The Effect of Drought Stress on Nitrogenase and Antioxidant Enzymes Activities in Nodules Formed from Symbiosis of Chickpea with Two Strains of *Mesorhizobium ciceri*

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**Abstract:** Chickpea plants were inoculated with two strains of *Mesorhizobium ciceri*: C-15 and CP-36 strains in order to evaluate activities of nitrogenase and antioxidant enzymes in nodules performance under drought stress as well as control condition after 15 days of imposition of drought stress. Drought stress decreased nitrogen fixation in both strains but the magnitude of decline in inoculated plants with CP-36 strain was higher than that of strain C-15. Under drought stress, nodular guaiacol peroxidase (POX) activity increased in both strains but was higher in nodules produced by C-15 strain. Ascorbate peroxidase (APX) increased significantly in nodules of symbiosis of chickpea with C-15 strain. These results showed that plants inoculated with C-15 strain had high tolerance for drought stress as compared to CP-36 strain which probably is related to increase antioxidant enzymes activities (APX and POX) under drought condition.

**Key words:** Chickpea • Drought stress • Nitrogen fixation • Antioxidant enzymes • *Mesorhizobium ciceri*

**INTRODUCTION**

Chickpea’s seeds are an energy-giving product and a protein source to human nourishment mainly in developing countries [1]. This legume is the third most cultivated grain legume in the world [2]. Chickpea is planted on 700000 hectares in Iran and ranks fourth in the world after India, Turkey and Pakistan [3]. Chickpea can fix atmospheric nitrogen through its symbiosis with rhizobia and increases the input of usable nitrogen into the soil and therefore can enable cultivation of crops in many nitrogen poor soils. Cultivation of this legume in rotation with cereal crops can also contribute to the total pool of nitrogen in the soil and improve yield of cereals [4]. Yield potential of this legume depends on the rhizobia association and plant genotype which together influencing the symbiotic performance [5, 6].

Nodules have a high capacity to produce reactive oxygen species (ROS), even though the concentration of free \( \text{O}_2 \) in the central zone is only 5-60 nM [7, 8]. This high capacity for production of ROS arises from the elevated rates of respiration in bacteroids and mitochondria, the abundance of leghemoglobin (Lb), \( \text{O}_2 \) labile proteins and non protein Fe, the presence of enzymes forming \( \text{O}_2 \) radicals and prooxides [9]. Nodules also possess an array of antioxidant metabolites and enzymes that prevent the generation of high oxidizing radicals in which damage lipids, proteins and DNA [1, 10-12]. In several studies have been suggested that antioxidant activities play a crucial role in the protection of nodule formation and the nitrogen-fixing mechanism [2,5,13,14]. Moreover, ROS generation can be enhanced under environmental stresses such as drought [15] which is considered as a major constraint of symbiotic nitrogen fixation and plant production. Drought causes premature senescence of nodules and disturbs the mechanisms of control \( \text{O}_2 \) that are essential for active nitrogen fixation [15]. Due to ability of nodules to generate activated oxygen and their high demand for antioxidant production to keep nodule functioning, this possibility exists that drought change the balance between production and scavenging of activated oxygen, leading to oxidative stress and consequently, affect symbiotic nitrogen fixation. Symbiosis response to oxidative stress includes morphological modifications such as the change of the nodule cortex structure [16] and biological adaptation such as the modulation of antioxidant enzyme expression in nodules. In several reports the relationship between enhanced antioxidant enzyme activities and increased symbiosis resistance to environmental stress has been noted [5, 11].

In present study, the effect of drought stress induced by PEG 6000 (-0.15 MPa) was evaluated on symbiosis of chickpea plants with two strains of *M. ciceri*. 

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The nitrogenase activity (ANA), H₂ production, and activities of antioxidant enzymes such as Catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and glutathione reductase (GR) under control conditions as well as under drought stress were analyzed.

