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Hydrogen Peroxide Mediated Oxidative Stress Endurance in *Azorhizobium Caulinodans* -ORS-571

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The process of symbiotic nitrogen fixation results in generation of reactive oxygen species such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The response of *Azorhizobium caulinodans* to these toxic oxygen species is an important factor in nodulation and nitrogen fixation. It has been found that pretreatment of *A.caulinodans* with low, non-lethal levels of a selective oxidant viz., H_2O_2 (200μM) imparts resistance to oxidative mortality. The catalase activity was also found to be higher in H_2O_2 pre-treated cells than untreated controls.

Keywords: *Azorhizobium caulinodans*, Catalase, Hydrogen peroxide.

Azorhizobium caulinodans, a composite of both diazotrophic bacteria and symbiotic rhizobia avidly fixes N_2 both in culture and in plants(Dreyfus and Dommergues, 1981) for these two disparate N_2 fixation processes, dissolved O_2 optima vary some orders of magnitude: 10µM in culture versus 10mM in plants (Bergersen *et al.,* 1986; Buckmiller *et al.,* 1991). As Biological N_2 fixation is extremely O_2 sensitive, N_2-fixing organisms have evolved O_2 detoxification and oxidative avoidance mechanisms.

Cellular metabolism of molecular oxygen produces reactive and potentially toxic oxygen species such as superoxide, hydroxyl radicals and hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1989). To defend the reactive oxygen species, microorganisms contain antioxidants and enzymes that prevent or repair oxidative damage. Catalases are haem-containing enzymes, disassociating H_2O_2 to O_2 and H_2O. These enzymes play an important role in reducing the formation of the highly reactive hydroxyl radical, which arises from the degradation of H_2O_2 via the Fenton reaction (Halliwell and Gutteridge, 1989). The response of bacteria to initiate oxidative stress has been most extensively studied in *Escherichia coli*(Demple and Brook 1983). Catalase activity is often described as essential for aerobic life and recently it has been realized that there are atleast three unrelated sequence families that code for enzymes with catalase activity. These are true catalase, catalase peroxidase and Mn-catalase peroxidases that are a distinct subclass of the haem peroxidase super family of enzymes (Melonder, 1991).

In the early stage of interaction between rhizobia and legumes, reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) superoxide anion (O_2) and hydroxyl radical (OH) are generated. This ROS generation is similar to that seen when pathogens infect host plants (Lambert and Dixon, 1997; Santos *et al.,* 2000). It has been confirmed that H_2O_2 plays a supportive role in nodule initiation by mediating nod factor responses between *Sesbania rostrata* and *Azorhizobium caulinodans* (Haeze *et al.,* 2003) and through the analysis of mutations in catalase genes, H_2O_2 has also been found to inhibit

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infection thread formation and nodule development (Herouert,1996; Sigaud *et al.,* 1999) *Azorhizobium caulinodans* ORS-571 obtained from IMTECH Chandigarh, India was used in this work and grown in YEM medium (Vincent, 1970) consisting of (gl^{-1}) yeast extract (0.4), K_2HPO_4 (0.5), NaCl (0.1) $MgSO_4.7H_20$ (0.2) mannitol (10), or TY medium comprising ($g1^{-1}$) bacterialtryptone (5), yeast extract (3), $CaCl_2.6H_2O$ (1-3) for solid medium.

Azorhizobium caulinodans was inoculated into YEM broth and grown with shaking at 30° C to OD 600 0.2 (4×10^6 cfu. ml^{-1}) aliquots (5ml) of the culture were transferred into sterile tubes and H_2O_2 was added to the desired final concentration and incubated as before. Samples were taken immediately prior to and periodically after H_2O_2 addition, diluted in YEM minus mannitol and plated into TY agar to monitor the cell viability. Colonies were counted after 2 days incubation at 30°C. For H_2O_2 adaptation experiments overnight cultures were used to inoculate 50ml YEM to a final OD 600 0.01 (2×10^5 cfu ml^{-1}) when the culture had grown to OD 600 0.2, (4×10^6 cfu. ml^{-1}) H_2O_2 was added to a final concentration of 100,200 and 300μM after that the pretreated cells had been incubated with 3mM $H_2O_2$for 2h, a sample was taken at periodic intervals for viable counting.

For assay of catalase activity, culture samples were centrifuged at 10000 x g for 10 min and the resultant cell pellet was washed twice in 50 mM potassium phosphate butter (pH 7.0) before being re-suspended in the same buffer prior to the assay. Catalase activity was determined by measuring O_2 evolution from cell suspensions following addition of 0.5 mM H_2O_2 as described by Perth and Jensen (1967) using a clark-type oxygen electrode. Catalase units are expressed as mmo1 O_2 evolved min^{-1} (mg protein)$^{-1}$.

Protein was determined as described by Markwell *et al*., 1978, using bovine serum albumin (2mg ml^{-1}) as the standard.

All experiments were performed at least three times (unless indicated otherwise) and were highly reproducible. Therefore, data from one replicate is presented below. Data were subjected to Duncan's Multiple Range Test (DMRT) using SPSS7.0 statistical pakage.

The adaptation of various concentration of H_2O_2 on the viability of *Azorhizobium caulinodans*, cultures were examined by treating the exponentially growing cells with non-lethal concentration of H_2O_2 100, 200 and 300 μm for 120 minutes, and then testing it viability against 3mM H_2O_2

The results pertaining to the Table 1 shows that the pre-treated cells with 200 H_2O_2 were more resistant to 3mM H_2O_2 as compared to the untreated cells. Cells treated with 200 H_2O_2 in the presence of protein synthesis inhibitor chloramphenicol did not acquire any resistance to 3mM H_2O_2 (data not shown) indicating that protein synthesis is required for adaptation of H_2O_2.

In this study we have shown that, pretreated *A. caulinodans* adopts to normally lethal levels of the stress (3mM H_2O_2), this

Table 1. Adaptation of *Azorhizobium caulinodans* to pretreatment of different concentrations of H_2O_2 survival population

Pretreatment concentration of H_2O_2	Time in hrs during challenge inoculation with H_2O_2 0.5	1.0	1.5	2.0	2.5
Control	3.0±0.26x10^{6}d	1.8±0.22 x10^{6}d	8.0± 0.64x10^{5}d	2.0±0.18 x10^{5}d	1.0±0.20x10^{4}d
100 mm	3.60.32x10^{6}b	2.8±0.26 x10^{6}b	3.0±0.30 x10^{6}b	3.2± 0.34x10^{6}b	4.4±0.40 x10^{6}b
200 mm	3.8±0.34x10^{6a}a	3.2± 0.30x10^{6}a	3.4± 0.32x10^{6}a	3.6±0.44 x10^{6}a	3.8±0.36 x10^{6}a
300 mm	3.2±0.36 x10^{6}c	2.4± 0.24x10^{6}c	2.6±0.26 x10^{6}c	3.0± 0.40x10^{6}c	3.4±0.32 x10^{6}c

*Initial inoculation load 4.0x10^6

Values are mean ±SD 0f for three replicates from one representative experiment and each experiment was carried out 3 times, and similar results were obtained each time, within a column different letters after values indicate that there is a significant difference at p value of 0.05, as determined by one way analysis of variance followed by DMRT.

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