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Natural and dark-induced nodule senescence in chickpea: nodule functioning and H_2O_2 scavenging enzymes

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Abstract

An investigation was carried out on chickpea (*Cicer arietinum* L.) cv. C-235 inoculated with *Rhizobium* sp. *Cicer* strain cv 4 Az^r. Nodule functioning was monitored at 15 d intervals starting from 45 days after sowing (DAS) and inoculation in order to study nodule development and senescence under natural and stress conditions (dark treatments of 18 and 66 h). Maximum rate of N_2-fixation was observed between 50 - 60 DAS. After this acetylene reducing activity (ARA) fell and it was negligible 75 DAS. This decline in ARA with ageing of plants and nodules was accompanied by a decline in leghemoglobin content and greening of the nodules. When 60 % of the nodule tissue had turned green 75 DAS, a sharp increase in nodule peroxidase activity (3.7 fold) was observed whereas the catalase activity was reduced by 50 % in comparison with the control. The glutathione-reductase and ascorbate-peroxidase activity followed a trend parallel to that in N_2-fixation, but the variation was much smaller. The changes in the total soluble carbohydrates, cytosolic proteins and nitrogen content per se were not expressive. Dark treatments induced premature senescence of the nodules as was evident from the marked decrease in ARA. However, the decline in leghemoglobin content was relatively small as compared to ARA. The changes in cytosolic proteins, total soluble carbohydrates, peroxidase activity, catalase activity, glutathione reductase activity and ascorbate peroxidase activity of nodules under dark-induced nodule senescence were almost parallel to those observed under natural senescence.

Additional key words: acetylene reducing activity, ascorbate peroxidase, catalase, *Cicer arietinum*, leghemoglobin, peroxidase.

Introduction

In legumes, atmospheric nitrogen is fixed by the root nodules having a limited functional span which determines their contribution to the host plant nutrition in

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Abbreviations: ARA - acetylene reducing activity, ASC - ascorbate peroxidase, DAS - days after sowing, GSSG - glutathione, lb - leghemoglobin, TSC - total soluble carbohydrates.

terms of fixed nitrogen. The duration of this active functional period depends on legume plant species, rhizobial strain, nodule structure and environmental conditions. It comes to a close with the onset of nodule senescence accompanied by a decline in N_2-fixing activity and decrease in leghemoglobin content of nodules. However, decrease in nitrogenase activity which usually precedes leghemoglobin degradation during nodule senescence is accompanied by a change in colour from pink to green. Greening of the nodules has been ascribed to conversion of leghemoglobin to choleglobin, impairing its function (Virtanen and Miettinen 1963).

Little information exists about the causes of senescence in nodules. Among various possibilities the source-sink relations during redirection of nutrients, changes in hormonal control, or changes of the energy status of the cell (Thimann 1980, Thomas and Stoddart 1980) are the most frequent. The accumulation of free radicals was claimed to occur in ageing leaves by Leshem (1981). An accumulation of H_2O_2 in senescing nodules has been reported (Becana 1988, Dhandi 1994) which might hamper N_2-fixation and induce nodule senescence. Therefore, it was considered worthwhile to study the changes in activities of some enzymes responsible for scavenging H_2O_2 with reference to the pattern of senescence in nodules of plants subjected to dark treatments and in naturally senescing nodules.

Materials and methods

Investigations were carried out on chickpea (*Cicer arietinum*) cv. C-235. The plants were grown under natural light in unglazed earthen-ware pots (25 × 25 cm) each containing 6.5 kg of dune sand. Before sowing seeds were surface sterilized and inoculated with specific rhizobial culture [*Rhizobium* sp. *Cicer* strain cv 4 Az^r (azide resistant mutant of effective strain cv 4) supplied by the Department of Microbiology, CCS Haryana Agr. Univ., Hisar]. The plants were supplied with nitrogen-free nutrient solution (Wilson and Reisenauer 1963) except for an initial starter dose of nitrate nitrogen. In order to study the developmental and functional changes in nodules leading to nodule senescence the samplings were made at 15 d intervals starting from 45 days after sowing (DAS). In the second experiment, 45-d-old plants were subjected to dark treatments of 18 and 66 h after 9-h exposure to natural day light in greenhouse.

