

**ECOLOGY AND EPIDEMIOLOGY OF FLUORESCENT
PLANT ROTTING PSEUDOMONADS IN AND
AROUND RIVER WATER**

**A THESIS
SUBMITTED TO THE
BIDHAN CHANDRA KRISHI VISWAVIDYALAYA
FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY**

**IN
PLANT PATHOLOGY**

**BY
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MARCH, 1990

In reverent Dedication

to

*My Parents & Family Members for
their Love, Inspiration & blessings*

Bidhan Chandra Krishi Viswavidyalaya

Dr. N. Mukherjee,
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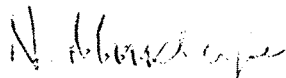
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C E R T I F I C A T E

This is to certify that the work recorded in the thesis entitled "Ecology and epidemiology of fluorescent plant rotting Pseudomonas in and around river water" , submitted by Shri Monoranjan Chatterjee in fulfilment of the requirements for the Degree of Doctor of Philosophy in Plant Pathology of the Bidhan Chandra Krishi Viswavidyalaya, is the faithful and bonafide research work carried out under my personal supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma. The assistance and help received during the course of investigation have been duly acknowledged.


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Date : 30.3.90
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(MONORANJAN CHATTERJEE)

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Chapter I

INTRODUCTION

INTRODUCTION

The components of any environment urban or rural carry number of bacteria of different biological nature along with other microorganisms. *Pseudomonas* form an important group among them. Fluorescent *Pseudomonads* in their turn are most important for different reasons. A large number of them, as reported, occur as saprophytes in soil or sometimes in water. Some others of such bacteria are occurring as epiphytes on plant surfaces particularly. Recent investigations (Schroth et al. 1977) have definitely established their wide-spread occurrence in agricultural areas. A kind of omnipresence of such bacteria on all natural surfaces including surfaces of different constructions and urban structures having some amount of moisture on them.

The urge for survey of such bacteria however, has grown from the facts that some such bacteria are parasitic on plants and animals under conditions of stress for the later. Opportunistic invasion of human body under conditions of stress like burning, injury and fractures by some such bacteria like *Pseudomonas aeruginosa* have actually been the cause of the urgency of investigation of such bacteria-their ecology and epidemiology.

The present work was planned for studying the extent of such bacteria in environments both urban and rural, existing around a very important river of northern India named 'Ganga' over a small

stretch of about 60 Km of the river, encompassing rural and agricultural and urban and industrial areas. Keeping in view the most important fact that such bacteria are known to occur and survive under conditions of high humidity and moist and wet surface conditions. Considering that the area under investigation is in the lower gangetic plains and receives quite high rainfall during the months from June to September and also the fact that accumulation of a large volume of water in and around the zones leads to submergence and flooding, the present work was considered to be urgent.

Investigations with regards to extent of such bacteria in nature has not been taken up in the country so far. Following standard methods of detection of the bacteria in or on soil, water and plant surfaces, the initial survey work was done. Based on the results of the survey, laboratory and out-door experiments were conducted for understanding the nature of the bacteria, their relationship with different components of the selected environments and their biological faculties. Some initial works on the prospect of management or control of such bacteria have also been included in the programme.

Chapter II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 The literature on green fluorescent bacteria as reviewed from published works seemed to be quite wide-spread particularly in urban environments having high density of population. With the interest of presenting the diverse situation under which such bacteria occur the present review has been made broad-based.

2.1 (a) Fluorescent bacteria in and around water bodies :

Water being a very choiced medium for such bacteria, number of reports of occurrence of some species of green fluorescent bacteria in water are there. Sugahara et al. (1984) isolated a green fluorescent bacterium from the coastal sea water of Singapore and reported that a Pseudomonas species producing fluorescent pigment was dominant. Ristanovic et al. (1980) made an investigation into the marine water to analyse the distribution of microorganisms and the physiochemical conditions therein. They isolated a fluorescent Pseudomonas species along with other bacteria. Robertson et al. (1983) also surveyed fifteen stations of NovaScotia coastal water in Canada during June to Sept. in 1981. They could isolate Pseudomonas aeruginosa, a fluorescent bacteria along with others. Identity of the green fluorescent bacteria commonly found in water was thus determined. Peterson et al. (1984) surveyed ten to twelve stations of the Naples Bay, Florida in USA and recovered the bacteria.

