DISCUSSION

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Polyamines have since long been known to be associated with growth processes in a wide variety of living organisms ranging from bacteriophages to animal systems (Bachrach, 1973). The level of polyamines is found to be elevated in stages of rapid growth, such as in tissues undergoing rapid cell proliferation (Ham, 1964), exponential growth phase of microorganisms (Cohen, 1971) and during the germination of seeds (Villanueva et al., 1978). Addition of exogenous polyamines leads to an increase in growth, as observed in Helianthus tubers (Cocucci and Bagni, 1968), wheat (Smith, 1971) and rice (Sen, 1983). In the present study, a similar enhancement of growth was obtained with polyamines both in light and dark grown radish seeds throughout the course of germination.

Polyamines have been suggested to serve as the sole source of nitrogen for explants of Helianthus tubers (Bagni, 1979). It may be possible that in the case of radish cotyledons also they may contribute as a nitrogen source. However, a similar increase in growth was observed even when the seeds were grown in the presence of nitrate. This increase by the compounds was over and above that brought about by nitrate alone. Also, the compounds increased growth in ammonium treated seeds as well, though ammonium

alone did not have any effect. Further, unlike nitrate or ammonium, polyamines and guanidines alone did not increase the level of GDH and GS, as would have been the case if they acted as a nitrogen source. Thus, it seems unlikely that they may enhance the growth by serving as a nitrogen source.

Polyamines have been suggested to play an important role in the growth processes of organisms and much of the current evidence supports this suggestion (Bagni and Serafini-Fracassini, 1974). At concentrations between 10-100 μM, they have been suggested to act as growth factors for plants. The growth of dormant tissues of Helianthus tuber explants could be initiated by the addition of a polyamine. This effect of polyamines was found to be similar to that observed on application of a growth hormone, NAA (napthyl acetic acid). Moreover, the application of IAA led to an increase in the level of polyamines (Bagni, 1966). Subsequent studies have shown that polyamine biosynthesis and content in the plant tissue rises following the application of other plant growth hormones like auxin (Bagni et al., 1981 a), gibberellin (Dai et al., 1982) and cytokinin (Suresh et al., 1978).

Similar relation of hormones and polyamines biosynthesis has also been observed in other systems

(Bachrach, 1973). Blockage of polyamine synthesis by various inhibitors abolishes the effect of the hormones as seen in auxin treated Helianthus tuber tissue (Bagni and Serafini-Fracassini, 1974). Application of canavanine and canaline, analogues of arginine, leads to an irreversible inhibition of auxin-stimulated growth of the explants. Thus, the action of hormones appears to be mediated via polyamines which may be termed as 'secondary messengers' analogous in their role to c-AMP. A recent report of polyamine-activated nuclear protein kinase that phosphorylates nonhistone protein (Atmar, et al., 1978) supports this view.

During germination of seeds, hormones including gibberellic acid have been found to increase (Yomo and Jinuma, 1966). Various dormancy breaking factors like light or chilling result in an increase in the amount of these growth regulators suggesting that germination may result from it. Similarly, exogenous application of certain growth regulators results in promotion of seed germination (Khan, 1979). A similar increase in the rate of germination by the application of polyamines to rice seeds has been observed (Sen, 1982). Endogenous polyamine content is known to rise during germination in pea and Phaseolus seeds (Villanueva et al., 1978).

These observations suggest that polyamines may play a growth regulatory role during germination and growth of the embryo.

Guanidines are structurally related to polyamines and have been shown to act antagonistically to polyamines in cress, barley and oat seedlings (Srivastava and Smith, 1982 a, b). They have also been found to reverse the effect of polyamines on membrane bound peroxidase (Srivastava and Rajbabu, 1983 a). However, in certain cases they have been shown to act in a manner similar to polyamines. Studies on senescing barley leaf have shown that both spermine and dodine retarded senescence (Srivastava et al., 1983) and also activated membrane bound ATPase in maize scutellum (Srivastava and Rajbabu, 1983 b). In the present study on radish seeds.also guanidines had an effect similar to polyamines, bringing about an increase in growth in seeds grown in light as well as dark.

Increase in growth by polyamines and guanidines suggested that they may do so by affecting the enzymes of either nitrogen assimilation or reserve mobilization in the cotyledons. However, the compounds inhibited nitrate reductase activity in light grown seeds. Both polyamines and guanidines inhibited only the light mediated increase in NR activity since the level of the inhibited enzymes was same as that in dark grown seeds.

