5. SUMMARY
Azotobacter vinelandii and Azotobacter chroococcum, both free living and nitrogen fixing bacteria, were chosen for the present study. Ceresan and monocrotophos, two pesticides widely differing in their chemical nature (former, a mercurial compound with two aromatic rings; latter, a phospho-organic aliphatic compound) have been used as chemical stresses upon the cells of the above mentioned strains. UV-radiation has been administered as the second variable (physical stress).

The objective of the present study is to provide a qualitative as well as quantitative information on the effects of these chemical and radiation stresses on the non-target beneficial bacteria. Furthermore, findings of this work would help to understand some of the physico-chemical characteristics of Azotobacter cells which are as yet less known.

Initially, growth patterns of both the strains were screened turbidimetrically on Burk's nitrogen free medium and optimal growth conditions were standardised. Under these conditions the growth rate of A. chroococcum cells reveals to be faster than A. vinelandii cells (A. chroococcum: g=1.9h, A. vinelandii: g=2.7h). Growth inhibition of bacteria increased with increasing concentration of ceresan. Also, the antimicrobial action of ceresan was more pronounced at lag phase for A. chroococcum (prolongation of lag upto 20h) whereas in case of A. vinelandii exponential phase was most adversely affected (lengthening of generation time, 200%).
Monocrotophos did not cause any alteration in the growth pattern of either of the strains nor was it seen to be degraded and used up as a sole nutrient by the bacteria.

Assessment of the lethal action of these stresses was based on their potency to impair the reproductive function of cells, and cell survival, through colony forming ability of the control as well as treated cells, was monitored by plating method. Logarithm of the survival fraction was plotted against concentration of ceresan or dose of UV-radiation from which the fifty percent lethal dose (LD$_{50}$) was obtained. The shape and slope of the survival curves provide important informations. In single treatments of UVC and ceresan *A. vinelandii* survival curves exhibited a prominent shoulder at low doses of stresses. This indicates the multitarget nature of the system and/or the presence of an efficient repair mechanism which especially mends the damages of the DNA. Inactivation of *A. chroococcum* cells caused by the same stresses followed a simple-kill-kinetics giving a straight line profile. *A. vinelandii* cells (LD$_{50}$, 1 ppm) were two fold more sensitive to ceresan compared to *A. chroococcum* cells (LD$_{50}$, 2.2 ppm). With UV-C, *A. vinelandii* (LD$_{50}$, 12 Jm$^{-2}$) exhibited more than two folds resistance as compared to *A. chroococcum* (LD$_{50}$, 5 Jm$^{-2}$).

Over the range of UV-light (260 -340 nm) used, 280 nm light showed a dominant inactivation peak in both the strains of bacteria. Inactivation of both the species of *Azotobacter* caused by UV-B (280-320 nm) is qualitatively same but quan-
titatively *A. vinelandii* (LD$_{50}$, 31.5 Jm$^{-2}$) is one and half times more resistant than *A. chroococcum* (LD$_{50}$, 18 Jm$^{-2}$).

UV-C and ceresan seemed to share some common sites of action during the cell killing viz. A-T base pairs of DNA. Membrane damage, too, is demonstrated to contribute to ceresan inac-
tivation of organisms.

During UV-B (280 nm) inactivation of cells the main target is membrane whereas in UV-C treatments cell killing occurs primarily through DNA lesions and membrane appeared as a secondary target.

To investigate the effect of both the stresses in combina-
tion - one followed by the other - on the experimental or-
ganisms, two kinds of combined treatment studies on survival were conducted. One, in which the cells were preexposed to
low dose (sublethal dose) of first stress agent and sub-
sequently treated with wide range of second stress doses. Second, bacteria pretreated with a high dose (that lies beyond the shoulder region) of first stress and subsequently exposed to various doses of second stress. It is interesting to note that in combined stresses where pretreatment dose of first stress was low, a marked decrease in slope of the survival curve was recored. This indicates the antagonistic interaction of the two stresses on microorganisms. On the other hand, in second batch where pretreatment dose of first stress was high, an increase in the slope of the survival curve has been obtained. This has been interpreted as indicating the synergistic interaction of the stresses used.
on the bacteria. The observed antagonism is ascribed to induction of repair processes of cell which normalise the DNA lesions caused by UV-C and ceresan to a greater extent than in their single treatments. Synergism is explained as being based on the assumption that large dose of the first stress either impairs the repair proteins, or repair proteins are in short supply. Hence all the lesions caused by two stresses are not completely attended to. The third possibility could be that the frequencies of DNA lesions on both the strands is so high that lack of template activity occurs which puts a blockade on de novo DNA synthesis during the repair process.