Four pots constituting four replicates of two plants each were sampled. Nitrogenase activity (EC 1.18.6.1) was measured using nodules attached to the excised roots. For other biochemical estimations, the nodules were removed from the roots immediately after sampling and washed free of sand. The nitrogenase activity was determined by incubating isolated roots bearing nodules in 10 % C_2H_2 for 10 min at room temperature in 100 cm^3 bottles fitted with suba seals. The acetylene reduction assay was carried out according to Hardy *et al.* (1973). 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 a standard. Total soluble

carbohydrates were measured by the method of Yemm and Willis (1954) using anthrone reagent.

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 nM 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 NADPH oxidation. The non-enzymatic rate was negligible.

Ascorbate peroxidase (L-ascorbate:hydrogen-peroxidase oxidoreductase, EC 1.11.1.11.) was measured by a modified spectrophotometric procedure 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.

The catalase (EC 1.11.1.6) activity was measured by the method of Sinha (1972). The reaction mixture in 3 cm^3 contained 0.5 cm^3 extract, 0.1 M phosphate buffer (pH 7.0) and 0.1 M H_2O_2. The reaction was carried out at 37 °C for 5 min after which the enzyme activity was stopped by addition of 4 cm^3 of 5 % dichromate in acetic acid. A control was run at the same time in which enzyme extract was added after the addition of 5 % dichromate acetic acid.

The peroxidase (EC 1.11.1.7) activity was assayed as described by Shannon *et al.* (1966). The reaction mixture consisted of 20 mM sodium acetate buffer (pH 5.0), *o*-dianisidine (1 % in methanol), 3.0 cm^3 H_2O_2 and 0.01 cm^3 of enzyme extract. The change in absorbance at 470 nm was measured.

The data were statistically analysed for standard error of means and the results presented are thus mean ± SEM of four replicates.

Results and discussion

The development of the plant towards reproductive phase enhanced greening of the nodules and led to decline in nodule leghemoglobin (lb) content and acetylene reducing activity (ARA) which were considered as indices of nodule senescence. The fresh mass of green nodules was 15 % of total at 45 DAS, 20 % at 60 DAS and 60 % at 75 DAS (Fig. 1*A*). Greening of the nodules has been attributed to oxidation of lb, through the associated molecular changes are not properly understood. They either involve breakdown or replacement of methine bridges with O_2 (Virtanen *et al.* 1947).

The maximum rate of N_2-fixation was observed between 50 and 60 DAS. After this a considerable fall in ARA was recorded and the activity reached negligible levels 75 DAS (Fig. 2*A*). Such decrease in ARA during plant ontogeny has been widely reported in the literature (Vance and Heichel 1991). The peak of N_2-fixation is attained at different times after sowing depending on the plant species. Dalton *et al.* (1986) reported that ARA reached a peak value 27 DAS in soybean. Usually the decline in nitrogenase activity has been associated with pod filling stage of the

legumes (Lawn and Brun 1974, Bethlenfalvay and Phillips 1977). Three different explanations could be possible for this pod filling decline: (*1*) the root nodules become carbohydrate limited, (*2*) senescence is induced by a signal from the shoot at the onset of pod-filling or (*3*) the decline and senescence are a result of

