Living organisms are under constant attack by the polluting agents in the environment. But the in-built ability present in them, resists these onslaughts and also corrects the damages caused by these agents. Both physical agents like UV radiation and chemical pollutants like pesticides act against agriculturally important organisms. In a given ecosystem action of more than one stress agent on the living organisms is not uncommon. In the present study two such environmental pollutants, UV radiation and ceresan (an organomercurial fungicide) have been chosen for their interaction with a free living nitrogen fixing organism, *Azotobacter vinelandii*. The action of these agents individually or in combination would

1. unravel the alterations brought about in the physiology of *A. vinelandii*,
2. elucidate the mode of action of these agents,
3. locate the exact site of action of these agents, and
4. identify the repair mechanisms present in the cell.

Studies relating to the interaction of ceresan and UV with cell free DNA of *A. vinelandii* has been carried out in this laboratory by Saini (1987). But the problems regarding the physiological modulations, particularly nitrogen fixing ability of *A. vinelandii* remained unresolved. In the present study an attempt has been made in this direction.

Initially experiments were conducted to assess the growth patterns of *A. vinelandii*. Cells were treated with ceresan and UV radiation individually and in combination.

The study was performed using two different concentrations of ceresan, 1 and 2ppm. The results revealed that the growth of the organism was inhibited at both the concentrations. The generation time (G.T) noticed for 1 and 2ppm ceresan treated cells
were 3.88 and 5.18 hrs respectively. It is noteworthy here that the growth retardation increased with increasing concentration of ceresan but this increase was not found to be linear to the increase in ceresan concentration. The growth inhibitory action of ceresan was seen at log phase. The growth of the organism is generally correlated with the biosynthesis of macromolecules particularly DNA. Keeping this view in mind, DNA biosynthesis in ceresan treated cells was studied using radioactive uracil as a tracer. A remarkable inhibition in DNA biosynthesis was noticed at both the concentrations of ceresan used. These results suggest DNA as one of the putative targets of action for ceresan. In addition, protein biosynthesis was also monitored in ceresan treated cells using radiolabeled leucine as tracer molecule. In these experiments the translational process was found to be greatly inhibited. Furthermore, experiments conducted to assay the nitrogenase activity in cells collected from mid log and stationary phase cultures grown under ceresan stress (1 and 2 ppm) revealed reduced level of the enzyme activity. All these results provide the following mode of action of ceresan in a bacterial cell: Ceresan interacts with DNA biosynthesis and thus leads to reduced level of translation. This in turn brings down the titre of the enzyme nitrogenase. These processes, cumulatively, retard the growth of the bacterial cells.

In order to elicit information on the effect of UV radiation in A. vinelandii the cultures were exposed to UV (25.5 and 51 J/m²) in the presence of light and also in the dark. Growth patterns studied in the cells treated with UV (25.5 J/m²) and grown in the presence of light and also in the dark exhibited G.T. 3.07 and 2.83 h respectively. On increasing the UV dose to 51 J/m² the G.T. noticed in the presence of light and dark were 3.27 and 2.95 h respectively. These results reveal growth inhibition under both light and dark conditions. DNA biosynthesis studies in cells which underwent similar treatment regimen were undertaken. In the presence of light 20 and 30% inhibition in DNA
synthesis was noticed at 25.5 and 511J/m² respectively. On the contrary, in the cells which received the same dose of UV in dark conditions, the rate of inhibition was found to be only 7.3 and 11.5% respectively. Living organisms possess DNA repair mechanisms such as photoreactivation and dark repair pathways which can correct the damages inflicted to the genetic material by UV radiation. Greater degree of growth retardation and inhibition in DNA biosynthesis in the presence of light when compared to the cells treated under dark conditions suggest that dark repair mechanism is more efficient than photoreactivation repair. Protein biosynthesis studies among the cells which were treated as above also showed a similar type of inhibitory pattern. Nitrogenase activity, assayed in the mid log phase cells exposed to both the doses of UV (25.5 and 511J/m²) under visible light was inhibited by 23.94 and 30.44% respectively. Similar doses, under dark conditions, inhibited the nitrogenase activity by only 12.51 and 16.92% respectively. Furthermore, when nitrogenase activity was assayed in the stationary phase cultures irradiated with UV in the presence of light as well as in the dark, the percentage inhibition was greater in UV-light regimen than that of UV-dark treatment.

