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Growth and N_2 fixation in *Sesbania rostrata* by H_2O_2 pretreated *Azorhizobium caulinodans* and it's effect as green manure on lowland rice

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The response of *Azorhizobium caulinodans* to reactive oxygen species such as hydrogen peroxide (H_2O_2) plays an important role in nodulation and nitrogen fixation. It has been found that pretreatment of *A.caulinodans* with a selective oxidant viz., H_2O_2 (200µM) has enhanced the osmotic, thermal, and desiccation tolerence. The survivability in different carrier materials was found to be enhanced by H_2O_2 pretreatment as compared to untreated controls. A pot culture study was conducted to compare the efficiency of H_2O_2 pretreated cells for nodulation, plant dry wt and ARA activity in *Sesbania rostrata*, followed by the *Sesbania* incorporation as green manure for rice grown under pot culture condition. The crop was studied for certain plant growth and yield components, such as plant ht, plant dry wt, no of panicles, total "N" uptake. The results of our present study have showed that the plant inoculated with H_2O_2 pre-treated cells of *A. caulinodans* positively augmented an increase in the growth and yield in both *Sesbania rostrata* as well for rice grown under flooded conditions in the pot culture experiment.

Key words: *Azorhizobium caulinodans*, hydrogen peroxide, carrier material, rurvival, rice

INTRODUCTION

Nitrogen is one of the key nutrients that most frequently limit the rice production. Biological nitrogen fixation (BNF) is a fascinating phenomenon, which involves a highly specialized and intricately evolved interaction between soil microorganisms and higher plants, harnessing the atmospheric elemental nitrogen. One of the most exciting recent advances in Biological Nitrogen Fixation (BNF) is the development of an annual tropical African legume shrub, *Sesbania rostrata*, which grows rapidly in the wet season, producing high levels of biomass even in flooded conditions and exhibits higher rates of nitrogen accumulation, which makes this species one of the most valuable green manure (Ndoye et al. 1988, Boivin et al. 1997) *Sesbania rostrata* estabilishes a highly specific interaction with genus *Azorhizobium caulinodans* (Dreyfus et al. 1988), which induces effective nodules on the stem and root of *Sesbania rostrata* and also posses a unique capacity among rhizobia to fix N_2 in the free living state in culture and in plants (Dreyfus and Dommergues 1981, Dreyfus et al. 1988). It has been found that for these two disparate N_2 fixation processes, dissolved O_2 optima may vary some orders of magnitude: 10µM in culture versus 10mM in plants (Bergersen et al. 1986, Buckmiller et al. 1991).

Biological N_2 fixation is extremely O_2 sensitive, since the 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 these reactive oxygen species, microorganisms produce certain antioxidants and enzymes that prevent or repair oxidative damage. One among them are catalases, which are haem-containing enzymes, disassociating H_2O_2 to O_2 and H_2O, which play a role in the reduction of 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).

In the early stage of interaction between rhizobia and legumes, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH) are generated and 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 the nodule initiation by mediating the nod factor responses between *Sesbania rostrata* and *Azorhizobium caulinodans* (Haeze et al. 2003). Although there has been numerous reports regarding the H_2O_2 mediated catalase enzyme induction in rhizobium and its subsequent effect on the nodulation in leguminous crops (Santoz et al. 2001, Jamet et al. 2002, Carmen-Vergas et al. 2003). Little is known about the survival of H_2O_2 adapted symbiotic nitrogen fixing bacteria under adverse soil conditions and its role on nodulation in legumes(Graham 1992, Kitts and Ludwig 1994).

Our previous study (Joe and Sivakumaar 2007) has showed that pre-treatment of *A. caulinodans* cells with 200µM H_2O_2 has imparted a higher resistance to further H_2O_2 exposure i.e., up to 3mM H_2O_2 treatment as compared to the untreated control. It has been also demonstrated that these pretreated exponential cultures also typically showed a moderate increase in catalase activity as compared to the untreated control. This fascinated us to undertake the present study to explore the ability of pretreated H_2O_2 cells with enhanced

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catalase activity for survival under different created stress conditions and also in different carrier materials. Additionally we assessed the ability of the H_2O_2 preated cells of *Azorhizobium caulinodans* for its nodulation, plant dry wt and ARA activity in *Sesbania rostrata*, followed by incorporation of the same as green manure for rice grown under pot culture condition.

MATERIALS AND METHODS

Culture and Growth conditions

Azorhizobium caulinodans ORS-571 obtained from IMTECH Chandigarh, India was used in this work and grown in YEM(Yeast Extract Manitol) broth(Vincent, 1970) consisting of ($g1^{-1}$) 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.

