BIOLOGIA PLANTARUM 41 (3): 435-443, 1998

Nodule structure and functioning in *Cicer arietinum* as affected by nitrate

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Abstract

Chickpea (*Cicer arietinum* L.) cv. C-235 inoculated with *Rhizobium* sp. (*Cicer*) strain cv4Az was raised in sand culture under natural conditions with nitrogen-free nutrient solution. 45-d-old plants were treated with 20 and 50 mM KNO_3 and samplings made 2 and 6 d after treatment. KNO_3 application induced premature nodule senescence. Light microscopic investigations showed that KNO_3 treatments resulted in structural degradation of the central bacteroidal tissue. The mass of green nodules increased by 35 % under these treatments. This was accompanied by a rapid decline in leghemoglobin (Lb) content of the nodules being 51 - 67 % lower than in control. The total soluble nodule proteins showed relatively minor changes under KNO_3 treatments thus suggesting preferential degradation of Lb. These changes were associated with a rapid decline in N_2-fixing activity. However, the decline in total soluble sugars was relatively minor as compared to acetylene reducing activity, thus indicating that sugar deprivation is not the cause of decreased nitrogen fixation ability. Glutathione reductase and ascorbate peroxidase activity showed a 10 - 20 % decrease in comparison with the control. Accumulation of H_2O_2 and structural degradation of the nodular tissue are considered to be the factors leading to nodule senescence under nitrate treatments.

Additional key words: chickpea, hydrogen peroxide, leghemoglobin, N_2 fixation, senescence, soluble saccharides.

Introduction

In legumes, atmospheric nitrogen is fixed by the root nodule having a limited functional life span which determines their contribution to the host plant in terms of fixed nitrogen. The duration of this functional period depends on legume plant species, rhizobial strain, nodule structure and environmental conditions.

Received 12 January 1998, *accepted* 1 August 1998.

Abbreviations: ARA - acetylene reducing activity, ASC - ascorbate, DAT - days after treatment, GSSG - glutathione, Lb - leghemoglobin, TSS - total soluble saccharides

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As symbiotic N_2-fixation is an adaptive mechanism, excess of combined nitrogen in the rooting medium not only inhibits new nodule formation, but also limits the functional span of already existing nodules. The study of nitrate induced senescence of legume nodules is rather complex because of the involvement of nitrate metabolism and oxygen relations (Dalton *et al.* 1991). Nodules function under a paradoxical situation. In order to fix nitrogen, bacteroids inside the nodules must be supplied with O_2 at a sufficiently fast rate to meet their respiratory requirement for efficient production of ATP, but at the same time, level of free oxygen inside the nodule must be kept low to protect nitrogenase which is highly O_2 labile. Leghemoglobin facilitates O_2 diffusion inside dense nodular tissue maintaining a constant supply at low tensions of free O_2. However, it can perform this function only if heme iron is present in ferro form; when it is present in ferric form it is unable to bind molecular O_2 (Appleby 1984).

During senescence the pH of nodules becomes more acidic (pH approximately 5.5) (Pladys *et al.* 1988) and this acidic pH favours autoxidation of oxygenated Lb to ferric Lb yielding superoxide radical $O_2^{\cdot}$. The $O_2^{\cdot}$ radical can also be formed by oxidation of key proteins of bacteroids such as ferredoxin, hydrogenase and nitrogenase (Dalton 1995). The superoxide radical and H_2O_2 are also formed when the O_2 consumed during respiration of bacteroids and nodule mitochondria is reduced to water. $O_2^{\cdot}$ is converted to H_2O_2 in the presence of the superoxide dismutase. Increased H_2O_2 production might hamper N_2-fixation as it leads to lipid peroxidation and is the precursor of the hydroxyl radical ($HO^{\cdot}$) which is highly toxic for biological systems (Becana and Rodriguez-Barruco 1989). Becana *et al.* (1988) reported that nitrate treatment led to H_2O_2 accumulation in legume nodules. Therefore, the present investigations were conducted to study the effect of nitrate on nodule functioning and some H_2O_2 scavenging enzymes in chickpea.

