Effect of nitrate and aminoethoxyvinylglycine on *Cicer arietinum* L. nodules

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Abstract

The chickpea (*Cicer arietinum* L.) cv. HC-1 was raised in earthen pots filled with dune sand in screenhouse. At vegetative stage, *i.e.* 40 - 45 d after sowing, 10, 20 and 40 mM NO_3^- was applied through rooting medium. After 24 h of NO_3^- treatments an ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG) in concentration 5 μM was given. A conspicuous increase in (5 - 9 fold) ethylene evolution in nodules was noticed after NO_3^- treatments. This rise was parallel to the increase in 1-aminocyclopropane-1-carboxylic acid (ACC) content and ACC oxidase activity. On the contrary, a sharp decline in ACC content, ACC oxidase activity and ethylene evolution was observed when AVG was given. A decrease of in acetylene reduction assay (ARA) with NO_3^- treatments was associated with decline in cytosolic pH (from 6.12 to 5.45), leghemoglobin (Lb) content, accumulation of H_2O_2 and with the loss of membrane integrity. The lipid peroxidation, followed as MDA production and electrolyte leakage increased with NO_3^- treatments, however, the level of MDA was brought down in AVG-treated nodules. Results confirm that ethylene might be involved in mechanism by which the functioning of nodules is adversely affected by NO_3^-.

Additional key words: chickpea, ethylene, 1-aminocyclopropane-1-carboxylic acid, ACC oxidase, N_2-fixation, membrane integrity, cytosolic pH.

Introduction

Plants are exposed to a wide range of different stresses which can originate either from human activities or may have natural causes, such as drought, salinity, low temperature, high irradiance, and nutritional factors. As plants have only limited mechanisms for stress avoidance they require flexible means for adaptation to changing environmental conditions (Polle and Rennenberg 1993). One such stress factor can be high nitrate concentration in soil. Nitrate adversely affects specifically nodule functioning in legumes. The hypotheses proposed for applied NO_3^- induced nodule senescence include carbohydrates deprivation (Vassileva and Ignatov 1996), inhibition of leghemoglobin function (Nandwal *et al.* 1993), as well as competition for reductants and decreased permeability for O_2 diffusion (Vessey and Waterer 1992, Escuredo *et al.* 1996, Matamoros *et al.* 1999). The nitrate stimulated ethylene production in young roots of legumes that can have a negative effect on nodule formation has been reported by many authors (Fearn and La Rue 1991, Ligero *et al.* 1991, Caba *et al.* 1998, Schmidt *et al.* 1999). Nodule production both in the absence of NO_3^- and in the presence of inhibitory concentration of NO_3^- (Ligero *et al.* 1991, Caba *et al.* 1998) can be stimulated with AVG or Ag^+ as inhibitors of ethylene formation and perception, respectively. While several studies have investigated the effect that ethylene has on nodulation in plant-*Rhizobium* association, very few have looked on nodule functioning. To find out the role of this phytohormone in the functioning of nodules after application of nitrate, this study was initiated. We wanted to establish whether during NO_3^- induced nodule senescence, the nodules are the source of ethylene and what could be the link to the changes in pH, H_2O_2 content and membrane integrity.

Received 5 January 2000, *accepted* 23 June 2000.

Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; ARA - acetylene reduction assay; AVG - L-α-(2-aminoethoxyvinyl) glycine; Lb - leghemoglobin; MDA - malondialdehyde.

Acknowledgement: The authors thank to Indian Council of Agricultural Research (ICAR), New Delhi, India, for providing the funds for carrying out this investigation [No. 16(10)/93-FC-II].

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Materials and methods

Chickpea (*Cicer arietinum* L.) cv. HC-I seeds were raised in earthen pots filled with 5 kg of acid-washed river sand. The seeds were surface sterilized and inoculated with *Rhizobium* culture Ca-181. The plants were supplied with nitrogen-free nutrient solution at required intervals. KNO_3 at concentrations 10, 20, and 40 mM was added to the pots at the vegetative stage (40 - 45 d after sowing). L-α-(2-aminoethoxyvinyl) glycine (AVG; *Sigma*, St. Louis, USA) as ethylene biosynthesis inhibitor was given 24 h after nitrate treatments at a concentration of 5 μM to rooting medium. Three days after the NO_3^- treatment plants were sampled for described assays.

