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Characterization of macromolecular oxidative damage in native *Agrobacterium isolate*

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As a part of systematic study of nature genetic engineer mediated transformation of plants.Experiments were designed to simulate the conditions of rhizospheric environment and related molecular changes in a native soil isolate agrobacterium (Doon GE1). Agrobacterium feels oxidative stress at and above 0.15% H_2O_2 or low pH values. Further, agrobacteria were evaluated for oxidative stress mediated damage in DNA and proteins macromolecules.

Key words : Agrobacterium, Oxidative damage, DNA/Proteins

INTRODUCTION

Agrobacterium comes under the rizobiaceae family. It is a gram negative rod shaped soil bacteria of 0.6 -1.0 µm width and 1.5 -3.0 µm length and occurs singally or in pairs. It is a causative agent of crown gall disease on dicotylendonous plants. Agrobacterium is frequently exposed to reactive oxygen species from the environment (rizosphere) and from those generated by aerobic respiration as a result of the partial reduction of molecular oxygen. The toxicity of this ROS arises from it's ability to oxidize and leach iron from iron sulphure clusture (4 Fe -4s) containing proteins and there by inactivating engymes such as aconitase , 6-phosphogluconate, dehydratase, fumarase and dihydroxy acid dehydratase of common biosynthetic pathways. Clusture $(4\ fe\text{-}4s)^{2+}$ is utilised as a lewis acid to catalyse substrate dehydration. The solvent exposure of the clusture allows it to be accessible to o^{2-} for it univalinatily oxidation. The resultant $(4fe\text{-}4s)^{3+}$ clusture is unstable and disintegrate into $(4fe\text{-}4s)^{+}$ form, which is catalytically inactive with loss of a ferrous iron atom to the cytosol. The iron accumulates in the cytosol and could participate in the 'fenton reaction', leading to increased production of the highly deleterious hydroxyl radicals which can cause damage to macromolecules including DNA, Protein and lipids (Saenkham *et al.*,2007).

In response to reactive oxygen ,agrobacterium differs from most other bacteria having Fe-superoxide dismutase (fe SOD) iso-metalloenzyme, catalyzing the dismutation of superoxide anions to form H_2O_2 , a substrate of catalase and molecular oxygen. Chromosome gene Kat A and Cat E encode catalase which is known to detoxify H_2O_2, a major component of oxidative stress, imposed on a cell. Kat A plays the 1^0 role and Cat E acts synergistically in protecting agrobacterium from H_2O_2 toxicity during all phases of growth. Therefore, in the present study, '*in vitro*' experiments were designed, in terms of oxidative superoxide anion stress, to simulate the conditions of rhizospheric environment during agrobacterial infection.

MATERIALS AND METHODS

Soil samples were collected from the fields of S.B.S.P.G.I. campus, Balawala Dehradun for the isolation and characterization of agrobacterium as per the standard identification tests and finally acridine induced plasmid curing was performed on the L.B. media containing 0.1% kanamycin. Toxic effects of the H_2O_2 and low pH were seen on the different growth stages by plate sensitivity and oxidative damage of macromolecules (DNA and Proteins) was assessed electrophorectically.

Isolation of genomic DNA from bacterial cell was done by taking 2ml of bacterial cell suspension, centrifuged at 5000 rpm for 10 minutes, added .2ml of extraction buffer and .2 ml of SDS buffer for rupturing the bacterial cells then centrifuged for 5 minutes at 5000 rpm at 4^0C. 0.2ml of SDS buffer was used to dissolve the pellet. Allowed to stand at room temperature for 10 minutes.Deprotonise the solution by adding .2ml of chloroform : isomyl alcohol and then immulsify by gentle inversion. Centrifuged at 10,000 rpm for10 minutes and pippeted out the upper layer for adding the equal amount of chilled isopropanol. Incubation was done for 30 minutes at -20^0 C, again centrifuged at 10,000 rpm for 10 min. .2ml of chilled ethanol was added on the supernatant

centrifuged at 10,000 rpm for 5 minutes, discarded the supernatant, air dried pellet suspended in 50 µl of TE buffer and visualized through agrose.

Isolation of cytoplasmic proteins was done from 10 ml of bacterial culture broth centrifuged at 10,000 rpm for 10 minutes then the washing of the pellet was done twice by standard distilled water.5ml of extraction buffer was added and boiled in water bath at 100^{0}C for 5 minutes and then quantitative and qualitative study of protein was done as per the Lowry *et al.* (1951) and SDS-PAGE, respectively.

RESULTS AND DISCUSSION

Agrobacterial growth on kanamycin containing L.B medium confirms the presence of kanamycin resistant gene in agrobacterium. Absence of agrobacterial growth at and beyond 48 hrs of kanamycin (0.1%) plus acridine treatment indicates the acridine induced elimination of kanamycin resistance gene(s),containing plasmid from bacteria (Fig. 1).

