

Cycloheximide, a known inhibitor of protein synthesis completely inhibited the enzyme activity if it was supplied for the complete 4 hours. If it was supplied for 2 hours (either first 2 hours or last 2 hours), 80% of inhibition of the enzyme activity was observed. This indicates that

the enzyme was synthesized on cytoplasmic ribosomes.

Inhibition of enzyme activity with actinomycin D and cycloheximide gives an idea that increase in nitrate reductase activity under continuous far-red light is due to _de novo_ synthesis of enzyme. The use of inhibitors although suggest possible involvement of RNA and protein synthesis, but no means provides a proof that such a mechanism is operative _in vivo_. Infact, cycloheximide shows too many side effects _in vivo_ .e.g. inhibition of ion uptake, respiration (Ellis and MacDonald, 1970) and many other metabolic processes (Mc Mahon, 1975), to justify its direct role in protein synthesis.

SUMMARY

The nitrate reductase activity in Zea mays is regulated by phytochrome (P_{fr}). A brief irradiation of red light to excised leaves lead to enhancement in the nitrate reductase activity in excised leaves, whereas the similar period of irradiation with far-red light was found to be ineffective. Phytochrome participation in this photoresponse was established by reversibility of red light effect by far-red light. The kinetics of the nitrate reductase activity was followed after irradiation of red light for 5 minutes and under continuous far-red light, where phytochrome operates through "high irradiance reaction". The enhancement in nitrate reductase activity was preceded by 30 minutes lag and reached maximum at 4 hours in both the cases. The kinetics of enhancement in nitrate reductase activity was essentially that of photomodulation, as it depended on continuous presence of P_{fr} in the system. Exgenously supplied acetylcholine enhanced nitrate reductase activity in dark.

The role of transcription and translation in phytochrome mediated enhancement was also investigated. Cycloheximide, an inhibitor of protein synthesis in cytoplasm and actinomycin D, an inhibitor of RNA synthesis repressed the enhancement of nitrate reductase activity

by 100% and 93% respectively. These results point
that that phytochrome possibly regulates nitrate
reductase activity at transcriptional and trans-
lational levels.

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