Combined treatments of UV-B and ceresan either have brought about additive effect on bacterial survival or sensitivity remained unaltered as compared to their single treatments. On the contrary, UV-B pretreated cells of *A. chroococcum* showed surprisingly high tolerance towards the increasing concentration of ceresan. No significant change in the survival of cells belonging to either of the species was recorded even at substantially high doses of monocrotophos (upto 150 ppm).

DNA being the carrier of genetic informations, priority was given to monitor its behaviour at molecular level under the above mentioned environmental stresses. To reduce the large number of unaccountable factors *in situ* and to make the analysis more decisive an *in vitro* study of cell free DNA was preferred. For this, DNA was extracted from log phase
cells of \textit{A. chroococcum} following Marmur's method with a few modifications. Influence of radiations and pesticides on DNA helix stability was studied using $T_m$ value as the main parameter. The GC content of \textit{A. chroococcum} DNA was computed to be 71 percent. DNA dissolved in dilute saline citrate showed an increase in Tm values with increasing concentration of ceresan (by $2.4^\circ C$, at $D/P = 4$, $D$ and $P$ are the molar concentrations of drug and DNA respectively). The result showed that ceresan stabilized DNA helix through binding with nitrogen bases (intercalation mode). In order to compare the effects of ionising, (e.g., gamma) radiation vis-a-vis non-ionising radiation (UV), similar studies on gamma-irradiated cell-free DNA have also been carried out. Both UV-C and gamma were found to cause damage and destabilization in DNA helix (exhibited by lowering of $T_m$ values). Gamma radiation inflicted much more damage to DNA than did UV-C radiation. Strand breaks - both single and double - were the main lesions in DNA irradiated with gamma. Such damage has been clearly demonstrated using electron microscopic techniques on DNA molecules. The combined interactions of two stresses (ceresan and UV-C/gamma radiation) on DNA stability were also studied. UV-C pretreatment of DNA prevented the binding of ceresan to nitrogen bases of DNA probably due to thymine dimerization and other local distortions in the helix. Thus increase in free ceresan concentration in the solution due to its failure in binding with DNA bases led to the distortions of hydrophobic inter-
actions associated with DNA, which, in turn, resulted in a drastic decrease in $T_m$ values with increasing D/P ratios. Similar effect was obtained in gamma pretreated DNA complexed with ceresan at higher D/P ratios (-4). Ceresan pretreated DNA showed resistance against UV-C light to some extent while its sensitivity remained unaltered towards gamma radiation. It is inferred from the study of DNA treated with combined stresses that ceresan has a base specificity for A-T base pairs of DNA in its intercalative mode of binding. That monocrotophos had no effect on the stability of DNA helix was noticed through its inability to alter the $T_m$ value of DNA upto a ratio of D/P = 4.

Biological membranes essentially required to maintain their integrity for the viability of an organism were identified as the next critical target of action by chosen stress agents in the present study. Transport of radio-labelled L-leucine across the membrane of *Azotobacter* cells was taken as the criterion for the above objective. Basically, filtration method was employed to assay the transport activity. The computation of $K_m$ values of L-leucine in both the strains was done following Lineweaver-Burk equation. This is the first kind of report to establish the presence of an energy dependent amino acid uptake system in *Azotobacter* cells. *A. vinelandii* cells have more efficient leucine uptake system than *A. chroococcum* cells. Initially the patterns of leucine accumulation in control cells of both the strains were standardised (at 150 sec, *A. vinelandii* :
40 nmoles/mg protein, *A. chroococcum*: 10 nmoles/mg protein). Monocrotophos (500 ppm) and ceresan (1 ppm) grown cells (in single treatments) of *A. vinelandii*, demonstrated decreased activity of leucine transport. *A. vinelandii* cells exposed to UV-B/UV-C (51 Jm⁻²) showed an inhibition in leucine uptake. Monocrotophos/ceresan grown cells of *A. vinelandii* when exposed to UV-B/UV-C exhibited an enhanced uptake activity.

A non-specific permeability alteration resulting into membrane leakage was observed in *A. chroococcum* cells when exposed to UV-B/UV-C radiation. Monocrotophos and ceresan grown cells (in single treatments) of *A. chroococcum* scored more accumulation of leucine as compared to control cells. Monocrotophos grown cells of *A. chroococcum* were protected from membrane leakage caused by exposure to UV-B while UV-C still caused the damage. Ceresan grown cells of *A. chroococcum* did not exhibit any such leakage due to UVB/UVC light exposure.

It appeared that membrane of *A. chroococcum* cells is much more fragile as compared to that of *A. vinelandii* cells. Hence, leucine transport was mainly affected through the perturbation in membrane permeability in the cells of the former species. In *A. vinelandii* the leucine uptake activity was mainly altered through the influence of environmental stresses on permease proteins.

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