For combined treatment, overnight grown cultures were first irradiated with UV at 25.5 or 511J/m² and then grown in the medium containing ceresan (1 and 2ppm). The results revealed that growth of the organism was severely hampered at both the doses of UV and ceresan. DNA biosynthesis was also found to be drastically inhibited under the combined treatment of the cells with both the stress agents. The cultures irradiated with UV at 25.5J/m² and post-treated with 1 and 2ppm ceresan exhibited 43 and 60% inhibition respectively in DNA biosynthesis. But the cultures treated singularly with 25.5J/m² UV or 1 or 2ppm ceresan demonstrated 20, 23 and 35% inhibition respectively in DNA biosynthesis. On increasing the dose of UV to 51J/m² the percentage of inhibition increased which was again found to be much greater than those of single treatment of UV and ceresan. This type of inhibition is possible only if both the stress
agents interact synergistically i.e., enhance each others effect. These results indicate the possibility of DNA being the prime target of both ceresan and UV radiation. Protein biosynthesis studies carried out in cells treated with UV (25.5 and 51J/m²) followed by ceresan supports the view that both the agents interact synergistically in A. vinelandii. In the mid log phase cells subjected to UV (25.5J/m²) irradiation and then treated with 1 or 2 ppm of ceresan, the nitrogenase activity decreased by 64.66 and 75.11% respectively. In a similar experiment when the UV dose was raised to 51J/m² and then treated with 1 or 2 ppm of ceresan, 71.35 and 79.18% inhibition in nitrogenase activity was noticed. A similar trend was noticed when nitrogenase activity was assayed in the stationary phase cultures treated as mentioned above. From these results it can be concluded that combined treatment of A. vinelandii cells with UV and ceresan results in synergism.

PART - B

Biological nitrogen fixation is an anaerobic process. The nitrogenase complex is irreversibly damaged when it comes in contact with oxygen. The nitrogen fixing organisms such as Azotohacter vinelandii have evolved different strategies to minimize the toxic effects of oxygen. Superoxide dismutase is believed to play an important role in the protection of nitrogenase from oxygen toxicity. Another important mechanism is the evolution of a higher respiratory rate. The present study describes the role of superoxide dismutase and various cytochrome components of the respiratory chain in the protection of nitrogenase under hyperbaric oxygen conditions. The hyperbaric conditions were created by treating the cells with methyl viologen, which in the semiquinone stage i.e. reduced form, reacts with oxygen and generates superoxide radicals (O₂⁻). Superoxide dismutase (SOD) scavenges O₂⁻. In the present study, results obtained with A. vinelandii cultures reveal that both the enzymes, nitrogenase and SOD, exhibit maximum activity
at the same period of growth (viz., between 16th and 20th hour of culture). This increase in SOD activity, parallel to the increase in nitrogenase activity, points to the possible involvement of superoxide dismutase in the protection of nitrogenase from oxygen toxicity.

Growth patterns of *A. vinelandii* under the hyperbaric oxygen conditions (methyl viologen treatment), were studied. Three different doses of methyl viologen 20, 50 and 80 μM were used. Growth was inhibited at all the three concentrations used. The growth retardation was found severe with increased concentration of methyl viologen.

Further, to get a better understanding about the involvement of SOD in the protection of nitrogenase from the toxic effects of oxygen, nitrogenase and SOD activities were determined in the cells treated with three different concentrations of methyl viologen. The results show that at low concentration of methyl viologen (20μM) nitrogenase activity was found to be the same as that of the control. But SOD activity was seen induced in these cells. At higher concentration of methyl viologen (50 and 80 μM), the nitrogenase activity decreased with a concomitant increase in SOD activity. These results provide a direct evidence that SOD plays an important role in the protection of nitrogenase when O$_2^-$ is present in high concentrations. It is important to note here that SOD produced was not enough to scavenge all the O$_2^-$ when they are present in higher amount. This results in inhibition of nitrogenase activity.