Materials and methods

Plants and growth conditions: Chickpea (*Cicer arietinum* L. cv. C-235) plants were raised in sand culture in earthenware pots (22.5 × 22.5 cm) each containing 6 kg of river sand. Before sowing, seeds were surface sterilized and inoculated with a specific pure *Rhizobium* sp. (*Cicer*) strain cv4Az obtained from Microbiology Department, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Plants were grown in a greenhouse under natural light and temperature. Except for an initial dose of nitrate nitrogen (250 cm^3 of 0.05 mM KNO_3) plants were supplied with appropriate amounts of nitrogen free nutrient solution (Wilson and Reisenauer 1963). 45 d after sowing, when the plants had developed sufficient number of nodules, the plants were divided into three sets: one served as untreated control, the other two were subjected to 20 and 50 mM KNO_3, respectively. The samplings were conducted 2 and 6 d after treatment. From every treatment four pots constituting four replicates of two plants each were sampled at appropriate intervals of time.

Nitrogen fixation: The rate of N_2-fixation was determined by incubating isolated roots bearing nodules in 10 % C_2H_2 for 10 min at room temperature (20 ± 5 °C) in 100 cm^3 bottles fitted with *Subaseals* (*Perfit*, India). The acetylene reduction assay was carried out according to Hardy *et al.* (1973).

Contents of leghemoglobin, total soluble proteins, total soluble saccharides and nitrogen: Immediately after sampling, the nodules were removed from the roots and washed free of sand. Nodules were excised and processed for various estimations without any further delay. Leghemoglobin content was determined as described previously (Swaraj *et al.* 1986). The protein content was measured by the method of Lowry *et al.* (1951) using the Folin-Ciocalteau reagent with bovine serum albumin as standard. Total nitrogen content of nodules was estimated by microKjeldahl's method with little modification using *Kjeltac Autoanalyser 1030* (Sweden). Total soluble saccharides were measured by the method of Yemm and Willis (1954) using anthrone reagent.

Enzymes: The enzyme extract was prepared by homogenizing 0.8 g of nodules in 5 cm^3 of 0.1 M phosphate buffer (pH 7.0), containing 0.05 % bovine serum albumin in a prechilled pestle and mortar. The homogenate was centrifuged in a refrigerated centrifuge (*REMI G30*, India) at 10 000 *g* for 10 min and the supernatant obtained was used for determination of activity of GSSG-reductase and ASC-peroxidase. All the steps in the preparation of enzyme extract were carried out at 4 °C.

Glutathione reductase (NADPH: oxidised glutathione oxidoreductase, EC 1.6.4.2) was assayed by measuring the decrease in absorbance at 340 nm due to NADPH oxidation (as described by Dalton *et al.* 1986). Reaction mixture consisted of 0.2 mM GSSG, 0.125 mM NADPH, 50 mM Tricine (pH 7.8), 0.5 mM EDTA and 0.05 cm^3 of extract in a final volume of 2.0 cm^3. Rates were corrected for GSSG-independent oxidation. The nonenzymatic rate was negligible.

Ascorbate peroxidase (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) was measured by a spectrophotometer (*Spectronic - 21*, *Bausch & Lomb*, USA) based on the rate of decrease in absorbance of ascorbate at 265 nm during ascorbate oxidation (Nakano and Asada 1981). The assay was performed in a 3 cm^3 quartz cuvette containing 0.5 mM ascorbate, 0.1 M phosphate buffer (pH 7.0), 2.0 mM H_2O_2 and 0.01 cm^3 of extract. Corrections were made for the low rates of ascorbate oxidation due to nonenzymatic H_2O_2-independent oxidation.