The ethylene production was measured by the method of Fearn and La Rue (1991). The nodules were placed in 15 cm^3 reaction vials containing wet cotton pad. The vials were made airtight with subaseal and kept in dark for 30 min at 25 °C. Then a 2 cm^3 gas sample was taken from each vial and assayed for ethylene production on gas chromatograph (*Nucon 5700*, New Delhi, India).

Free ACC content of nodules was assayed following the method of Miller and Pengelly (1984). Nodules (1 g) were ground in 2 cm^3 of 5 % (m/v) 5-sulfosalicyclic acid with a mortar and pestle and the extract was centrifuged at 30 000 *g* for 30 min at 4 °C. Then 0.4 cm^3 of supernatant and 0.2 cm^3 of 50 mM $HgCl_2$ was added to 0.6 cm^3 of 5 % 5-sulfosalicylic acid in 15 cm^3 reaction vials. The vials were made airtight with subaseal and 0.1 cm^3 of 2.6 % NaOCl in 5 M NaOH was injected into the vials. The vials were then vortexed for 5 s and incubated on ice for 15 min. Two cm^3 gas sample was taken from each vial and assayed for ethylene production.

The activity of ACC oxidase was measured by the method of Fearn and La Rue (1991). Nodules (1 g) were incubated in 15 cm^3 reaction vials containing 2 cm^3 of 20 mM ACC. The vials were made airtight with subaseal and kept in dark for 4 h at 25 °C. Then gas samples were taken and analysed for ethylene production.

Nitrogenase activity of nodules was determined by acetylene reduction essay (ARA) of Hardy *et al.* (1968) whereas leghemoglobin content was estimated by the method of Hartree (1955). H_2O_2 content of nodules was determined by a modified method of Patterson *et al.* (1984). 500 mg of nodules were homogenized with 0.2 g of activated charcoal and 10 cm^3 of 5 % trichloroacetic acid (TCA). The homogenate was filtered through *Whatman No. 1* filter paper and used directly for assay. A 0.2 cm^3 of the extract was added to 4 cm^3 100 mM K-phosphate buffer (pH 8.4). Then 2 cm^3 of colorimetric reagent was prepared by mixing 0.6 mM M_4-(2-pyridyl-azo) resorcinol and potassium titanium oxalate in 1:1 (v/v) ratio. The mixture was kept on ice until use. H_2O_2 was determined from the difference in absorbance at 508 nm between sample and blank.

The lipid peroxidation in nodules was measured in terms of malondialdehyde (MDA) content by thio-barbituric acid (TBA) reaction with little modifications of Heath and Packer (1968). 500 mg of nodules were homogenised with 5 cm^3 of 0.1 % TCA. The homogenate was centrifuged at 8 000 *g* for 15 min. One cm^3 of supernatant was precipitated by 4 cm^3 of 20 % TCA containing TBA. The mixture was heated in a water bath shaker at 95 °C for 30 min and quickly cooled in an ice-bath. The absorbance was read at 532 nm after centrifugation at 8 000 g for 10 min and the value for non-specific absorption at 600 nm was subtracted.

The membrane injury in nodules was evaluated by Sullivan's test (1972). The cytosolic pH of the nodules was determined by the method of Klucas and Arp (1977).

Complete randomized design (CRD) was used in the experiment arrangement where each observation was replicated thrice and critical difference (CD) among variants was calculated at $P = 0.05$.