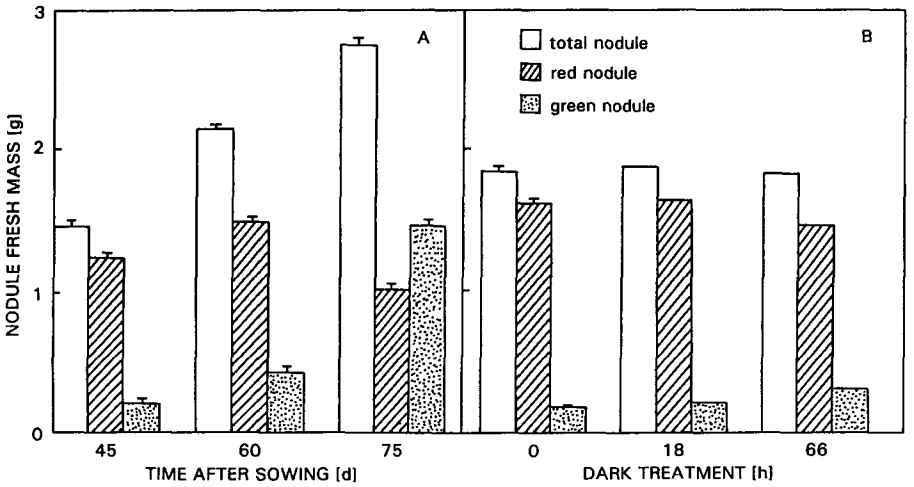

Fig. 1. Effect of natural and dark-induced senescence on nodule mass: *A*. Nodule mass at 45, 60 and 75 DAS; *B*. Nodule mass after exposing the plants to 0, 18 and 66-h darkness.

autonomous nodule ontogeny. In our studies, the total soluble carbohydrate (TSC) content of nodules did not show any decline (Fig. 3*A*) so the first explanation could be ruled out. Ontogenetic nodule development appears to be the most plausible

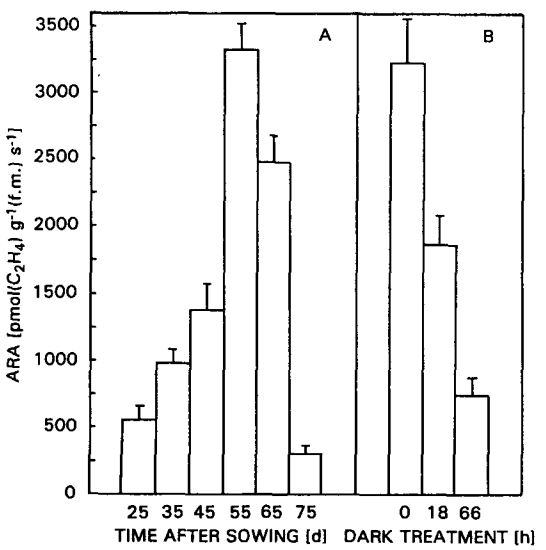

Fig. 2. Effect of natural senescence (*A*) and dark-induced senescence (*B*) on acetylene reducing activity (ARA).

explanation as during pod filling, annual legumes enter the ontogenetic stage of general (monocarpic) senescence. Wilson *et al.* (1978) and Schweitzer and Harper (1985) have reported in their work with male sterile soybeans that pod development was not required as a stage for triggering root nodule senescence. This is also evident from our data on nodule greening, where 15 % of nodule tissue had turned green at 45 DAS, well before flowering. In addition, many workers have shown recently that there was a significant decline in nitrogenase activity at early pod-filling stage in a number of plants (Zapata *et al.* 1987, Ismande 1989, Vikman and Vessey 1992, Vessey 1992).