Light microscopy: The nodules were fixed in FAA (formaldehyde, acetic acid, alcohol and water in the ratio of 10:1:2:7), passed through ethanol-xylene series for dehydration, infiltration and embedded in wax. Sections (8 - 10 μm thick) were stained with 0.05 % (m/v) aqueous toluidine blue and observed under a light microscope.

Results

Application of KNO_3 to the plants accelerated the rate of nodule senescence as is evident from the ratio of red to green nodules (Table 1). Two days after 20 and

50 mM KNO_3 treatment (DAT) acetylene reduction activity (ARA) decreased considerably (60 - 65 %) (Fig. 1). ARA decreased further with increase in duration of nitrate treatments, the value dropped by 82 - 83 % although 50 % of the nodules were still red. The decline in leghemoglobin (Lb) content was relatively slow (Fig. 1). However, as the duration of the treatment increased to 6 d, the Lb content decreased by 50 %. Nodule cytosolic proteins showed a relatively minor decline under nitrate treatments (Fig. 1) reflecting that Lb was more sensitive to KNO_3 as compared to other soluble proteins. The total soluble saccharide (TSS) content also showed minor decline under nitrate treatment (Fig. 1) as compared to the sharp decline in ARA. The nitrogen content, however, increased by 25 % at 20 mM KNO_3, but it was retained near control level at 50 mM KNO_3 (Fig. 1).

Table. 1. Effect of KNO_3 on total nodule mass, green and red nodule mass and nodule volume.

KNO_3 [mM]	DAT	Total nodule mass [g]	Red nodule mass [g]	Green nodule mass [g]	Nodule volume [%]
0	2	1.80 ± 0.30	1.54 ± 0.36	0.29 ± 0.11	1.90 ± 0.30
	6	2.00 ± 0.40	1.64 ± 0.25	0.35 ± 0.04	2.00 ± 0.20
20	2	1.68 ± 0.40	1.30 ± 0.35	0.38 ± 0.05	1.70 ± 0.30
	6	1.05 ± 0.20	0.54 ± 0.11	0.51 ± 0.01	1.00 ± 0.20
50	2	1.10 ± 0.10	0.75 ± 0.10	0.35 ± 0.06	1.10 ± 0.10
	6	0.94 ± 0.04	0.46 ± 0.04	0.48 ± 0.03	1.03 ± 0.05

Among the H_2O_2 scavenging enzymes of the glutathione ascorbate cycle, a considerable decrease in ascorbate peroxidase (ASC-peroxidase) activity was observed. A 23 to 27 % decline was registered 2 DAT at 20 and 50 mM KNO_3 (Fig. 2). As the DAT increased from 2 to 6, the ASC-peroxidase activity further declined by 32 to 63 % of the control. A lower decline in glutathion (GSSG)-reductase activity was observed ranging from 10 to 20 % with 20 mM KNO_3 2 and 6 DAT, respectively (Fig. 2). Increasing the concentration of KNO_3 to 50 mM did not further affect the GSSG-reductase activity.

As compared to the control, nodules harvested 6 DAT with 20 mM KNO_3 had smaller regions of meristematic activity. At 50 mM KNO_3 (6 DAT), the meristematic activity was reduced to almost zero and the outer cortex was damaged. Investigations of the central bacteroidal tissue from control nodules showed that the number of uninfected cells were less. In the infected cells, the nuclei were prominent and the bacteroids were densely packed (Fig. 3*a*). At 20 mM KNO_3 (6 DAT) no nuclei were seen and the bacteroids were packed less densely. The rhizobia appear to be contained within packet-like structures (Fig. 3*b*). Similar results were observed with 50 mM KNO_3 (Fig. 3*c,d*), where the bacteroids appeared shrunken and distorted, surrounded by a darkly staining material. As the nitrate concentration increased, a steady increase in the disorder in infected cells and the ratio of number of uninfected cells to infected cells were observed.

