REGULATION OF NITRATE REDUCTASE ACTIVITY IN RICE (*ORYZA SATIVA* L.) BY GROWTH REGULATORS

REGULÁCIA AKTIVITY NITRÁTREDUKTÁZY U RYŽE (*ORYZA SATIVA* L.) RASTOVÝMI REGULÁTORMI

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ABSTRACT

The effect of three growth regulators, namely kinetin, 6 benzyl adenine, 2 chloro ethyl trimethyl ammonium chloride at three concentrations (10-6 M, 5 x 10-5 M 10-4 M) was studied on the catalytic activity of nitrate reductase in green and etiolated seedlings. A concentration of 5 x 10-5 M was optimal for all the growth regulators treatments. All the growth regulators stimulated nitrate reductase activity effectively at 5 x 10-5M concentration in both etiolated and green seedlings and had an additive effect when supplemented by NO-3 up to 140% to 160%. The 99.2% and 93.4% inhibition of nitrate reductase activity resulted in development of etiolated and green seedlings, respectively when treated with eukaryotic 80S ribosome protein synthesis inhibitor cycloheximide. Prokaryotic 70S inhibitor chloromphenicol did not have any effect on measured parameters. Actinomycin D, a RNA synthesis inhibitor also inhibited the enzyme activity as 80s inhibitors (Green 80%, etiolated 98%). One may suggest from this that both DNA and protein synthesis are involved in the induction of nitrate reductase activity. The differential effect of aminoacids was observed on enzyme activity in combination with growth regulators.

KEY WORDS: kinetin, 6 benzyl adenine, 2 chloro ethyl trimethyl ammonium chloride, protein synthesis inhibitor, RNA synthesis inhibitor

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INTRODUCTION

The first step in nitrate assimilatory pathway is the reduction of NO^-_3 to NO^-_2 catalyzed by the enzyme nitrate reductase, which is a rate-limiting step that regulates the inflow of inorganic nitrogen (NO^-_3) to organic form in plants [3]. Several studies indicated that the level of nitrate reductase in plant tissues is altered by exogenous application of phytohormones. Foliar spray of tobacco seedlings with either cytokinin alone or in combination with giberellic acid enabled the leaves to synthesize nitrate reductase in complete darkness [9]. Benzyladenine causes a marked increase in the activity of nitrate reductase in embryos of *Agrostemma githago* even when these are germinated in nitrate free medium [1]. Inhibitors of RNA and protein synthesis obviated the effect thus indicating that benzyladenine mediated response was dependent on transcriptional and translational processes [22]. In general, auxins have little effect on nitrate reductase, but much lower activity is recovered in tissues treated with abscisic acid. Requirements of light could be met to certain extent by providing appropriate concentration of cytokinin in dark [1] [16] Applied kinetin and 6-benzyladenine to dark grown cotton seedlings and observed 90 % increase in activity when treated with kinetin and 70 % increase when treated with 6BA in dark grown seedlings. The induction by cytokinins is usually higher in the presence of nitrate than its absence [4], although the two inducers do not seem to be acting synergitically. The plant hormone abscisic acid represses nitrate reductase gene expression in barley, which is partially recovered by the addition of equal concentration of benzyl adenine [20]. Kinetin increases nitrate reductase activity by increasing synthesis and activation of the enzyme [30, 5]. [27] suggested that nitrogenous base adenine or adenosine have no effect on nitrate reductase activity. The exact mechanism of induction of transcription by cytokinin is warranted [15] reported that in higher plants, especially in leaves the nitrate reductase is regulated by hormones and nitrogen supplies. Cytosolic enzymes are regulated by hormones [21]. Hence the present study has been aimed to study the effect of growth regulators such as kinetin,6-benzyl adenine and 2-chloro ethyl trimethyl ammonium chloride on the activity of cytosolic enzyme nitrate reductase *in vivo* and nitrate reductase enzyme protein in paddy seedlings grown under light and dark conditions [11]. To study the involvement and effect of transcription and translation in the presence of growth regulators the protein synthesis and RNA synthesis inhibitors were used [12]. The different effect of aminoacids were studied as described by [11] previously and the most effective three aminoacids, such as cysteine, glutamine and glycine were selected for the present study to see the effect of these aminoacids in combination with growth regulators.