MATERIALS AND METHODS

Biological Material and Growth Conditions: Chick-pea (Cicer arietinum L.) cv. Bivanch seeds were obtained from Dry Land Agricultural Research Institute of Iran. Seeds were sterilized in 80% ethanol for 30s, 5% sodium hypochlorite for 2 min and washed almost ten times in sterilized distilled water and then grown in pots contain fine quartz sand. Plants were inoculated with 1 mL of two Mesorhizobium ciceri strains grown on YEM to an approximate cell density of 10⁹ mL⁻¹. The C-15 strain (provided by Soil and Water Research Institute of Iran) and CP-36 strain (Obained from International Centre for Agricultural Research in the Dry Area (ICARDA) of Syria). The cell suspension of strains was applied to the sand at the base of the stem of each plant at three and six days after emergence. Plants were kept under controlled conditions (25°C/18°C, day/night temperature; 16h/8h, day/night cycles; 70% relative humidity and 360 μmol m⁻² s⁻¹ light intensity). Plants were kept at about 80% field capacity for 3 weeks by the addition of a nutrient solution without nitrogen included the following concentration of macro and micro-elements: CaCl₂ (3.3 mM), MgSO₄ (2.05 mM), K₂SO₄ (1.25 mM), KH₂PO₄ (0.35 mM), H₂BO₃ (4μM), ZnSO₄ (1.5μM), CuSO₄ (1.55 μM), MnSO₄ (6.6 μM), NaMoO₄ (0.12 μM), FeEDTA (40μM) (34). The pH was buffered with 0.25 mM MES [2-(N-morpholino)ethane-sulfonic acid] and adjusted to 6.8 by adding KOH. Three-week-old plants were carefully transferred to glass tubes (h=600 mm, inner diameter=20 mm) filled with nutrient solution without nitrogen as suggested by Vadez et al. [17]. The tubes were closed with a rubber stopper at the lower side. Plants were put through a hole in a rubber stopper at the upper side of the tube and held at their stem with sponge. Nutrient solution was changed daily and aerated with an air flow of 400 ml min⁻¹.

For induction of drought stress, four-week-old plants were subjected to PEG 6000 of water potential -0.15 MPa. For 0, 5, 10 and 15 days after drought stress ended.

H₂ Evolution Measurements: The measurement of H₂ evolution is an indirect parameter for the determination of N₂ fixation activity of legume nodules [8]. For the H₂ evolution measurement, the sealed root/nodule compartment was connected to an open-flow gas exchange measurement system that allowed the application of a mixture of N₂/O₂ (80/20, v/v) to the root/nodule compartment. A gas flow of 200 mL min⁻¹ (about 1.2 vols min⁻¹) was applied to the root compartment. A sub-sample (100 mL min⁻¹) of the out-flowed gas was taken, dried (ice trap and MgCl₂) and passed through an H₂ analyzer (SL121 Hydrogen analyzer, Qubit Systems, Canada). When a stable H₂ outflow from the root/nodule compartment was reached, this value was taken as the apparent nitrogenase activity (ANA). The amount of fixed nitrogen per unit time per plant was calculated on the basis of the ANA measurements [18]. H₂ evolution was measured at 0, 5, 10, and 15 days after drought stress imposed.

Preparation of Enzyme Extracts: Extracts were prepared by homogenizing 0.1 g of fresh nodules in a mortar with 1.5 ml of ice cold 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mΜ EDTA, 1 mM PMSF as a protease inhibitor and 10% (w/w) PVP. Extracts were centrifuged at 13,000g for 20 min and the supernatant was used to determine antioxidant enzyme activities. Protein content was measured according to the Bradford’s method [19].

Enzyme Assays: Peroxidase (POX, EC 1.11.1.7) activity was determined as described in Bergmeyer [20]. Catalase (CAT, EC 1.11.1.6) activity was assayed according to the method of Aebi [21]. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed using the method of Nakano and Asada [22] and glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Foyer and Halliwell [23].

Statistical Analysis: Data were analyzed using SPSS (version 13.0) by two-way analysis of variance (ANOVA) and significant differences among treatment means and the control groups were determined using LSD (P ≤ 0.05).