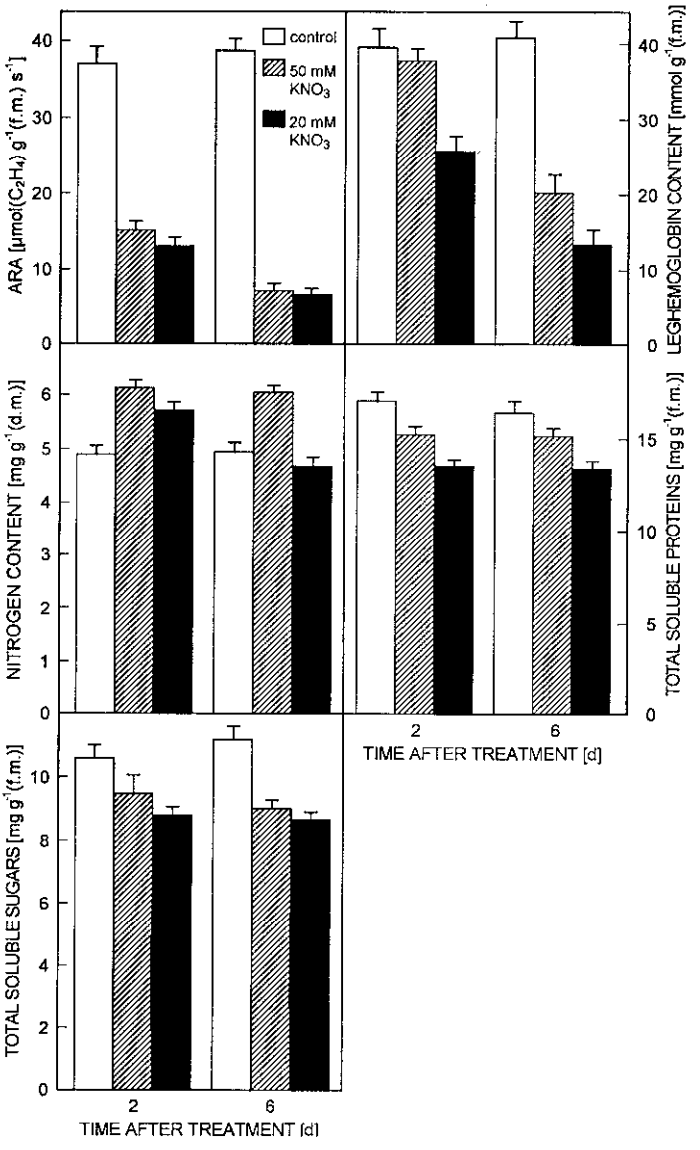

Fig. 1. Effect of KNO_3 on acetylene reduction activity, leghemoglobin, nitrogen, total soluble protein, and total soluble sugar contents. Lines above the bars indicate SEM of three replicates.

Discussion

Nodule senescence can be induced prematurely by treating the plants with KNO_3 (Swaraj *et al.* 1993, Escuredo *et al.* 1996). Becana and Sprent (1987) have reviewed that nitrate application induced premature greening of nodules, which is an

established index of nodule senescence. In the present investigations, treatment with 20 and 50 mM KNO_3 increased the green nodule mass which was accompanied by a considerable decrease in Lb. It was also observed that Lb was more deleteriously