NR is an inducible enzyme and a regulatory step in nitrate assimilation. Light is known to enhance its activity in a number of ways. It is suggested that light enhances the uptake of nitrate (Sasakawa and Yamamoto, 1979) in the cell and redistributes the nitrate present within the cell so that more of it is available to the enzyme (Aslam et al., 1976). Light has been proposed to act specifically on NR via phytochrome, since the effect of light was found to be red/far-red reversible (Jones and Sheard, 1975). Phytochrome modulated responses are believed to be due to an association of the phytochrome with cell membranes, resulting into an alteration of membrane permeability (Smith, 1974). Phytochrome may thus enhance nitrate reduction by altering the compartmentation of nitrate in the cells (Wagner, 1975) and regulating the nitrate flux among intracellular nitrate compartments. Recent studies in our laboratory (Srivastava and Rajbabu, 1984) have shown that polyamines reversed the effect of red light mediated increase in peroxidase activity in maize scutella. However, the effect of the compounds was observed only on the increase in membrane bound peroxidase activity, while the increase in cytosolic enzyme by the light was not reversed, suggesting that polyamines act by interacting with membranes. Moreover, the effect of phytochrome on the enzyme activity was observed only in the presence of embryo. The phytochrome

response thus appears to be perceived by the embryo and transmitted to the scutellum to elicit its response on the enzyme.

In the present study also, the compounds appear to inhibit only the light-mediated increase in NR activity, while there was no effect in dark grown seedlings. In addition to these specific effects on nitrate uptake and assimilation, light may also indirectly enhance nitrate reduction by bringing about an increase in polysome content and hence protein synthesis (Travis and Key, 1971) or by increasing the energy charge by photosynthesis (Klepper et al., 1971). Light has also been proposed to regulate NR level by affecting the conformation of the membrane on which the subunits of NR are subject to assembly and disassembly (Hewitt et al., 1979). Polyamines and guanidines may probably counteract with one or more of these effects caused by light leading to an inhibition of NR activity in light grown seeds.

Both the groups of compounds are known to affect membrane stability and function and it was possible that they affected the uptake of nitrate by the tissue and hence NR activity. However, the nitrate content of seeds grown in nitrate in the absence or presence of spermine did not differ significantly suggesting that the compounds did not

inhibit NR activity by inhibiting the uptake of exogenous nitrate. Also the compounds had no effect on the enzyme activity when added to the assay *in vitro*.

To investigate the mechanism of action of these compounds, the effect of polyamines and guanidines was tested during short term induction experiments using excised cotyledons of seedlings grown in the absence of a nitrogen source. Putrescine, which was found effective during germination, did not show any effect during induction. Polyamines and guanidines inhibited NR induction in both light as well as dark grown seeds, in contrast to their effect during germination. Since all polyamines and guanidines appeared to have a similar effect on NR activity, further experiments were carried out using spermine and GAA as representative test compounds of the two groups.

As observed earlier during germination, spermine did not affect the uptake of nitrate even during the induction studies. However, the metabolic pool of nitrate was decreased by 50% in the presence of spermine probably leading to a decreased NR level. The concept of two separate pools for nitrate within the cell was proposed by Ferrari *et al.* (1973). They suggested that most of the nitrate taken up by the cell is stored in a pool unavailable for reduction, termed as storage pool, while a smaller pool called the

metabolic pool is available to the enzyme and is responsible for the induction of the enzyme. However, Aslam (1981) reported that the cessation of nitrite production may be due to the leakage and hence exhaustion of NO_3^- from the cell into the medium. Recently, Hog et al. (1983) have shown that the sizes of the respective pools are highly dependent upon the oxygen tension in the assay, as well as the extent of leakage of nitrate from the cell. They suggested that the exact assessment of the two pools is difficult. However, Granstedt and Huffaker (1982) and Martinoia et al. (1981) have conclusively shown that in leaf protoplasts vacuole may serve as the major site for nitrate accumulation and as much as 58-99% of the nitrate may be present in the vacuole. Granstedt and Huffaker (1982) suggested that the proportion of nitrate in the vacuole may be affected by several factors. Light effectively releases nitrate from the storage pool. When external supply of nitrate is stopped the nitrate not reduced is accumulated in the vacuole with time. Thus, the accumulation of nitrate in the storage pool of vacuole is a function of the nitrate uptake and the rate of nitrate reduction. Since polyamines and guanidines do not affect the uptake of nitrate, the lowered metabolic pool as measured by the in vivo nitrate reduction may reflect an inhibited NR activity in spermine treated tissue leading to an increased accumulation of

nitrate in the tissue. This view is supported by the observation that tungstate, a specific inhibitor of NR also leads to a similar accumulation of nitrate in the tissue. Since the compounds did not affect the enzyme activity when added to the assay *in vitro*, they may be affecting the synthesis and/or breakdown of NR.

The effect of spermine on the induction of NR activity was compared with that of cycloheximide. The maximum inhibitory effect of spermine was observed when it was added at the start of induction and there was no effect when added at later stages. A similar pattern was obtained with cycloheximide suggesting that spermine probably interferes with the *de novo* synthesis of NR.