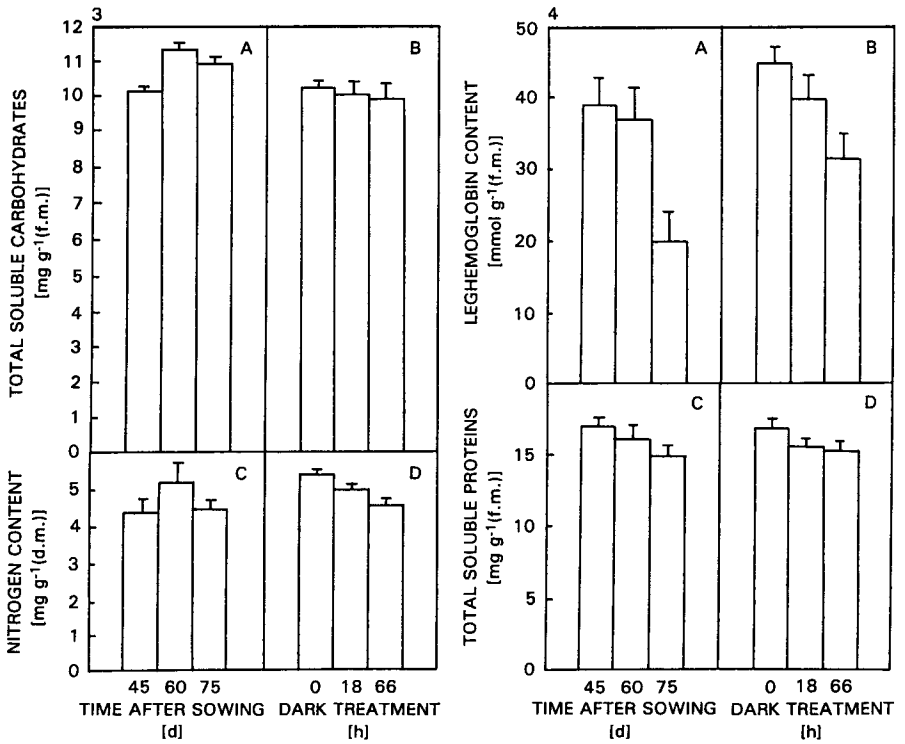

Fig. 3. Effect of natural (*A, C*) and dark-induced (*B, D*) senescence on total soluble carbohydrate content (*A, B*) and nitrogen content (*C, D*).

Fig. 4. Effect of natural (*A, C*) and dark-induced (*B, D*) senescence on leghemoglobin content (*A, B*) and total soluble protein content (*C, D*) in nodules.

The nitrogen content of the nodules in our experiment also showed a peak value around 60 DAS (Fig. 3*C*). However, changes in nodule nitrogen per se probably were not of much consequence as far as functioning of nodule and senescence were concerned.

Peaks of leghemoglobin (lb) content were observed at 45 and 60 DAS (Fig. 4*A*). We observed that during 60 - 75 DAS when plants have shown extensive flowering, lb content decreased by 50 % when 60 % of the nodules had turned green and ARA fell to negligible levels. From data on lb and nodule cytosolic proteins it was

concluded that lb was preferentially degraded during nodule senescence (Fig. 4*C*). Pfeiffer *et al.* (1983) also did not observe significant decrease in the bacteroid proteins of soybean nodules at physiological maturity.

In addition to ageing, adverse environmental conditions like light stress induce premature senescence of the legume root nodules. Dark treatment of plants induce metabolic and structural changes in nodules which are characteristic of their senescence (Paau and Cowles 1981, Pfeiffer *et al.* 1983, Andreeva *et al.* 1986). A 9 % decrease in the percentage of red to green nodules on the fresh mass basis (Fig. 1B) was observed after 66-h dark treatment. Dark treatments induced a 5 to 11 % increase in nodule volume under 18- and 66-h dark treatment, respectively (data not given). Although the dark treatments induced very little greening in the nodules yet the decline in ARA was considerably steep (Fig. 2*B*).

Dark treatment of plants has been reported to bring about a rapid decline in the rate of N_2-fixation (Romanov *et al.* 1980, Paau and Cowles 1981, Pfeiffer *et al.* 1983, Swaraj *et al.* 1985, 1986, 1988, 1994). Siddique and Bal (1991) studied the N_2-fixation in peanut and cowpea nodules under dark conditions. Peanut nodules maintained their levels of ARA activity. On the other hand, cowpea plants infected with the same strain of *Bradyrhizobium* showed greater than 40 % decrease in ARA within 12-h. Reports on soybean (Ching *et al.* 1975), chickpea (Swaraj *et al.* 1986), and cowpea (Swaraj *et al.* 1988) showed similar declines in ARA value when the plants are kept in dark. The decline of N_2-fixation is, in general, attributed to the limited photosynthate availability (Murphy 1986). However, data on nodule TSC obtained from present investigations did not support this hypothesis. Relatively minor decrease in nodule TSC was observed when plants were exposed to a dark period of 18 - 66 h (Fig. 3*B*). Swaraj *et al.* (1986) also reported that with prolongation of the dark period to 48 h, though the ARA decreased to 10 % of the control values, TSC showed only 25 - 40 % decrease. Prolonged maintenance of N_2-fixation in peanut in the absence of photosynthesis could be explained in terms of metabolic reserves in the form of lipid bodies in contact with the peribacteroid membrane (Siddique and Bal 1991). However, photosynthate supply may not be the only regulator of ARA but some other factors may also be involved. Nelson *et al.* (1984), Dabas *et al.* (1988) and Aggarwal *et al.* (1991) also could not detect any correlation between the nitrogenase activity and gross levels of carbohydrates present in the nodules. Schweitzer and Harper (1980) and Rainbird *et al.* (1983) attributed dark-induced decline in N_2-fixation at night to lower temperatures. If temperature was kept constant during the dark period no decline in ARA values was observed. Contrary to these observations, Siddique and Bal (1991) reported dark induced decline of ARA in cowpea kept under constant temperature regime.