H_2O_2 pretreatment of *Azorhizobium caulinodans* for adaptation experiments

Azorbizobium caulinodans was inoculated into YEM broth and grown with shaking at 30° C to a value of 0.2 at OD 600 (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 of 300µM 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. The surviving colonies after 2 days incubation at 30°C were used for the further study. The protocol was repeated about three times and the surviving cells were used for further study.

Desiccation resistance and thermal tolerance

The desiccation resistance of H_2O_2 pretreated cells was determined as per the methods as determined by Bleaky et al. 1998 with a slight modification.. The cells of *Azorhizobium* (1 X 10^9 were suspended in YEM (Yeast extract manitol) medium placed in a desiccator oven for 24 h at 25˚C and then subjected to an 8-day old incubation at 30˚C.

Initial cell numbers, before and after the drying treatment were determined by plate count. For enumeration of the cells surviving the desiccation 2.0ml of minimal salts solution was added to each plate, and the cells from the dried film were suspended with scraping and mixing and then the enumeration of the bacteria was done. For thermal tolerance experiment 1ml cell suspension (1 x 10^9 cfu/ml) of culture was kept in a water bath 45˚ for 60 min and the survival per cent of bacteria was determined.

Osmotic tolerance and Osmotic shock

The sensitivity of the cells to osmotic tolerance and osmotic shock were done as per the methods as determined by Kadouri et al.(2003) in which 25-ml portion of 4M glucose solutions were added of bacterial suspensions, The final glucose concentration were adjusted to 2M. The bacteria were incubated at 30˚ for 24 h. Sensitivity to osmotic shock was determined by adding 25ml of Tris-glycerol solution(0.05M tris, 4M glycerol; pH 7.6) to 25 ml of cell suspension and incubating the preparation for 30 min at 30˚C. The cells were then centrifuged (4000 x g, 10 min) and then re-suspended in 50ml of distilled water. Bacterial viability was determined by plate count.

Survivability in different Inoculant carriers

The survivability in different carier materials were tested as per the methods of Falik and Okon (1996). One ml bacterial suspension was mixed with one of the following autoclaved carriers, such as vermiculte, lignite and peat. Inoculants were stored in sterile flasks at 30˚C. After 30 days incubation, the carriers have undergone desiccation to different extends. One gram of each sample was added to potassium phosphate buffer (0.06M, pH 6.8) and stirred at 200 rpm for 2 h at 30˚C and their bacterial viability was determined.

Pot culture experiment

The experiment was conducted in the Department of Microbiology, Faculty of Agriculture, Annamalai University in the period of Oct to Dec-2006.

The soil were sieved through a 20-mesh, thoroughly mixed, and placed in clay pots (15cm diameter), which were kept continuously flooded. Each pot was given a basal dose of triple super phosphate (37.5mg P_2O_5), murrate of potash (25mg K_2O) and ammonium molybdate (0.625mg), before *S.rostrata* crop.

Treatment

Two cycles of alternate *S. rostrata* and rice were grown under continuously flooded condition during Sep – Dec 2006. In both the experiments the H_2O_2 pre treated cells were compared for their efficiency against the vegetative cells of *A. caulinodans* six replications were maintained for each treatment.

The treatments were maintained in two sets to meet the above two objectives of the study. In the first set the treatments were compared in order to investigate the effects of H_2O_2 pretreated cells on nodulation, acetylene reduction activity and plant biomass of *S. rostrata.* In the second set of the treatment the effect of the pretreated cells were evaluated for their efficiency in rice for yield and N balance.

Pot culture and green manure incorporation experiment

Seed priming with A. caulinodans

Seeds of *S. rostrata* coated with either vegetative cells of *S. rostrata* or H_2O_2 pretreated cells of *A. caulinodans* were sown. The plants were thinned to three per pot after 5 days of planting. The shoots of *S. rostrata* 45 days after incorporated in to the soil were cut into small pieces (3-6 cm) and mixed thoroughly along with roots into the soil. Fifteen days old seedlings(excluding 15 days in seed bed) of rice were transplanted into each pot 5-10 days after incorporation of green

manure, the rice plants were grown for an average of 90 days

Azorhizobium inoculation

For seed treatment the seeds were treated with either H_2O_2 pretreated or vegetative cells at 10 ml per pot (Minimum inoculation load of 1 x 10^9) mixed with lignite, 5 ml of rice gruel to enhance the adhesiveness.

Acetylene Reduction Activity, Dry wt and N determination

The acetylene reduction activity, dry wt of stem and root nodules, dry wt and N content of the plant were measured 45 days after emergence. Four replicates pots of each treatment were randomly uprooted and the soil carefully washed off. The plant were excised at about 2-3 cm above the crown and the aerial stem portions of three plants from each pot were separately pooled in a plastic bag (15 x 30 cm) and the acetylene reduction activity was carried out as per the methods as determined by Ladha et al. (1992).