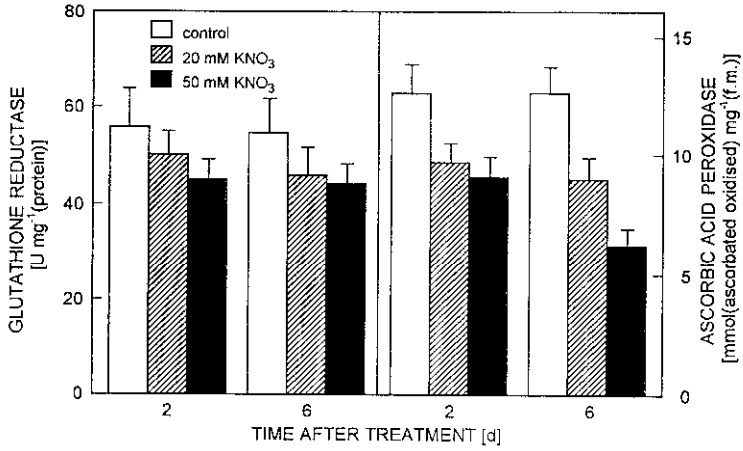

Fig. 2. Effect of KNO_3 on glutathione reductase and ascorbate peroxidase activity. Lines above the bars indicate SEM of three replicates.

affected by the presence of nitrate than the total soluble proteins indicating that it is particularly sensitive. This was also found in alfalfa, cluster bean and pea nodules (Becana *et al.* 1988, Swaraj *et al.* 1993, Escuredo *et al.* 1996). In nitrate treated nodules, Lb may be inactivated by oxidation of the heme Fe, formation of a complex with NO_3^- or degradation of the globin or the heme group (Becana *et al.* 1991, Becana and Klucas 1992, Jun *et al.* 1994). The decline in Lb content may be also ascribed to degradation of the hemoprotein by acidic proteases with high affinity for Lb (Pladys and Rigaud 1985), a process that is favoured by the low pH of nitrate-treated nodules (Pladys *et al.* 1988).

Nitrate-treated plants resulted in a considerable inhibition of ARA within 2 to 6 DAT. Comparable results have been reported (Minchin *et al.* 1989, 1992, Faurie and Soussane 1993, Swaraj *et al.* 1993). One possible explanation is that the utilization of reducing equivalents to support nitrate assimilation would deprive the nodules of their sugar supply and cause the nitrogenase activity to decrease (Oghogorie and Pate 1971, Hardy and Havelka 1976, Taylor *et al.* 1988). Contrarily, in our studies the total soluble saccharide content of nodules showed a relatively minor decline as compared to ARA. There are many reports supporting our observations (Streeter 1981, 1985, Wasfi and Prioul 1986) where no decrease in total soluble saccharide content of the nodules was observed after NO_3^- supply.

Little is known about H_2O_2 metabolism in legume nodules during their senescence. Nodule cytosol contains high level of the ASC-peroxidase (Dalton 1995). In our studies the activities of ASC-peroxidase decreased by 30 to 50 % with 20 and 50 mM KNO_3. The decline in GSSG-reductase activity was lower ranging from 10 to 20 %. Escuredo *et al.* (1996) reported a 50 % decrease in ASC-peroxidase activity 4 d after treatment with 10 mM KNO_3 in pea nodules. Swaraj *et al.* (1994) reported

approximately 30 % decline in ASC-peroxidase activity in cluster bean with 20 and 50 mM nitrate. Dalton (1995) and DeLorenzo *et al.* (1994) also reported a decline in the operativity of the ASC-GSH cycle.