The participation of the respiratory chain in the protection of nitrogenase activity under oxygen stress conditions was also studied. To achieve this, membrane particles containing respiratory chain components were isolated by differential centrifugation. The role of individual cytochrome components under oxygen stress conditions was studied by difference spectroscopy.
The difference spectroscopy data obtained reveal that cytochromes c<sub>4</sub>+c<sub>5</sub> in the reduced form showed characteristic fused peak at 551 nm. But this peak disappeared when the spectrum was recorded in the presence of methyl viologen (O<sub>2</sub><sup>-</sup> condition). Instead, a shoulder at 580 nm appeared. This shoulder has been assigned to an oxygenated compound which is formed by the interaction of O<sub>2</sub><sup>-</sup> with the reduced form of cytochromes c<sub>4</sub>+c<sub>5</sub>. The oxygenation of the reduced form of cytochromes c<sub>4</sub>+c<sub>5</sub> also demonstrated the formation of a shoulder at 580 nm (oxygenated compound).

The purified form of cytochrome o exhibits its absorption peak at 557 nm. Interestingly cytochrome b<sub>1</sub> also has its absorption peak at 559 nm. Since the concentration of cytochrome b<sub>1</sub> is many folds higher than that of cytochrome o, the 559 nm peak overshadows the absorption peak of cytochrome o. Therefore to get a clear picture about the role of cytochrome o in the protection of nitrogenase from the toxic effects of oxygen, CO reduced minus reduced spectroscopy was performed. The reduced form of cytochrome o showed troughs at 551 and 520 nm, and shoulders at 575, 538 and 470 nm. But the spectrum recorded in the presence of O<sub>2</sub><sup>-</sup> showed the broadening of the trough between 551 and 560 nm. This could probably be due to the formation of an oxygenated compound. The difference spectroscopy performed for cytochrome b<sub>1</sub> showed a shoulder at 559 nm which remained unaffected even during the generation of O<sub>2</sub><sup>-</sup>.

All these results point to the fact that under oxygen stress conditions cytochromes c<sub>4</sub>+c<sub>5</sub> → o → O<sub>2</sub> pathway is switched off. During this process following changes take place:

(i) Cytochromes c<sub>4</sub>+c<sub>5</sub> is converted into the oxygenated form,
(ii) Cytochrome o is converted into its oxygenated form, and
(iii) Cytochrome b<sub>1</sub> does not show any change.
Under these conditions, cytochrome $b_1$ which is located at the branching point of the respiratory chain can direct the flow of electrons to cytochrome $d$. This view has been corroborated by the results obtained from reduction kinetics studies. The reduction kinetics experiments indicate a considerable decrease in the absorption of cytochromes $c_4+c_5$ whereas the absorption of cytochrome $b_1$ does not change.

In order to determine the role of cytochrome $d$ in the protection of nitrogenase under the hyperbaric oxygen conditions, the rate of succinate oxidation was studied during the generation of $O_2$ using an oxygen electrode. The results showed a very high $K_m$ value of 1.8uM and $V_{max}$ of 28nM/minute/mg protein as calculated from Lineweaver-Burke plot. The high $K_m$ and $V_{max}$ values of succinate oxidase under hyperbaric oxygen condition suggest that the major flux of electrons are oxidised by cytochrome $d$. The Hofstee plot obtained for succinate oxidase was found to be monophasic. This again lends support to the view that under oxygen stress conditions cytochrome $d$ is the only functional oxidase in the respiratory chain of *A. vinelandii*.

Cytochrome $d$ has been shown to be a cyanide resistant oxidase whereas cytochromes $c_4+c_5 \rightarrow o \rightarrow O_2$ pathway is highly sensitive to even low concentrations of cyanide. Keeping this in view cytochromes $c_4+c_5 \rightarrow o \rightarrow O_2$ pathway was blocked and the role of cytochrome $d$ in the succinate oxidation during the generation of $O_2$ was determined. It was found that the oxidation of succinate by cytochrome $d$ continued even in the presence of cyanide. But at higher concentration of cyanide (4mM) succinate oxidase activity was inhibited by 63%. This shows that cytochrome $d$ loses its cyanide resistant nature at this concentration. The residual 37% activity has been attributed to a third minor pathway as proposed by Kauffman and Van Gelder (1974).