Results and discussion

ACC content in nodules of NO_3^- treated plants was 2 - 3 fold higher than in nodules of control plants (Fig. 1*A*). Similarly, ACC oxidase activity in nodules increased by 64 - 73 % when 10 to 40 mM NO_3^- was given in the medium (Fig. 1*B*). These two factors resulted in a conspicuous increase (5 - 9 folds) in ethylene production in nodules (Fig. 1*C*). NO_3^- have been found to induce ethylene biosynthesis in tomato plants (Corey and Barker 1989). Application of AVG decreased ACC content to 23 - 36 %, ACC oxidase activity to 33 - 50 % and ethylene production to 58 - 69 % of the nitrate induced levels (Fig. 1). These results are in agreement with the findings of Ligero *et al.* (1991) and Rawal *et al.* (1994) in *Medicago sativa* and *Cicer arietinum*, respectively. It was surprising to note that NO_3^- being a nutrient and not a stress factor induced ethylene biosynthesis.

There is a common agreement that the stress-induced enhancement of ethylene synthesis is due to the stress-promoted synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) which is converted to ethylene by ACC oxidase. In NO_3^- affected cells of chickpea the increase in ACC contents in nodules may be due to the increased synthesis of ACC and *de novo* synthesis of ACC synthase which are involved in regulation of ethylene biosynthesis and have been used for promotion of ethylene biosynthesis by stress (Abeles *et al.* 1992). Caba *et al.*

(1998) also reported increase in ACC oxidase activity and ethylene evolution under high NO_3^- concentration. In NO_3^- affected nodules the formation of ethylene depends also on a degree of membrane injury (Table 1). Inhibition of ACC oxidace activity and decrease in ACC content by AVG led to the inhibition of ethylene evolution. However, Kacperska and Kubacka-Zebalska (1993) have reported that stress promoted synthesis of ethylene in cells depends on the availability of ACC and on lipoxygenase-mediated production of lipoperoxides which facilitate the non-enzymatic conversion of ACC to ethylene. The ACC oxidace activity depends on membrane integrity and is inhibited in tissues subjected to membrane destabilizing factors (Yang and Hoffman

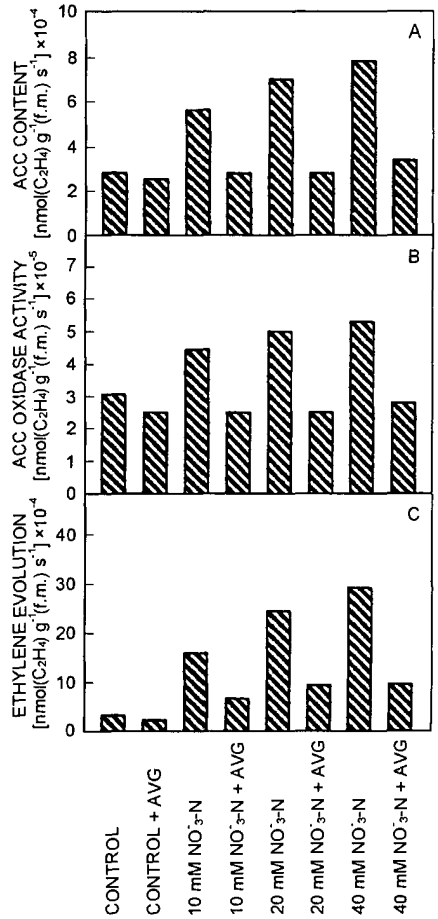

Fig. 1. Effect of NO_3^- and AVG on ACC content (*A*), ACC oxidase activity (*B*), and ethylene evolution (*C*) in chickpea nodules [CD (P = 0.05): 1.02 (ACC content), 0.56 (ACC oxidase), and 3.61 (ethylene evolution)]

1984). However, in chickpea inhibition of ACC oxidase activity by NO_3^- application did not occur though loss of membrane integrity was noticed. In NO_3^- treated plants when AVG was applied, a decrease in ethylene evolution in the consequence of a drop in the activity of ACC oxidase was observed but the production of ethylene was still higher than in the control. This shows that in addition to activity of ACC oxidase, free radical system is also involved for the synthesis of ethylene.

As expected, N_2-ase activity (determined by ARA) declined sharply with the application of NO_3^- (Table 1). Similar decrease in ARA on exposure to nitrate was reported with several legumes (Chamber-Perez *et al.* 1997, Matamoros *et al.* 1999).