RESULTS

Effect of Drought Stress on Nitrogenase Activity (ANA): In order to evaluate the effect of drought stress on nitrogen fixation, apparent nitrogenase activity (ANA) was measured using H₂ evolution values in non-stress and stress plants (Fig. 1). Under control conditions, ANA in chickpea plants inoculated with C-15 strain was 38% greater than ANA in chickpea plants inoculated
Fig. 1: Effect of drought stress on symbiotic nitrogen fixation as measured by apparent nitrogenase activity (ANA) in chickpea plants inoculated with *M. ciceri* C-15 (A) and CP-36 (B). ANA was measured at four times, namely: beginning of the drought (day 0), 5, 10 and 15 days after drought stress imposition. In parallel, ANA was measured in plants grown under control conditions in both symbioses. Values are the means±SD of three replicates. An asterisk (*) represent significant differences with the corresponding control value at P≤0.05.

Fig. 2: Effect of drought stress on antioxidant enzyme activities: CAT, APX, POX and GR in chickpea plants inoculated with *M. ciceri* C-15 (A, B, C and D) and CP-36 (E, F, G and H). Nodule samples were harvested at Four different times, namely: beginning of the drought (day 0), 5, 10 and 15 days after drought stress imposition. In parallel, plants grown under control conditions in both symbioses were harvested. Values are the means±SD of three replicates. An asterisk (*) represent significant differences with the corresponding control value at P≤0.05.
with CP-36. In drought conditions, ANA was affected with different extent according to the inoculated rhizobia strain. In chickpea plants inoculated with C-15 strain, ANA decreased 15.5, 27.3 and 43.1% after drought imposed for 5, 10 and 15 days respectively (Fig. 1A), whereas, in plants inoculated with CP-36 strain, ANA declined 31, 46 and 72% for 5, 10 and 15 days respectively (Fig. 1B).

**Response of Antioxidant Enzymes to Drought Stress:**
The antioxidant enzymes assay in nodules under drought stress showed that the rate of enzymes activities were different depending on strain and the drought stress levels were used (Fig. 2). Under control conditions, at the beginning of drought stress CAT and APX activities in chickpea plants nodules inoculated with C-15 strain were 16.5 and 62% higher than those inoculated with CP-36, respectively. However in both strains, FOX and GR activities under control condition were similar. Drought stress imposed for 5, 10 and 15 days did not affect on CAT activity in nodules of C-15 strain (Fig. 2A) in contrast, the nodules of CP-36 strain, CAT activity decreased after 5, 10 and 15 days of drought stress imposed. However the difference in the mean values of CAT activities in CP-36 strain were statistically significance for only 15 days of drought stress imposed (Fig. 2B).

In nodules of CP-36, APX activity did not change compared to the control nodules after application of 5, 10 and 15 days drought stress (Fig. 2F). In symbiosis of chickpea plants with C-15 strain, APX activity did not change after 5 days of drought but increased 19.6 and 29.4% relative to the control nodules at 10 and 15 days of drought, respectively (Fig. 2B). In both strains, FOX activity were increased under drought stress but the rate of activity was higher in C-15 strain that CP-36 (Fig. 2 C, O). The plants exposed to drought stress for 5, 10 and 15 days, the FOX activity in nodules of C-15 strain increased 14.4, 25 and 38% compared to the control plants respectively (Fig. 2C). The rate of activities were less for CP-36 but the rates were increased to 10.5, 18.5 and 22.6% in 5, 10 and 15 days of drought respectively in C-15 strain (Fig. 2G).

GR activity in nodules of two strains did not differ under drought stress compared to control nodules (Fig. 2D, H).

**DISCUSSION**

Selection of suitable Rhizobia strain is an important factor in effectiveness of symbiosis. In several reports has been noted the high contribution of inoculated rhizobial strain to the variance of nitrogen fixation activity [11, 24, 25]. The results of present study showed that nitrogenase activity (ANA) under control condition in chickpea plants inoculated with C-15 strain was higher than that in chickpea inoculated with CP-36 strain. Consequently, it could be concluded that C-15 is a better homolog to chickpea than CP-36.