The results of H_2O_2 toxicity and low pH on different growth phase and macromolecules (Proteins and DNA) in *Agrobacterium* are presented as Table (1, 2 and 3) and Fig. (2, 3 and 4.)

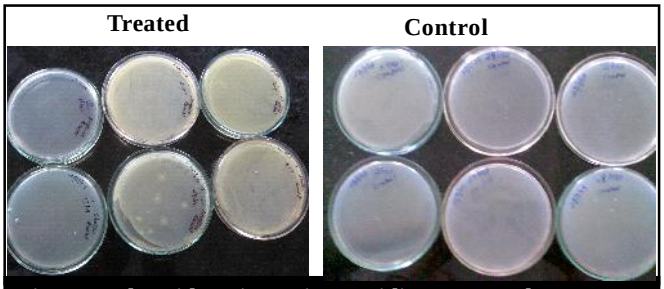

Fig. 1 : Plasmid curing using acridine orange dye

Table 1 and 2 studied agrobacterial ability to cope up induced oxidative stress only upto the level of 0.15% H_2O_2 not beyond where it was unable to survive and

Table 1 : H_2O_2 Tolerance of Agrobacrterium

H_2O_2 (%) in Media	Agrobacterial resistance (survival)
0.07	Abundant
0.08	Decreased
0.09	Decreased
0.1	Decreased
0.2	Least
0.3	Almost Nil
0.6	Almost Nil

Table 3 : Agrobacterial Protein content (Lowry *et al.*,1951)

Sample	Protein content (µg/ml) bacterial culture
Control	85.5
H_2O_2 (.3%)	98.1
H_2O_2 (.6%)	91.8

Table 2 : Agrobacterial growth pattern at H_2O_2 toxicity

H_2O_2(%) in media	Average no. of cell survival (x10^7) as colony	
0.3	Exponential phase	Stationary phase
	47	254

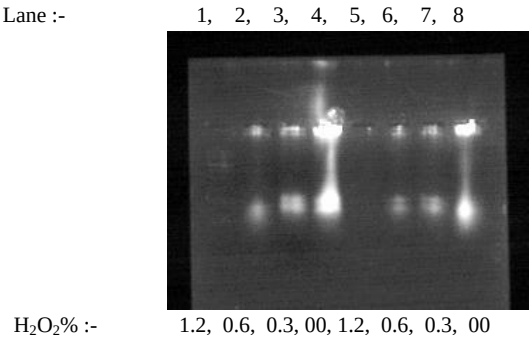

Fig. 2 : Agrose gel electrophoretograms of H_2O_2 treated

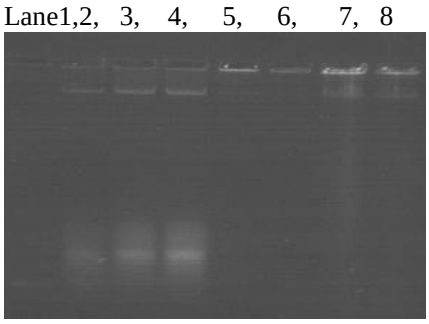

Fig. 3 : AGE profiles of bacterial culture grown on varying pH of A.B. medium

exponential phase colonies were found more sensitive as compared to stationary phase colonies. The expression pattern of two catalase reflects their physiological role in protection against H_2O_2 toxicity. Agrobacterium possess two catalase for the detoxification of H_2O_2, A bifunctional catalase – peroxidase Kat A and monofunctional catalase Cat E. In stationary phase culture, total catalase activity

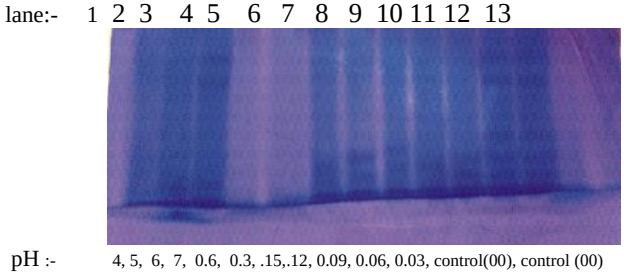

Fig. 4 : SDS-PAGE (7.5%) profiles of agrobacterial proteins at varying pH values and H_2O_2 percentage

increased two fold. Thus, increase in H_2O_2 resistance was detected with the entry of cells in stationary phase.In Fig. 2, 3 and 4, the oxidative damage of DNA and protein was seen because of the participation of ROS in fenton reaction and formation of OH^- radical which is highly deleterious and cause damage to these macromolecules. (Keyer and Imlay,1996; Saenkham *et al.*, 2007; Richter and Ronald, 2000)

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