Sugahara et al. (1984) in subsequent studies isolated the same bacterium from coastal waters of the tropical areas and expressed their view that this bacterium possibly played an important role in such situation in the way of recycling of matter. Seyfried et al. (1985) made a through epidemiological microbiological study of 10 beaches in Ontario, Canada and isolated the bacterium. The bacterium was also present in bathing sea-beaches. This has revealed from the studies of Sherry, J.P. et al. (1979) who surveyed 4 bathing beaches of Ontario Lake and found the bacteria to be present there. Terai and Takahiro (1983) surveyed the Kijaki lake water and isolated the bacterium. Mishra and Tewari (1983) studied the water of the mesotrophic lakes and isolated the bacterium. They marked the seasonal variation of the population in such places. Seyfried et al. (1985) in another study on lake water isolated the bacterium from the sediment samples of lake. This finding appeared to be another facet of the ecology of the bacterium concerned. Some sort of a Universty in their occurrence has been expressed when the bacteria were isolated from the water of the dams of the river Lozoya in Spain by Alonso et al. (1985). They considered this as an indication of heavy amount of pollution of such water bodies.

Khalaf and Tahir (1985) isolated the bacterium from the unsterilized wells and springs in the Khazir Gomel basin in northern Iraq. They claimed presence of such bacteria in these water bodies caused pollution of drinking water. Another situation under which this bacterium occurred was the ground water. Vanderkooij, D. (1978) isolated

the bacteria from the ground discharged water through a tap. This report indicated that the bacteria may be present in the ground water and contaminate the drinking water coming through taps (Dott, 1984).

Young Viola *et al.* (1977) reported that the bacteria were present in the sewage water. Wheeler *et al.* (1980) studied sewage water of domestic and hospital areas and observed that the percentage or number of such fluorescent bacteria was higher in hospital water than that in the domestic area.

Selyavko and Lambina (1985) reported distribution of micro-organisms including Ps. aeruginosa and Staphylococcus aureus in the disposal system of sewage waters of residential area.

Ilyaletdinov *et al.* (1983) reported that the bacteria were present in different types of sewage water and were responsible for different types of pollution.

Besides these natural water bodies the bacterium has been found in swimming pools also. Schindler *et al.* (1979) sampled from different water bodies of number of swimming pools of schools, public municipalities, hostels, boarding houses, hospitals, blocks of residential, flats and dwelling houses and successfully isolated the bacterium. Jentsch *et al.* (1980) also isolated the bacterium analysing water samples from different swimming pools. The bacteria

were also found to be present in the private swimming pools in Melbourne, Australia. Millis et al. isolated the bacterium in 1981. Esterman et al. (1984) made a survey to study the microbiological characteristics of South Australian swimming pools and detected the bacterium in such water with others.

Sampling and study of different types of recreational water by Brenniman et al. (1981) revealed that the bacteria were present in them to degrade the water quality of the pools. Simman et al. (1977) isolated the bacterium by sampling from the plastic joint at the bottom of a swimming pool. Alvarez et al. (1981) by sampling and studying recreational water in the north central Florida in U.S. A. isolated the bacteria along with other fungi. These were recorded in both summer and winter seasons (Alvarez et al. 1983). Fox Alissa B. and George, W. Hambrick (1984) observed that 117 persons were infected by these bacteria due to their bathing in an indoor swimming pool).

Vasilevskaya et al. (1984) observed that even the distilled water used for the noninjectional medicines, also contained 14-600 fluorescent bacterial cells and the majority of them were Pseudomonas aeruginosa. Martino et al. (1985) found that the water supply in the hospital wards was a source of Pseudomonas aeruginosa. Carbone et al. (1985) tested the microbiological characteristics of mineral waters on free distribution at Naples in Italy and found the presence of Pseudomonas aeruginosa to a high level.

Kosatsky and Kleeman (1985) reported superficial and systemic illness of human being related to hot tub-bath, and it was found to be due to presence of Pseudomonas aeruginosa in the contaminated hot tub-water.

2.1 (b) Fluorescent bacteria in Soil:

Green et al. (1974) isolated the bacteria from different soils and they concluded that soils are also possible sources of Pseudomonas aeruginosa. Schroth et al. (1977) observed that the soil of the pots growing ornamental plants carried the bacteria. He also described the soils bearing crop plants on them and virgin soil as a reservoir of the bacteria. Marques et al. (1979) isolated Pseudomonas aeruginosa from soil and reported that the bacteria were resistant to antibiotics as well as heavy metals. Zechman and Casida (1982) reported the presence of the bacterium in the natural soil under non-sterilized conditions.

Ilyaletdinov et al. (1983) observed that the bacterium is present in activated sludge and helped in the process of mercury concentration in sewage. Gupta et al. (1984) reported the presence of Ps. aeruginosa in the Chandigarh sewage samples both from domestic, industrial and PGI sources.