Nitrate reductase in addition to reducing nitrate, also shows diaphorase activity *in vitro* involving the reduction of artificial electron acceptors such as 2,6-dichlorophenol indophenol, ferricyanide and cytochrome c (Hewitt, 1975) as well as quinone analogues like dibromothymoquinone (Jawali *et al.*, 1979). It also reduces *in vitro* nitrate to nitrite utilizing artificial electron donors like reduced methyl viologen or FMN. The two components showing partial activities are presumed to be linked by cytochrome b_{557} (Hewitt *et al.*, 1976). Several compounds have been reported to affect the partial activities differentially. Cyanide

and tungsten inhibit only the methyl-viologen-NR, while cytochrome c reductase activity is not affected (Hewitt, 1975; Hewitt et al., 1979). Sulfhydryl reagents however, inhibit cytochrome c reductase but not methyl viologen-NR (Wray and Filner, 1970). Hydroxylamine is found to affect neither the diaphorase nor the methyl viologen-NR activity but interacts with reduced cytochrome b_{557} (Jawali et al., 1978; Jawali and Sane, 1984). Also, plants grown in the absence of molybdenum were found to synthesize the diaphorase component but not the molybdenum containing component (Hewitt et al., 1979). In the present study, spermine however, appeared to inhibit both the partial activities, suggesting that the synthesis of the whole NR complex was inhibited.

Polyamines have been earlier reported to promote protein synthesis, by stabilizing ribosomes (Hardy and Turncock, 1971), nucleic acids (Ames and Dubin, 1960) and accelerating almost every step in protein biosynthesis (Bachrach, 1973). However, recently polyamines have been implicated in inhibiting macromolecular synthesis (Apelbaum et al., 1982). It was proposed to be a mechanism whereby polyamines prevented ethylene biosynthesis and hence senescence of the apple tissue. There is also a report where polyamines as well as putrescine and cadaverine caused a partial inhibition of incorporation of ^{3}H -

leucine into proteins in rat hepatocytes (Auberger et al., 1983), suggesting a major regulatory mechanism.

The effect of the compounds was also studied on the breakdown of NR during storage, since the NR activity obtained is a result of the balance between the synthesis and degradation processes. NR was found to be more stable in vivo, during the storage of intact cotyledons, than the homogenate. Thus, at the end of 6 hr 82% of the original activity was retained in the cotyledons stored in dark as opposed to 47% in the homogenate. The faster decay observed in the homogenate may be a result of the destruction of the compartmentalization in intact tissue, resulting in a degradation of NR by a specific NR inactivating system or by proteases within the cell. Light brought about an increase in activity upto 2 hours in the intact cotyledons during storage. After this the activity declined, the rate of decline being same as that in the cotyledons stored in the dark. The increase by light was not observed in the stored homogenate of light induced NR probably due to the disruption of organelles and protein synthesizing machinery during homogenization.

Spermine did not alter the rate of decay in the homogenate from the cotyledons treated with spermine either in light or dark or when the spermine was added to the

homogenate during storage. However, when added during storage of intact cotyledons the level of NR in spermine treated tissue was less as compared to the control at the end of 10 hr of storage. Light did not increase the activity in the control tissue and lower level of enzyme was observed in both light and dark. However, the rate of decline of the enzyme level did not differ significantly in the control and spermine treated tissue. This suggests that spermine affects the synthesis but not the breakdown of NR. The fact that the effect of spermine is not observed in the homogenate supports this possibility. Spermine is found to increase protease activity during induction as well as storage as was seen earlier during the germination of seeds and this may also contribute further to the lower level of NR observed in the presence of spermine. The NR inactivating system may also contribute to the decay of the enzyme. An inhibitor of NR has been earlier reported in radish cotyledons (Stulen et al., 1971). It was noncompetitive with respect to nitrate and was phenolic in nature. In the present study, the inhibitor detected was a dialyzable, heat-stable moiety. The inhibitory effect was increased when the inhibitor was pretreated with NADH and was reversed when pretreated with ferricyanide. The inhibitor possibly inhibits NR by reducing it to an inactive form and its inhibitory effect is abolished when oxidized by ferricyanide.

The increased inhibition in presence of NADH may be due to increased level of reduced inhibitor. NR is known to be present in oxidized and reduced forms which are inter-convertible. The active form corresponds to the oxidized state of the enzyme which is converted into an inactive form when reduced. A reversible inactivation of nitrate reductase through a redox mechanism has been demonstrated in spinach (Relimpio et al., 1971), maize scutella (Wallace, 1975) and maize leaf (Echevarria et al., 1984). In contrast, there are reports where NADH exerted a protective and/or stimulatory effect on nitrate reductase from rice (Gandhi et al., 1973), wheat and maize (Datta et al., 1983). In these tissues it is proposed that the reduced form of the enzyme is the active form. In the present study, the oxidized form of the enzyme appears to be the active form. The inhibitor does not seem to be a phenolic compound since extraction of the enzyme in the presence of PVP led to a decrease rather than an increase in NR activity. Spermine however, did not affect the NR inactivating system.