Nitrogen fixation process is a very sensitive to environmental stresses [26, 27]. In several studies have shown that nitrogen fixation declines significantly under stress conditions such as salt stress [11, 28] and osmotic stress [25, 29]. The result of this study showed that drought stress decreases the nitrogenase activity in chickpea inoculated with both strains but the magnitude of decline in C-15 strain was higher than that in CP-36. Therefore, the contribution of the rhizobia strain on variation of an effectiveness system, even though in drought condition, suggested that selection of suitable strain could lead to the enhancement of chickpea production in soils with nitrogen deficiency. Similar reports have been noted same suggestion for some other legumes [26, 27].

Drought stress induces oxidative stress in nodules [15] that causes premature senescence of nodules and declines the nitrogen fixation [13]. Therefore, an efficient antioxidant system is critical for N fixation in symbiosis. There is a correlation between CAT activities and the nitrogen-fixing capacity [11] and therefore CAT may be involved in having a better system of nitrogen fixation. However in several studies have been reported that CAT activity decreases under stress conditions [5, 22, 25]. The results of this study indicated that CAT activity in control condition was higher in plants inoculated with C-15 strain than that in CP-36, in which this was in parallel to higher nitrogen-fixing capacity in C-15 strain. In drought condition the reduction of nitrogenase activity in CP-36 strain was similar to the decline of CAT activates. Therefore this result confirms the important role of CAT activity in nodule functioning and also suggests that CAT can act as biochemical marker of symbiotic efficiency [5, 25]. The same conclusions were reported in *Sinorhizobium meliloti-Medicago sativa* symbiosis [30] and chickpea-Rhizobia associations [5, 25].

POX is an important enzyme in the antioxidant defense of nodules which is critical for scavenging of H$_2$O$_2$ [31]. The induction and protective role of POX under osmotic stress [25, 29], salt stress [5] and low temperature [32] were reported in legume-rhizobia nodules. Our data indicated that drought stress stimulated POX activity in both strains and POX was negatively correlated with inhibition of nitrogen-fixing capacity (ANA). POX was stimulated more in plants inoculated with C-15 strain.
which they are more tolerant to drought condition, compared to the plant inoculated with CP-36. Therefore, POX may play an important role in antioxidant response of plants against drought stress and also its induction could provide a sensitive marker for recognizing of tolerant symbioses to drought. The similar results were obtained under water stress condition for \textit{M. sativa} [23] and \textit{P. vulgaris} [33].

An important antioxidant mechanism in the nodules is ascorbate-glutathione cycle, which resulting detoxification of H$_2$O$_2$ in expense of NAD(P)H [16]. This cycle should be active during nitrogen fixation, since nitrogenase activity increases the production of H$_2$O$_2$ and superoxide radicals [16]. Reports show that enzymatic (GR and APX) activities of this cycle were higher in nodules with higher nitrogen fixation capacity [11]. In this study, under control conditions, APX activity in nodules formed by C-15 strain which showed the best symbiotic efficiency was higher than that of the nodules of non-local strain. Therefore, there is a positive relationship between nodule functioning and APX activity. Results of present study indicated that in symbiosis of C-15 strain with higher nitrogenase activity (ANA) than that in nodules of CP-36 strain under drought stress, APX activity was stimulated significantly whereas, APX activity did not induce under drought conditions in CP-36 strain. Induction of APX activity and its protective role under stress conditions have been widely reported for different plants [13, 22, 31, 32, 34].

GR is responsible to generate ascorbate and glutathione which are the main antioxidant in legume nodules [13, 35]. However the GR activity in stress condition is varied. In some reports has been mentioned that GR activity decreases in stressed nodules [11, 16]. In contrast, Hernandez-Jimenez \textit{et al.} [31] observed an increase in GR activity in stressed nodules. Results of present work showed that drought stress did not affect GR activity in both strains.

In conclusion, this study confirmed the relation between enhanced APX and POX and increases symbiosis tolerant to drought stress. Therefore antioxidant defense seems to be obtained primarily by guaiacol and ascorbat peroxidase (POX and APX) activities.

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