The plant dry wt, the number of root and stem nodules of the *Sesbania* plant used for assay and the rice growth and yield parameters such as plant ht, number of tillers, number of panicles, grain and straw yield were determined using the standard methods. The total N content of the plant was determined using the Microkheldal assay

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and the treatment means were compared relative to control following a post hoc test or least significant difference (LSD) test. Unless indicated otherwise, differences were only considered when significant at P<0.05.

RESULTS AND DISCUSSION

It has been clearly evident from the Table 1 that H_2O_2 preated *Azorhobium caulinodans* cells exhibited a higher thermal tolerance, desiccation tolerence, osmotic tolerance and shock as compared to the untreated vegetative cells of A. *caulinodans.* The results of our present study has clearly demonstrated that, pretreated cultures of *A. caulinodans* adopts to normal levels of created stress, this adaptive response is similar to those previously described for enteric bacteria Christman et al. (1989), in *B. subtilus* (Dowds et al. 1987) and in yeast (Jamieson 1992).

The H_2O_2 pretreated *Azorhobium caulinodans* cells have exhibited a higher survival percentage as compared to the untreated vegetative cells in different carrier materials. The fact that H_2O_2 pretreated *Azorhobium caulinodans* cells exhibited increased stress endurance is of greater importance for commercial bacterial inoculants. Stress endurance varies according to the inoculant preparation and the storage conditions (Kadouri et al. 2003). The survivability of inoculated bacteri has been significantly reduced after a six months storage period (Falik and Okon 1996). This reduction in the inoculated population of bacteria may probably due to the stress that developed during storage under suboptimal conditions, such as lack of moisture, stress and available nutrients. The role of catalase in enhancing the survival during starvation periods has been reported in various bacteria (Jamieson 1992, Christman et al. 1989).

Table 1. Studies on the desiccation, thermal and osmotic tolerance of H_2O_2 pretreated cells of A. *caulinodans*

Treatment	Number of viable cells/ ml after different stress challenge*			
	Desiccation tolerence**	Thermal tolerance**	Osmotic tolerance***	Osmotic shock***
Control	(4.2±1.02) x $10^{6\,b}$	(2.2± 0.84) x $10^{5\,b}$	(3.9 ±1.21) x $10^{4\,b}$	(4.1±1.10) x $10^{5\,b}$
H_2O_2 Pretreated	(7.9 ±1.53) x $10^{8\,a}$	(3.5±0.75) x $10^{8\,a}$	(7.2±1.42) x $10^{7\,a}$	(4.3±1.24) x $10^{8\,a}$

Initial inoculation load 1.0 x 10^9

** assayed according to Bleaky et al. (1998)

*** assayed according to Kadouri et al. (2003)

Values are Mean ±SD of 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 a post hoc test.

This higher degree of tolerance exhibited by H_2O_2 pretreated *A. caulinodans* may be attributed to the fact that a number of proteins and their genes, which form a part of the oxidative stress response have been identified including the superoxide dismutase as well as DNA repair enzymes such as exonuclease IV, DNA polymerase, Rec B nuclease, and Rec A, which are important in repairing the DNA damage under adverse conditions (Demple 1991, Farr and Kogoma 1991). Genes involved in the control of the oxidative stress response have also been identified for example the induction of nine proteins to H_2O_2 stress is under the positive control of Oxy R gene product (Nystrom 1993) H_2O_2 adapted enteric bacteria become resistant to heat and osmotic challenge and there has been overlap between the proteins synthesized during the oxidative stress and that induced by carbon or nitrogen starvation in E. *coli* (Demple and Brook 1983).

Table 2. Survival of H_2O_2 pretreated *Azorhizobium* cells in different carrier materials

A.Caulinodans isolates	Vermiculite Lignite	Lignite	Peat
	Per cent survivability** in different carriers		
Control (wild strain)	44 ± 2.0 ^{b}	32 ± 1.5 ^{b}	38.89 ± 1.81 ^{b}
H_2O_2 Pretreated	74.32 ± 3.42 ^{a}	64.66 ± 3.42 ^{a}	68.34 ± 3.84 ^{a}

Initial inoculation load 1.0 x 10^9

** Survival population after 30 days of incubation at 30 ± 2 °C

Values are mean +SD of 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 a post hoc test.

In our present study the H_2O_2 pretreated cells were also tested for its efficiency to form effective nodules in *Sesbania rostrata* plants and also for its ARA activity , dry wt and "N" content in *Sesbania rostrata* plants. The pretreated *Sesbania* plants were also incorporated as green manure for the rice crop. It has been clearly evident from the Table that the H_2O_2 pretreatment has positively augmented the increase in the number of nodules, plant dry wt, ARA activity (Table 3) the data regarding the role of H_2O_2 pretreated cells of *Azorhizobium caulinodans* in *Sesbania* has been scarce.