As compared to ARA, the dark-induced decline in lb content was relatively small (Fig. 4*B*). An 11 to 28 % decline was observed with 18- and 66-h dark treatment, respectively. On the other hand, Roponen (1970) reported a rapid greening of nodules. Pfeiffer *et al.* (1983) reported a slight decrease in lb after 4-d dark treatment of soybean, but after 8 d of continuous darkness the lb content was below the detection limit. However, a decline in total nodule soluble proteins in their study was

relatively faster than that of lb. In our studies, the decline in total soluble proteins (Fig. 4*D*) was even less than that of lb.

Production of activated oxygen species is a normal process inside the nodules because they are rich in lb, which serves as an oxygen carrier within the nodules and can function only if present in the reduced ferro form. This protein can undergo autooxidation producing superoxide ion (O_2^-) and ultimately H_2O_2 (Dalton *et al.* 1986). Other sources of active oxygen species in nodules are the proteins ferredoxin, uricase and hydrogenase (Dalton *et al.* 1986). In addition, ageing of the plant tissue is accompanied by increase in the rate of production of O_2^- and H_2O_2 (Harman 1981). The change in H_2O_2 level is not the outcome of the ageing process but rather the factor which triggers it. In senescing nodules Becana *et al.* (1988) and Dhandi (1994) have reported accumulation of H_2O_2. Therefore, in the present investigations attempts were made to establish a correlation, if any, between H_2O_2 scavenging enzymes and nodule senescence.

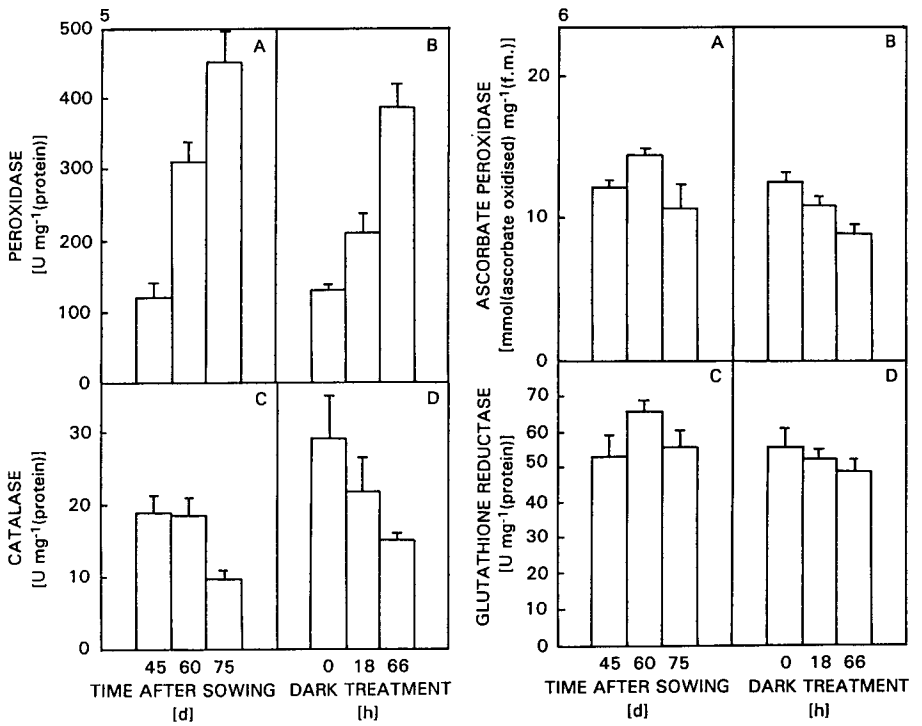

Fig. 5. Effect of natural (*A, C*) and dark-induced (*B, D*) senescence on peroxidase (*A, B*) and catalase activity (*C, D*) of nodules.

