

**Insights and Pitfalls of the Methodology for Free Radical Research in Legume Nodules: Hemoglobins as a Case Study**

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Reactive oxygen (ROS; superoxide radicals and H<sub>2</sub>O<sub>2</sub>) and nitrogen (RNS; NO and nitrosothiols) species are of paramount importance in biology. In plants, ROS and RNS are generated in most cellular compartments. At the light microscopy level, the production of superoxide radicals, H<sub>2</sub>O<sub>2</sub> and NO may be detected based on their reactions with nitroblue tetrazolium, diaminobenzidine and 4,5-diaminofluorescein diacetate, respectively. Additional techniques are the HyPer bioprobe for H<sub>2</sub>O<sub>2</sub> and electron paramagnetic resonance (EPR) spectroscopy for free radicals. However, strict controls must be used to ensure specificity, discard interferences and minimize artifacts during tissue extraction. In the rhizobia-legume symbiosis, all three reactive species were detected in the infection threads and in mature and senescent nodules. Leghemoglobins (Lbs) are heme proteins found at millimolar concentrations in the cytoplasm of infected cells, where they transport and deliver O<sub>2</sub> to the symbiosomes. We have used two experimental approaches involving Lbs to localize ROS and RNS in nodules and identify potential pitfalls of the methodology. The first approach was to detect the nitrosyl-Lb complex by low temperature EPR of intact soybean nodules formed by wild-type and mutant bradyrhizobia deficient in denitrification enzymes. A comparison was made between the EPR and the fluorescent probe to detect NO. We conclude that bacteroid nitrite and NO reductases are essential to keep nodule NO homeostasis, that NO accumulates in both the inner cortex and infected zone, that Lbs may scavenge NO *in vivo*, and that NO is artifactually generated in nodule extracts but not in intact nodules. The second approach was to localize ROS in mutant *Lotus japonicus* nodules completely lacking Lbs. We found a drastic increase in superoxide production caused, at least in part, by induction of NADPH oxidases and changes in superoxide dismutase composition. The mutant nodules also showed an increase of H<sub>2</sub>O<sub>2</sub>. Taken together, our observations suggest that Lbs contribute to control the nodule concentrations of ROS and RNS.