MATERIALS AND METHODS

The paddy seeds (*Oryza sativa* L. cv. ADT 42) used in the current investigation were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore and from Rice Research Station, Aduthurai, India.

The seeds were washed with distilled water and then the seed surface sterilized with mercuric chloride for one minute and again washed with distilled water and used for sowing. For the present study, seedlings were raised in petriplates. The seeds were grown in petridishes covered with three layers of filter paper and moistened with a half strength Hoagland solution. Twenty five seedlings were sown in each petriplate and the growth regulators, such as kinetin, 6 benzyl adenine, 2 chloro ethyl trimethyl ammonium chloride were applied to the plants at three concentrations (10^{-6} M, 5 x 10^{-5} M 10^{-4} M) after ten days of emergence through irrigation as well as through foliar spraying on alternate days upto 21st day [12].

To study the effect of light and dark some plants were grown under dark conditions and the growth regulators were applied similarly to plants grown under light conditions. On 21st day after planting, 10 mM KNO_3 was added to the medium to see the effect on nitrate reductase activity and the leaves were harvested after 24 hours of NR induction by nitrate. To study the effect on protein synthesis, following inhibitors, such as cycloheximide (80S inhibitor) and chloromphenicol (70S inhibitor) and the RNA synthesis inhibitor (actinomycin D) were applied so that the seedlings were transferred to the medium contained these protein and RNA synthesis inhibitors in concentrations 50 $\mu g.ml^{-1}$.

To study the effect of aminoacids in combination with growth regulators, the aminoacids, such as cysteine, glycine and glutamine (10 mM) were added to the medium. The leaves were harvested from the control and treated seedlings [16]. After the nitrate reductase enzyme assay [7], purification of the nitrate reductase enzyme [7], activity staining the purified enzyme protein [25] and western blot analysis [19, 23] of the purified protein were done.

RESULTS AND DISCUSSION

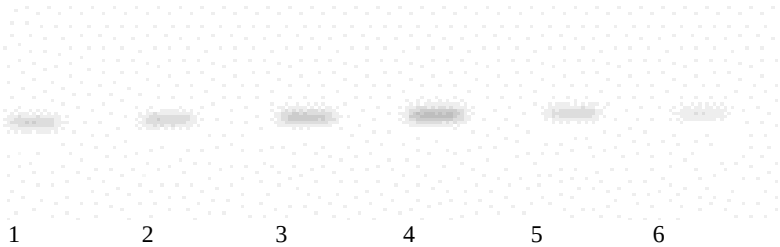
The effect of three growth regulators, namely kinetin, 6 benzyl adenine, 2 chloroethyl trimethyl ammonium chloride at three concentrations (10^{-6} M, 5 x 10^{-5} M 10^{-4} M) was studied on the catalytic activity of nitrate reductase in green and etiolated seedlings. A concentration of 5 x 10^{-5} M was the most optimal for all the growth regulators treatment (table 1 and figure 1).

Table 1: Effect of growth regulators on nitrate reductase activity under light and dark conditions

Growth regulators	Nitrate reductase activity (µmol NO_2^- produced mg^{-1} protein hr^{-1})			
	-KNO_3		+KNO_3	
	Light	Dark	Light	Dark
Control	2.5	1.5	2.5	1.5
Kinetin				
10^{-6}	3.4	1.9	3.9	2.9
5 x 10^{-5}	3.8	2.2	4.1	3.1
10^{-4}	3.6	2.0	3.9	3.0
6BA				
10^{-6}	3.2	1.8	3.9	2.8
5 x 10^{-5}	3.7	2.1	4.0	3.0
10^{-4}	3.4	1.9	3.9	2.9
CCC				
10^{-6}	3.2	1.4	3.6	2.7
5 x 10^{-5}	3.5	1.6	3.8	2.8
10^{-4}	3.3	1.5	3.7	2.7

Kinetin 6BA and CCC at 3 concentrations (10^{-6}M, 5 x 10^{-5} M, 10^{-4} M) were added to see their effect on nitrate reductase activity in the presence and absence of KNO_3 (25mM) in green and etiolated leaves. Values expressed are mean of 3 separate experiments.