Polyamines and guanidines also inhibited the light-mediated increase in GS activity. However, they had no effect when added to the assay in vitro. The activity of GS is found to increase during germination of seeds. It is proposed to play a key role in the assimilation of

ammonia into glutamine due to its low Km for ammonia. The enzyme is suggested to be located in the cytoplasm. However, the GS previously absent in the proplastids is known to rise in chloroplasts on illumination. The enzyme is absent in etiolated tissues. Chloroplastic GS is suggested to be involved in the reassimilation of ammonia released during the photorespiratory cycle (Nishimura et al., 1982). It is possible that polyamines inhibited the rise in the chloroplastic GS and hence inhibited only the light mediated increase in GS activity. Subcellular fractionation of the enzyme showed that the compounds did not affect the cytoplasmic GS and only the chloroplastic GS was inhibited.

Earlier reports have correlated increasing nitrate assimilation with increasing growth. However, from the above studies, it does not appear that the increase in growth obtained with polyamines and guanidines is associated with an increase in nitrogen assimilation since the compounds decreased the activity of the two key enzymes, NR and GS.

The compounds were, however, found to increase protease and AAT activity. The activity of protease is generally low in dry seeds, which increases with the start of imbibition and remains high during the early period of germination (Chrispeels and Boulter, 1975). The increase

in activity obtained may result from activation of the enzyme. The development of protease in the present study was found to differ with the nitrogen source supplied. The level of protease was high during the early period of germination when seeds were grown in the absence of a nitrogen source or in nitrate. Ammonium grown seeds, however, had a lower protease activity till the 2nd day of germination which increased by 3rd day. Similar pattern of enzyme activity was obtained in the presence of polyamines and guanidines. The increase in activity by the compounds was apparent during the early periods when seeds were grown in the presence of nitrate whereas the ammonium grown seeds showed the increase during the later periods.

Polyamines have been reported to inhibit protease activity and thereby prevent senescence (Kaur-Sawhney et al., 1982 a). It has been suggested that they may either inhibit the synthesis of proteases or may affect the release of the enzymes from the central vacuole or may bind to the enzyme thus rendering it less accessible to the substrate. In the present study, addition of spermine and GAA to the in vitro assay did not show any effect on protease activity suggesting that the effect was not due to their binding to the enzyme. The increase in protease activity was also not due to a de novo synthesis of the enzyme since the activating effect was observed even when the seeds

were grown in the presence of cycloheximide. These results indicate that polyamines and guanidines may affect protease activity either by activating the preexisting enzyme or by affecting its release. Proteases are known to be present in the cotyledons as zymogens which are activated during germination, or they may be synthesized de novo and transported into the protein bodies. The enzymes then degrade the storage proteins to produce polypeptides that are transported to the cytosol and then further degraded (Hara and Matsubara, 1980). Polyamines and guanidines may facilitate these processes of protein degradation. This apparently contradictory effect of polyamines and guanidines on protease observed in the present study could be due to hormonal interactions and the physiology of the plant. During germination, the cotyledonary reserve material is hydrolysed rapidly to provide amino acids to the growing embryo. Polyamines and guanidines, by enhancing protease activity, would thus increase the reserve protein mobilization and hence the growth.

The mobilization of reserve protein in the cotyledons results into an accumulation of amino acids which are supplied to the growing axis or transaminated to form other amino acids. The activities of AAT and other transaminases have been found to increase during germination. Much of the AAT activity is located in the cytosol, however, it is also

reported to be localized in chloroplast, mitochondria and peroxisome. In the chloroplast, enzyme activity increases during greening of the tissue (Hatch and Mau, 1973) and is involved in the synthesis of amino acids from glutamate which is the primary product of photosynthesis. Glutamate forms 33% of the free amino acid pool of the chloroplasts. Secondary transfer from glutamate results in the formation of aspartate and alanine and from these two amino acids other protein amino acids may be synthesized (Kirk and Leech, 1972). Both the groups of compounds increased the AAT activity of only the seeds grown in light, while that of dark ones was not affected. Subcellular fractionation showed that it enhanced the activity of only the chloroplastic enzyme. The increase brought about by spermine and GAA was abolished by cycloheximide, suggesting that the compounds affect the de novo synthesis of the chloroplastic enzyme.

The above studies thus show that in radish seeds, polyamines and guanidines may regulate growth during the early period of germination by enhancing the reserve protein mobilization in the cotyledons rather than the nitrogen assimilation process.

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