The present investigation showed a partial but significant recovery in NO_3^- induced decline in N_2-fixation when AVG was added. So, ethylene is proposed to be one of the factors leading to decreased ARA. The decline in ARA may also be due to more acidic pH (shifted from 6.12 to 5.45) under nitrate treatments which stimulates the oxidation and hydrolysis of leghemoglobin (Lb) and thus alters the N_2 fixation. A tremendous decrease of N_2-ase activity to 72 % was observed at a pH of 5.45 at 40 mM NO_3^- treatment. Similar results were also reported in *Phaseolus* nodules where 60 % inhibition of nitrogen fixation occurred at pH 5.0 (Pladys *et al.* 1988).

In present data, a sharp decline (29 - 74 %) in Lb content of nodules was observed under different nitrate concentrations (Table 1). These results are in consistence with the findings of De Lorenzo *et al.* (1994) and Escuredo *et al.* (1996). The decrease in the content of Lb paralleled to a decline in ARA. With increasing NO_3^- concentration H_2O_2 content of nodules increased markedly. It is possible that either senescence is initiated or wounded type of response to NO_3^- occurs which could lead to active oxygen formation. Therefore, the damage of Lb might be related to the accumulation of H_2O_2 (Table 1). Nitrate also initiates autoxidation of Lb and concurrent production of O_2^- radicals which led to the accumulation of H_2O_2 (De Lorenzo *et al.* 1994, Escuredo *et al.* 1996). Accumulation of H_2O_2 may induce a number of genes and proteins involved in stress defences (Morita *et al.* 1999) which still needs confirmation. Drop in Lb content of nodules following nitrate treatment may also be ascribed to degradation of the hemoprotein by acidic proteases at low pH of nitrate-treated nodules. The results are in agreement with the findings of Pladys *et al.* (1988). The application of AVG resulted in slight recovery in Lb content of nodules partially by removing cytosolic H_2O_2 and by increase in pH (Table 1).

The lipid peroxidation (in terms of MDA content) increased significantly with NO_3^- concentration (Table 1). On the contrary, Matamoros *et al.* (1999) have found decrease in the content of lipid peroxides in bean and pea nodules after 4 d at 10 mM nitrate. Our results suggest that increased lipid peroxidation is mediated by H_2O_2 accumulation. The content of MDA was decreased after application of AVG although it was still higher than in control plants.

Increased electrolyte leakage from tissues is usually an

expression of changed physical properties of cell membranes. This relationship was confirmed during present investigation as the leakage of ions increased about 2 - 4 times in nodules at high NO_3^- (Table 1). A sharp increase in membrane permeability (electrolyte leakage) is also a characteristic of a plant cell senescence. The electrolyte leakage was kept within a limit after the application of AVG. This again confirms that the effect of nitrate was mediated through the evolution of ethylene. The recovery of membrane integrity based either on MDA content or on the leakage of electrolytes in AVG treated nodules clearly illustrates that the damage to membrane integrity with nitrate was to some extent reversible.

Table 1. Changes in ARA [μmol(C_2H_4) g^{-1}(d.m.) s^{-1}], Lb [mg g^{-1}(d.m.)] and H_2O_2 [mmol g^{-1}(f.m.)] contents, lipid peroxidation [nmol(MDA) g^{-1}(f.m.)], membrane injury [%], and cytosolic pH in *C. arietinum* nodules in the presence of NO_3^- and AVG.

NO_3^- [mM]	0	0 + AVG	10	10 + AVG	20	20 + AVG	40	40 + AVG	$CD_{0.05}$
ARA	0.156	0.145	0.094	0.104	0.048	0.073	0.044	0.064	0.011
Lb content	7.63	7.70	5.39	6.31	4.37	6.23	2.02	2.99	1.17
H_2O_2 content	0.458	0.398	0.990	0.656	1.197	0.838	1.369	0.977	0.053
Lipid peroxidation	178.5	171.4	922.8	385.3	1163.0	420.8	1557.5	806.1	66.4
Membrane injury	98.50	98.86	129.85	99.42	237.00	204.83	385.88	207.76	55.35
Cytosolic pH	6.12	6.10	5.70	5.95	5.64	5.90	5.45	5.87	0.23

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