Table 3. Effect of H_2O_2 pretreated A. *caulinodans* inoculation on nodulation, ARA and total plant dry wt of *Sesbania rostrata* 30 days after emergence**

Treatment	No of nodules plant^{-1}	Dry wt of Nodules g plant^{-1}	ARA* activity of nodule (µml $C_2H_4h^{-1}$)	ARA* of Plant (µml $C_2H_4h^{-1}$)	Total Dry wt g plant^{-1}
Control	17 ± 2^c	0.18 ± 2^c	38 ± 2^c	5 ± 1^c	5.8 ± 1.2^c
Untreated	23 ± 3^b	0.28 ± 2^b	74 ± 4^b	11 ± 2^b	7.0 ± 2.0^b
H_2O_2 Pretreated	29 ± 3^a	0.36 ± 3^a	96 ± 4^a	16 ± 2^a	8.4 ± 2.4^a
LSD (<0.05)	3.12	0.07	4.02	2.04	1.04

*ARA is expressed as n moles of C_2H_4 reduced $h^{-1}g^{-1}$ nodule/plant fresh wt

**Observations at 60 days

Values are a mean of six replications. Each experiment is repeated three times and similar results are obtained each time. Means followed by different letters are differed significantly according to least significant difference test (P<0.05)

But there were numerous reports on the H_2O_2 pretreated cells of *Rhizobium* on Legumes. During early legume nodule development, invading rhizobia grow rapidly and proliferate extensively. As they migrate to the interior of the developing cortex, and as nodule bacterial counts mount exponentially, the rhizobia experience different O_2 environments (Carmen-Varges et al. 2003). In addition Luo et al. (2003) reported that in the early stages of interaction between rhizobia and legumes, hydrogen perioxide (H_2O_2), one among the reactive oxygen species is generated. Sigaud et al. (1996), reported that H_2O_2 has been found to inhibit infection thread formation and nodule development. Earlier reports by Crockford et al. (1995) has showed an increase in H_2O_2 was correlated with a two fold increase in the catalase activity in the *Rhizobium sp,* when treated with a non-lethal dose of H_2O_2. Our previous studies have showed a similar trend in *A. caulinodans*. It has well been documented that these enzymes play a major role in reducing the formation of these highly reactive hydroxyl radicals, which arise from the degradation of H_2O_2 via fenton reaction (Halliwell and Guttendrige 1986, D Haeze et al. 2000) In soyabean *Bradyrhizobium japonicum* symbiosis, the oxygen protective enzymes in the nodule tissue is positively correlated with the increase in the nitrogenase activity and leghaemoglobin content (Dalton et al. 1986)

Dalton et al. (1986) reported that soyabean Brady rhizobium japonicum symbiosis of oxidative protection enzymes in nodule tissue is positively correlated with the increase in nitrogenase activity and leghaemoglobin content. The data on the yield of rice variety ADT-42 incorporated with green manuring using the H_2O_2 preatreated *A. caulinodans* were summarized on the Table 4.

Table 4. Phyto-stimulatory effect on the incorporation of H_2O_2 pretreated A. *caulinodans* inoculated *Sesbania rostrata* on the yield components and N uptake of rice*

Treatment	Plant height (cm)	Grain yield (g plant^{-1})	Panicles (no plant^{-1})	Productive tillers (%)	Total Dry wt (g plant^{-1})	Total "N" uptake** (mg plant^{-1})
Control	73±3^c	4.8±0.4^c	7±1^c	70±4^c	17.6±1.2^c	16.0±2.0^c
Untreated	85±2^b	11.1±1.1^b	13±2^b	80±5^b	37.4±2.4^b	29.1±1.1^b
H_2O_2 Pretreated	91±3^a	16.2±2.2^a	17±3^a	94±4^a	46.4±2.4^a	38.5±2.5^a
LSD (<0.05)	4.12	1.24	2.0	4.14	3.10	3.05

*Observations at 90 day

** "N" uptake assayed according to Microkheldhel assay

Values are a mean of six replications. Each experiment is repeated three times and similar results are obtained each time. Means followed by different letters are differed significantly according to least significant difference test (P<0.05)

It has been found that incorporation of green manure of *S. rostrata* inoculated with H_2O_2 preatreated *A. caulinodans* has positively augmented the plant growth and yield components of rice such as plant height, grain yield, no of panicles, etc.

Meelu and Moris (1980) has previously reported a significant increase in the total N accumulation, grain, straw yield and total plant growth of rice by incorporation with green manuring by *S. rostrata.*

These findings open up the possibilities for investigation of genetic basis of effective association and colonization of H_2O_2 preatreated *A. caulinodans* with *S. rostrata* and other mechanisms involved.

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