SCIENTIFIC NOTE

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# Production of reactive oxygen species in peanut nodules

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**ABSTRACT**

In this study, it was investigated the reactive oxygen species (ROS) production in peanut nodules at three peanut growth stages (R): R1 (flowering), R4 (full pod) and R6 (full seed). Analysis of ROS production showed that superoxide anion and hydrogen peroxide contents decreased while lipid peroxidation and protein oxidation remained unchanged throughout nodule development. Furthermore, it was found that the inside of formed nodules was 100% red at (R1), 54% at (R4) and 39% at (R6), respectively. The total soluble protein content decreased while leghemoglobin content remained unaltered at different growth stages. Thus, our findings suggest that ROS production is not involved in the peanut nodule senescence indicating that this process would not be accompanied by an oxidative burst. It is possible to suggest that the antioxidant system would play an important role in the protection of peanut nodules against ROS production.

**Keywords:** Peanut, nodule, reactive oxygen species, senescence.

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## INTRODUCTION

Legume nodules are unique symbiotic organs that develop on the roots and, in a few species, also on the stems, after infection with rhizobia. The plant provides sucrose to nodule host cells, where it is oxidized to dicarboxylic acids and used as energy source by the bacteroids to fix atmospheric nitrogen. Nodules have a high potential for the production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^{\cdot}$). This is due, among other factors, to the acid pH (5.5–6.5) and high leghemoglobin (Lb) concentration (1–2 mM) in the infected cells, the elevated rates of bacteroid respiration, and the abundance of oxygen-labile proteins. The ROS molecules are highly toxic, since they are able to modify all the primary constituents of the cell such as lipids, DNA, carbohydrates, and proteins leading to senescence and cell death. However, nodules possess an array of antioxidant metabolites and enzymes that prevent the generation of highly oxidizing radicals and hence the damage of molecules. In addition, antioxidants regulate the intracellular concentrations of $O_2^{\cdot-}$ and $H_2O_2$, which serve useful purposes in signaling stressful situations and in activating defense genes (Mittler et al. 2004).

ROS are also involved in the nodule senescence process, and it has been proposed that these molecules and antioxidants interact with hormones such as abscisic acid in the orchestration of the natural aging process (Puppo et al. 2005). Nodule senescence is the result of a cluster of physiological, biochemical and morphological alterations induced by natural aging

and stressing conditions. In nodules of some legumes such as bean, soybean and lupine, it has been demonstrated that during the natural senescence process the nitrogen fixing and the antioxidant defense system activities decreased, while the ROS production and the oxidation of Lb increase, with the consequent damage of biomolecules (Matamoros et al. 2003). Unlike these legume nodules whose natural senescence process has been studied, no research on this process has been conducted in peanut (*Arachis hypogaea* L.) nodules.

Peanut is usually nodulated by slow-growing rhizobia of the genus *Bradyrhizobium* (Fabra et al. 2010). The symbiotic association between peanut and rhizobia is especially interesting because there are no cell-to-cell infection threads as in other legume–rhizobia symbiosis such as *Bradyrhizobium japonicun-soybean* and *Rhizobium tropici*–bean. Instead, infection through the epidermis involves intercellular penetration (crack entry), at the point of emergence of lateral roots. Thus, several of the phenomena that are essential in the infection thread mode do not occur in the crack entry infection process. Maiti et al. (2012) reported that, in terms of ROS production, nodule environment of crack entry legumes is different from those legumes where infection threads are formed. The authors propose that exchange of redox molecules and reactive chemical species is possible between the bacteroid and nodule compartment. Taking this into account, the objective of the present work was to investigate the reactive oxygen species production in peanut nodules at different growth stages.

## MATERIAL AND METHODS

Seeds of peanut cv Granoleico (Criadero El Carmen, General Cabrera, Córdoba, Argentina) were surface sterilized (Vincent 1970) and germinated for 96 h. After germination, seeds were transferred to pots with sterile volcanic sand. Seven days after sowing, plants were inoculated with 3 mL of *Bradyrhizobium* sp. SEMIA6144 ($10^8$ cells $mL^{-1}$) culture. Plants were grown in a controlled growth chamber (light intensity: 200 µmol $m^{-2}$ $s^{-1}$; 16-h day/8-h night cycle; 28 ºC; and a relative humidity of 50%) and periodically watered with Hoagland solution without nitrogen. The percentage of red and green nodules were determined from 5 plants which were taken at random and analyzed by cutting and observing at three growth stages R1 (flowering: approximately 30 days after planting), R4 (full pod: approximately 60 days after planting) and R6 (full seed: approximately 90 days after planting).