Figure 1:Western blot analysis of nitrate reductase protein isolated from the leaves grown under light and dark conditions and treated with growth regulators at 5 x 10^{-5} M concentration



1-NR enzyme protein isolated form seedlings grown under dark conditions treated with kinetin
2- NR enzyme protein isolated form seedlings grown under light conditions treated with kinetin
3- NR enzyme protein isolated form seedlings grown under dark conditions treated with 6 benzyl adenine
4- NR enzyme protein isolated form seedlings grown under light conditions treated with 6benzyl adenine
5- NR enzyme protein isolated form seedlings grown under dark conditions treated with CCC
6-NR enzyme protein isolated form seedlings grown under light conditions treated with CCC

The stimulatory effect was higher in seedlings treated with kinetin at 5 x 10^{-5} M concentration in green (3,8 µmol NO^{-}_{2} produced.mg^{-1} protein hr^{-1}) and etiolated (2,2 µmol NO_2 produced.mg^{-1} protein per hour) seedlings.

When the seedlings were supplied with KNO_3, an additive effect was observed and the efficiency of the enzyme activity markedly increased (green – 4,1 µ mol NO_2^- produced mg^{-1} protein.hr^{-1}; etiolated – 3,1 µ mol NO_2^- produced mg^{-1} protein.hr^{-1}). All the growth regulators caused a stimulated nitrate reductase activity in most effective way at 5 x 10^{-5} M concentration in both etiolated and green seedlings, and they had an additive effect when supplemented with NO_3^- up to 140 % to 160 %, although the magnitude of increase depends upon the species and concentration and nature of cytokinin [29]. Exogenous supply of nitrate elevated cytokinin zeatin riboside level in barley [27], revealing additive effect of KNO_3. It may be suggested that kinetin-mediated increase of nitrate reductase activity is the result of both synthesis and activation of the enzyme [5].

6BA treatment enhanced polyamine promoting nitrate reductase activity [8] as a result of increased endogenous benzyl adenine level. Growth regulators also increase the sugar content to bring about enhancement of nitrate reductase activity [2]. It is envisaged that increased carbohydrate supply results in the increased reductant and c-skeletons for promoting enzyme activity [24]. The 99,2 % and 93,4 % inhibition of nitrate reductase activity resulted in development of etiolated and green seedlings, respectively in case they were treated with eukaryotic 80S ribosome protein synthesis inhibitor cycloheximide (table 2).

Table 2: Effect of protein and RNA synthesis inhibitors on nitrate reductase activity in combination with growth regulators in leaves grown under light and dark

Growth regulators	Nitrate reductase activity (µ mol NO_2^- produced mg^{-1} protein hr^{-1})					
	Cycloheximide		Actinomycin D		Chloramphenicol	
	Light	Dark	Light	Dark	Light	Dark
Control	2.5	1.5	2.5	1.5	2.5	1.5
Kinetin						
10^{-6}	1.8	0.4	1.0	0.4	2.0	1.1
5 x 10^{-5}	1.2	0.7	1.1	0.7	2.2	1.2
10^{-4}	1.1	0.6	1.0	0.6	2.0	1.1
6BA						
10^{-6}	0.8	0.3	0.8	0.4	2.0	1.0
5 x 10^{-5}	1.0	0.5	1.0	0.6	2.2	1.1
10^{-4}	0.9	0.3	0.8	0.4	2.1	1.0
CCC						
10^{-6}	0.5	0.3	0.6	0.3	1.8	0.8
5 x 10^{-5}	0.7	0.5	0.8	0.5	2.0	1.0
10^{-4}	0.5	0.3	0.6	0.4	1.8	1.8

Kinetin, 6BA and CCC at 3 concentrations (10-6 M, 5 x 10-5, 10-4 M) were added. In addition to that cycloheximide, actinomycin D and chloramphenicol (50 µg ml-1) were added to see their effect in combination with growth regulators.

Values expressed are mean of 3 separate experiments.

Prokaryotic 70S inhibitor chloromphenicol did not have any effect, allowing to infer that nitrate reductase is synthesized in the leaf cell cytoplasm. The inhibition of cytoplasmic protein synthesis by cycloheximide resulted in decrease in nitrate reductase activity in maize by more than 85 %. However, it has been reported that nitrate reductase transcript accumulation was not affected by cycloheximide [26]. Hence, the inhibition by 80S inhibitor on enzyme activity on ion absorption is probably mediated via interference with energy and transfer and oxidative phosphorylation [21]. Chloramphenicol inhibits translation in ribosomes of mitochondria and plastids, but does not affect that in

cytosolic 80s ribosomes [13]. By contrast, cycloheximide inhibits translation of cytosolic ribosomes [22]. Actionmycin D, a RNA synthesis inhibitor also inhibited (table 3) the enzyme activity as 80S inhibitors (green 80 % etiolated 98 %). This suggested that both DNA and protein synthesis are involved in the induction of nitrate reductase activity. The accelerated nitrate uptake appears to be substrate induced and influences both DNA translation and transcription which is sensitive to respective inhibitors [14].