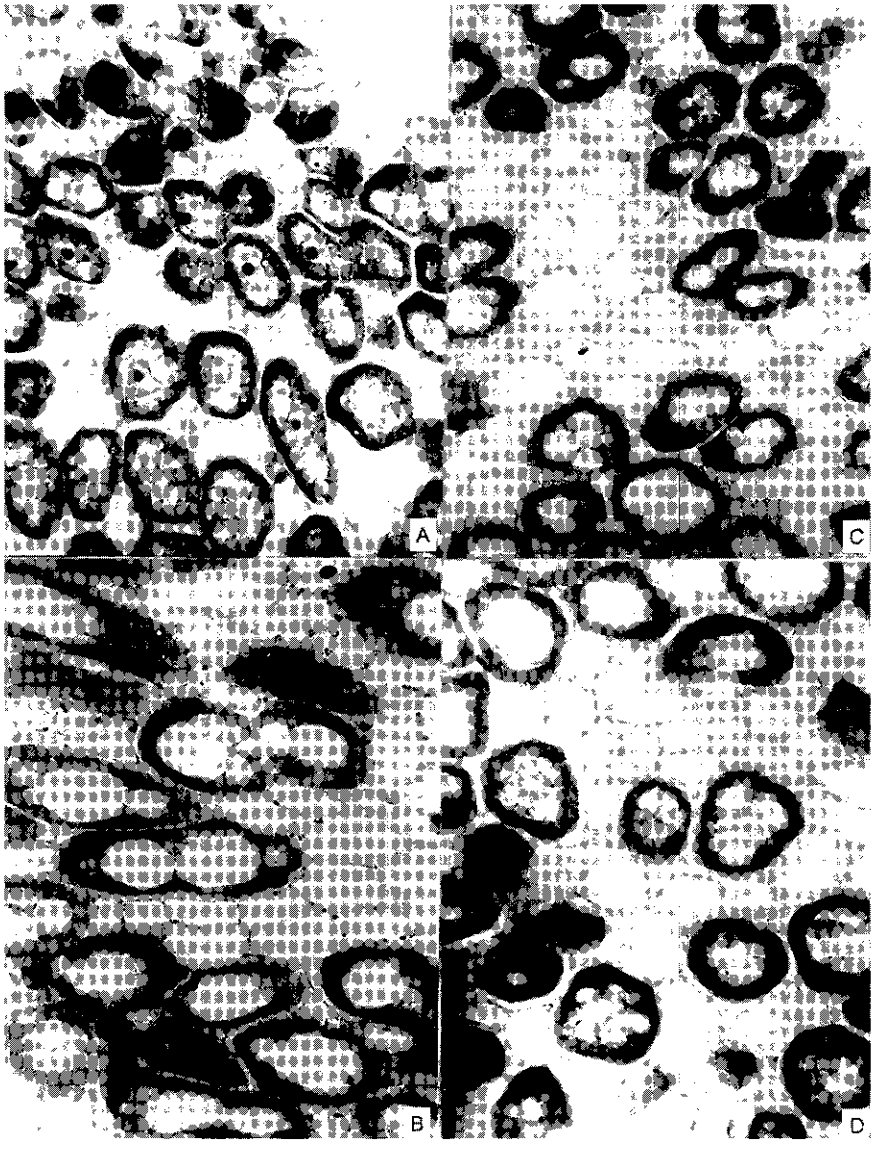

Fig. 3. Cross section of chickpea nodules showing the central bacteroidal region (×1200). *a* - control, *b* - 20 mM KNO_3, 6 DAT, *c* - 50 mM KNO_3, 2 DAT, *d* - 50 mM KNO_3, 6 DAT; UI - uninfected cells (with starch grains), IC - infected cells.

Nitrate treatments (20 and 50 mM KNO_3) adversely affected the nodule structure. The control (0 mM) nodules had a defined general structure of infected and

uninfected cells. As the nitrate concentrations increased to 20 mM the bacteroids were less densely packed. The rhizobia appear to be contained within packet like structures. When the concentration of nitrate was increased to 50 mM the bacteroids appeared shrunken and distorted, surrounded by a darkly staining material. Naisbitt and Sprent (1993) have also observed that at 20 mM nitrate the area around the bacteria was filled with fibrillar material and rhizobia appeared to be contained within packet like structures. DeLorenzo *et al.* (1990) also reported that the application of NO_3^- to nodulated plants gave rise to general structural degradation of the nodule.

The concomitant decrease in activities of ASC-peroxidase and GSSG-reductase suggests a link between N_2 fixation and some of the H_2O_2 scavenging enzymes. Our results do not support the sugar deprivation hypothesis, and accumulation of H_2O_2 due to decline in activities of ASC-peroxidase and GSSG-reductase and structural degradation of the nodular tissue may be the factors leading to nodule senescence under nitrate treatments.

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