ROS generation, protein and lipid damages, total soluble protein and leghemoglobin contents were also evaluated. Superoxide generation was measured following the method described by Frahry and Schopfer (2001). For the histochemical detection of $O_2^{\cdot-}$, the nodules were incubated in 10 mM citrate buffer-K (pH 6) containing 0.5 mM nitroblue tetrazolium (NBT). Hydrogen peroxide was measured spectrophotometrically after reaction with KI (Alexieva et al. 2001). For the histochemical detection of $H_2O_2$, a 1 mg $L^{-1}$ solution of 3,3´-diaminobenzidine (DAB) at pH 3.8 for 8 h under light at 25 °C was used (Orozco-Cardenas and Ryan 1999).

Lipid peroxidation (estimated as malondialdehyde) and protein oxidation (estimated as carbonyl groups) were used as reliable markers of oxidative stress. The level of lipid peroxides was determined as malondialdehyde (MDA) content by the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Protein carbonyl group content was measured by derivatization with 2,4-dinitrophenylhydrazine according to Levine et al. (1990) with some modifications. The total soluble protein content was determined by the method of Bradford (1976), using bovine serum albumin as standard (1 mg/mL). Leghemoglobin content was measured by the method of pyridine-hemocrome (Appleby and Bergersen 1980).

Differences among treatments were analyzed by one-way analysis of variance, and $P < 0.05$ was considered significant according to LSD´s test.

## RESULTS AND DISCUSSION

The results obtained showed that the $O_2^{\cdot-}$ nodular content decreased significantly from R4 (Figure 1A) and this was correlated with its histochemical detection through blue formazan production resulting from the chemical reaction between NBT and $O_2^{\cdot-}$ (Figure 2A). The low $O_2^{\cdot-}$ concentration found in peanut nodules at R4 and R6 was expected, considering that this highly toxic anion is rapidly converted to $H_2O_2$ by superoxide dismutases of plant and bacteroid cells (Rubio et al. 2004). Also, the $H_2O_2$ nodular content decreased significantly at R4 compared with R1 (Figure 1B) and it was correlated with data obtained from the histochemical detection using the DAB reagent (Figure 2B). The lipid peroxides and protein carbonyl groups levels remained relatively constant throughout all the phenological stages evaluated (data not shown). Similar results were found by Groten et al. (2005). These authors reported that the superoxide or hydrogen peroxide content strongly declined with age and that the protein carbonyl groups were almost constant throughout pea nodule development.

It is well-known that senescence in nodules is visible by a color shift in the nitrogen-fixing zone from red, associated with the functional Lb protein, to green, and associated with the degradation of its heme group. A hallmark of nodule senescence is the triggering of a wide range of proteolytic activities that cause large scale protein degradation (Pladys and Vance 1993). Our results showed that the inside of formed nodules was 100% red at R1, 54% at R4 and 39% at R6, respectively (Figure 3A) with a decrease in the protein content (Figure 3B). Lb content remained unchanged at different growth stages (data not shown).

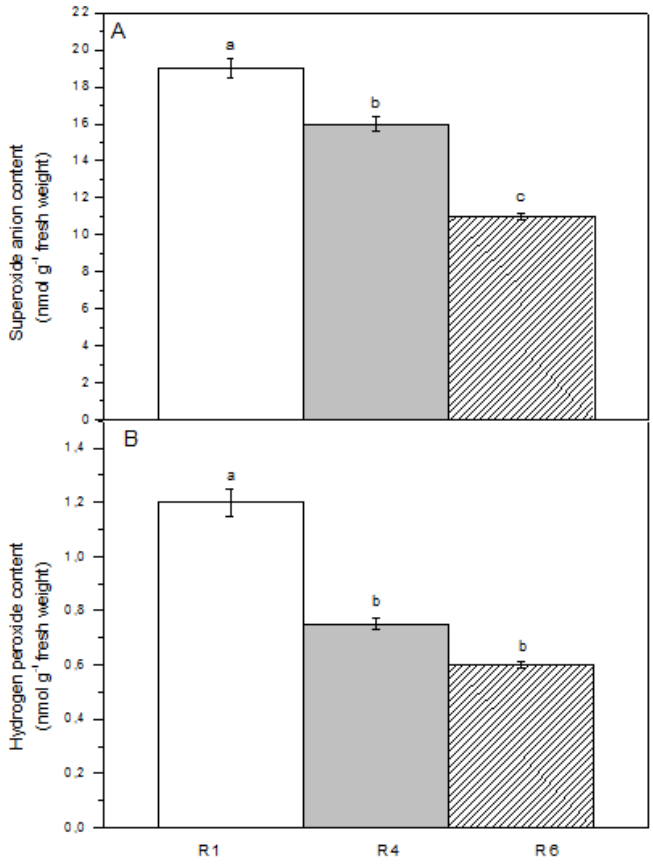


Figure 1. Superoxide anion (A) and hydrogen peroxide (B) contents in peanut nodules at different growth stages. Values are means ± SE (n=5). Different letters indicate significant differences at $P < 0.05$ according to LSD Fisher´s test.