Table 3: Effect of aminoacids on nitrate reductase activity under light and dark conditions treated with growth regulators

Growth regulators	Nitrate reductase activity (µ mol NO_2^- produced μg^{-1} protein hr^{-1})					
	Glutamine		Glycine		Cysteine	
	Light	Dark	Light	Dark	Light	Dark
Control	2.5	1.5	2.5	1.5	2.5	1.5
Kinetin						
10^{-6}	1.2	0.3	4.4	2.6	4.9	3.0
5×10^{-5}	1.5	0.5	4.5	2.8	5.2	3.3
10^{-4}	1.3	0.4	4.4	2.5	5.0	3.0
6BA						
10^{-6}	1.1	0.4	4.3	2.5	5.0	3.1
5×10^{-5}	1.3	0.4	4.4	2.7	5.0	3.2
10^{-4}	1.1	0.3	4.3	2.6	5.0	3.1
CCC						
10^{-6}	1.0	0.4	4.0	2.4	5.8	3.0
5×10^{-5}	1.1	0.4	4.2	2.6	5.9	3.1
10^{-4}	1.0	0.3	4.0	2.3	5.8	3.0

Kinetin, 6BA and CCC at 3 concentrations (10^{-6} M, 5×10^{-5} M, 10^{-4} M) were added. In addition to that glutamine, glycine and cysteine (10mM) were added to see their effect in combination with growth regulators. Values expressed are mean of 3 separate experiments.

It has been reported that 6 methyl purine, another RNA synthesis inhibitor also prevented nitrate reductase activity (83 %) [17]. The effect of aminoacids, namely glutamine, glycine and cysteine at 10 mM concentrations were studied in combination with growth regulators on nitrate reductase activity. Glutamine applied with CCC inhibited the activity maximum under green (1,0 µmol NO_2^- produce d mg^{-1} protein.hr^{-1}) and etiolated (0,1 µ mol NO_2^- produced mg^{-1} protein.hr^{-1}) conditions. High levels of glutamine resulted in inhibition of nitrate reductase activity and mRNA levels in leaf tissues [31, 6]. On the other hand, glycine (4,5 µmol NO_2^- produced mg^{-1} protein.hr^{-1} – green ; 2,8 µmol NO_2^- produced mg^{-1} protein.hr^{-1} – etiolated) and cysteine (5,2 µ mol NO_2^- produced mg^1 protein.hr^{-1} – green ; 3,3 µmol NO_2^- produced mg^{-1} protein.hr $-^1$ - etiolated) in combination with kinetin enhanced the activity maximum at 5 x 10-5 M concentration compared to other treatments (table 3). Thiol compounds and certain amino acids act to stabilize the tight form of the enzyme leading to increase in enzymic activity [17]. Cysteine contains thiol group and results in increased nitrate reductase activity. Kumar et al. (1988) observed the induction of nitrate reductase by glycine and concluded that this increment may be due to stabilization or increased supply of reducing power to the enzyme. For this an optimum concentration of 10 mM was suggested [28]. The differential effect of amino acids on nitrate reductase activity may be due to different functional groups present in different amino acids.

CONCLUSION

The growth regulators stimulated activity of the nitrate reductase enzyme even in the absence of light. Interestingly, an additive effect was observed when nitrate was supplied in the presence of growth regulators. Eventhough, the enzyme activity was stimulated by growth regulators in the presence of 80S protein synthesis inhibitor cycloheximide, RNA synthesis inhibitor actinomycin D, activity of the enzyme was inhibited, and this confirmed that the

location of the enzyme is in the cytosol and the enzyme activation is regulated at the level of transcription and translation. As observed in the previous study [12] here also the inhibitory effect of glutamine was observed even in the presence of growth regulators. The stimulatory effect of glycine and cysteine may be due to the supply of reducing power and stabilizing the tight form of the enzyme.

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