Fig. 6. Effect of natural (*A, C*) and dark-induced (*B, D*) senescence on ascorbate peroxidase activity (*A, B*) and glutathione reductase activity (*C, D*) of nodules.

A sharp increase in peroxidase activity was observed under natural and induced nodule senescence (Fig. 5*A, B*). Peroxidase activity increased 3.7 fold at 75 DAS (natural senescence) whereas under dark treatments a 1.5 and 2 fold increase was observed under 18- and 66-h dark treatment, respectively. Our observations regarding

increase in peroxidase activity under senescent-inducing conditions are difficult to explain. Probably nodule peroxidase is unable to detoxify H_2O_2 due to compartmentation within the nodule cells. An increase in peroxidase level has also been reported during fruit ripening and leaf senescence (Nooden 1984).

A 50 % decline in catalase activity was observed under natural and dark-induced nodule senescence (Fig. 5*C, D*). Catalase has been shown to have a positive correlation with nitrogenase activity. Francis and Alexander (1972) reported that effective white clover and soybean nodules contained more catalase than ineffective nodules. A decrease in catalase may cause increased peroxide levels during senescence (Becana *et al.* 1986). H_2O_2 can oxidise ferro-lb and can combine with O_2 to form the highly oxidant $OH^{\cdot}$. H_2O_2 can also lead to lipid peroxidation of membranes thus affecting the overall cell metabolism. Dhindsa *et al.* (1981) suggested that leaf senescence may be a consequence of cumulative membrane deterioration due to increasing level of lipid peroxidation probably controlled by, among other factors, activities of SOD and catalase.

The presence of an alternative H_2O_2 scavenging system, which is similar to that identified in chloroplasts, has been reported in soybean nodules by Dalton *et al.* (1986). This system involves the enzymatic reactions of ascorbate glutathione cycle that prevent peroxide caused damage. Two main enzymes of this cycle are ascorbate peroxidase (ASC peroxidase) and glutathione reductase (GSSG reductase) in nodules.

The activities of ASC-peroxidase and GSSG-reductase decreased during natural and induced nodule senescence. The activities of these enzymes were monitored at three stages of nodule development. A 17 % increase in ASC-peroxidase was recorded between 45 and 60 DAS. As the nodules progressed towards senescence the ASC-peroxidase activity declined by 14 % below the control value 45 DAS (Fig. 6*A*). Though quantitatively low, the activity was parallel to the increase and decrease in ARA (Fig. 2*A*). A 24 % increase in GSSG-reductase activity was recorded between 45 and 60 DAS. However, when the nodules progressed towards senescence between 60 to 75 DAS, the GSSG-reductase activity dropped to the initial level (45 DAS, Fig. 6*C*). Dalton *et al.* (1986) also reported that in soybean nodules, pattern of change in ASC-peroxidase ran parallel to that in lb concentration and ARA. However, in their studies the GSSG-reductase activity did not increase substantially as nodules matured.

ASC-peroxidase activity showed 12 and 29 % decrease when the plants were exposed to a dark period of 18 and 66 h (Fig. 6*B*). However, there was a relatively smaller decrease in GSSG-reductase activity (Fig. 6*D*). Correlation with the ARA (Fig. 2*B*) and with the lb content (Fig. 4*B*) can be observed.

A comparative study of natural and dark-induced senescence reveals that the both induce a sharp decrease in nitrogenase activity, however, dark-induced decline in nitrogenase activity is not accompanied by a simultaneous decrease in lb content. A parallel decrease in H_2O_2 scavenging enzyme catalase and an increase in peroxidase activity was recorded. The activities of the enzymes ASC-peroxidase and GSSG-reductase varied parallel to the change nitrogenase activity, however, the degree of variation was very small as compared to that in nitrogenase activity.

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