Loscos et al. (2008) reported the coexistence in a single bean plant of nodules at different senescence stages. Our results demonstrated that the peanut plant also contains two types of nodules (red and green) at R4 and R6. Ali and Bano (2008) showed that nodule senescence appears to be correlated with the protein content as well as the sugar assimilation in the host plant which in turn delay the degeneration of red bacteroid tissue of nodules and the nitrogenase activity. In this study, the decrease in total soluble protein content was the only parameter that indicated the state of nodule senescence, coinciding with the results obtained in soybean nodule by Nash and Schulman (1976). Thus, contrary to results found by Puppo et al. (2005) in soybean nodules which revealed that ROS are involved in the senescence process, our findings suggest that ROS production is not implicated in the peanut nodule senescence.

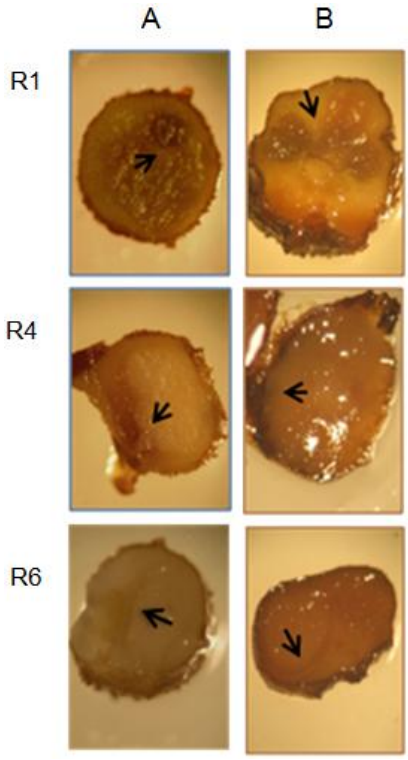


Figure 2. Superoxide anion histochemical detection (A), the arrow indicates formazan precipitated (blue). Hydrogen peroxide histochemical detection (B), the arrow indicates the brownish deposits resulting of the reaction between DAB and $H_2O_2$. Magnification 3.2X5.

## CONCLUSION

These results contribute to the current knowledge about the reactive oxygen species production in peanut nodules at different growth stages indicating that the senescence process would not be accompanied by an oxidative burst. It is possible to suggest that the antioxidant system would play an important role in the protection of peanut nodules against ROS production. Further studies are required to clarify this phenomenon that occurs during development in peanut nodules.

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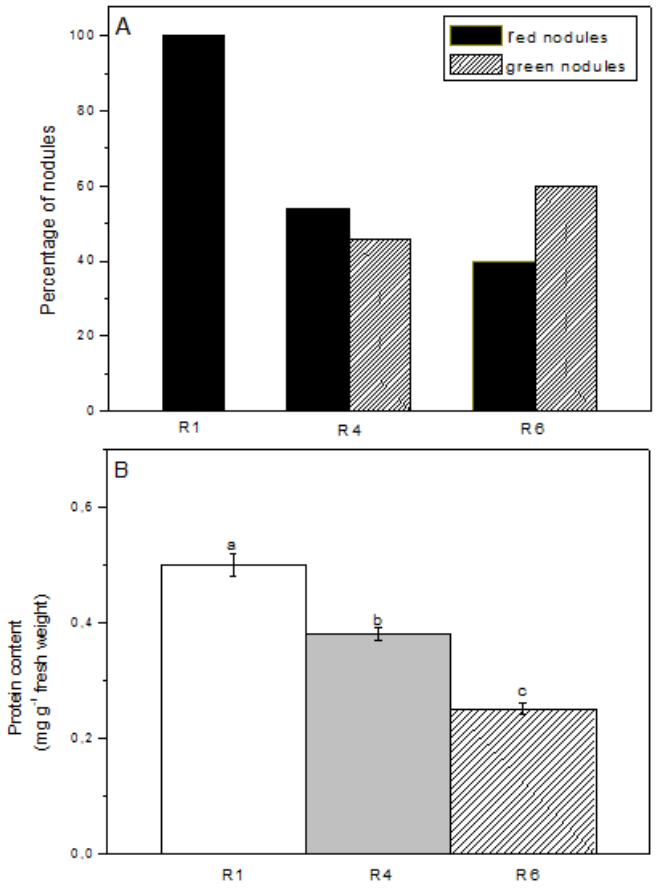


Figure 3. Percentage of red and green nodules at different growth stages (A); values are means of 5 plants. Protein content in peanut nodules at different growth stages (B); values are means ± SE (n=5). Different letters indicate significant differences at $P < 0.05$ according to LSD Fisher´s test.

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