2.1 (c) Fluorescent bacteria on different other surfaces :

Besides their wide-spread occurrence on water or soil, green fluorescent bacteria have been found to occur on different other surfaces possibly due to their coming on contact with water or soil.

Simmann and Jentsch (1977) examined for the existence of the bacteria on the elastic joints, made of plastic materials in the swimming pools. Exner et al. (1983) isolated the bacteria from the plastic tube surfaces of a dental unit. They also isolated the bacterium from different surfaces like plastic tubes possibly contaminated with water. Marrie and Coster (1983) made a scanning electron microscopic study of adherence of such bacteria on surfaces of plastic tube and found that the bacteria formed microcolonies in large numbers on the surfaces surrounded by an amorphous matrix. Frank Hann's Karl (1985) isolated the bacterium from the water, discharged of 22 electric water-jets which were made up of plastic materials.

Contamination of different types of clothes by the bacteria was studied by Uemura et al. (1985) and they found that under certain circumstances they do carry the fluorescent bacteria. Cetin et al. (1981) isolated the bacteria occurring as saprophytes, from the surfaces of different cosmetic preparations, creams and Suntan oils. Myers (1985) isolated the bacteria from the surfaces of several crude oils and observed that the isolates were pathogenic. Kumar et al. (1985) isolated the bacteria from the surfaces of antiseptic lotions and some of the sterilized materials even.

The prevalence of Ps. aeruginosa in the living environment among the semi-urban population of village Alipur, Delhi was studied and reported by Srivastava (1982), Kim (1982) isolated the bacteria from hospital environment and reported that the bacterium

is a common contaminating agent. Santos et al. (1980) isolated the bacteria from "Sant Tomas Hospital", the Philippines. Even in the intensive care unit this bacteria have been found to occur commonly (Mancini et al., 1985).

Graef and Eichhorn (1983) isolated the bacteria from the surfaces of hairs in the nose, on the head and auditory canals of the swimmers.

Kleiner and Profe (1985) studied how the bacteria survived on the surfaces of wood, steel, concrete plate, aluminium, plastics.

The reports referred has been successful to draw an image of wide distribution and occurrence of the bacteria under different situations having one common criterion that is the moist or wet nature of the surfaces. However, whether the bacteria have been studied in greater depth in India is not evident. A report only of its kind (Chatterjee et al., 1988) however has been successful to record and report such bacteria occurring around the Ganga.

2.2 Fluorescent bacteria on consumables :

The sort of universal presence of the green fluorescent bacteria extends to surfaces of different consumable materials.

Shooter et al. (1969) reported that the bacteria present in the hospital is transmitted through the supplied food and medicines. Shooter et al. (1971) isolated the bacteria from the hospital, canteens and schools supplying food and tiffin. Kominos et al. (1972)

reported that the bacteria present in the vegetables in hospital transmitted to the patients.

Rokoszewska et al. (1980) reported that these bacteria caused food poisoning by growing on the food particles and also in stew. This type of contamination take place due to the insufficient cleanliness of kitchen, vessels and tools.

Agarwal et al. (1982) collected wearing food like Dhal, rice or mixture of two in sterile containers and made bacteriological studies on them. They observed the presence of Ps.aeruginosa on many of the consumables. Alvarez and Ricardo (1982) reported the presence of these bacteria in the smoked mullet industries in Florida, U.S.A. They also collected commercial samples, marked smoked-mullet and found a high level of bacteria like Pseudomonas aeruginosa which causes health hazards.

Martinez et al. (1985) observed the localization of the bacteria with other microorganisms in fermented sausages like garlic sausage, salames and stufted sausages. Nandapurkar (1985) isolated the bacteria from different pharmaceutical preparation i.e. from tablets.

2.3 Fluorescent bacteria on living organisms :

Fluorescent bacteria are quite common on the body surfaces of both plants and animals. The relationship is not perfectly established however. Mostly they occur as epiphytes or as surface contaminants. However, it is apparent that they are more common on

surfaces which are wetted.

(a) Plants :

Benham, R.W. and Kester, B. (1932)'s work suggested that the bacteria are present on the surfaces of rose-bud and leaves of rose plants. Samish, Z. and Etinger - Tulezynska, R. (1963) observed the presence of the bacteria in the healthy tomato plant tissues. Barnes, E.H. (1965) reported the presence of the bacteria on leaf surfaces and in the intercellular leaf spaces. Stanghellini, M.E. (1972) reported that the seed pieces from plants having blackleg of potato are the carrier of the bacteria.

Green et al. (1974)'s investigation revealed that agricultural soils and potted ornamental plants are the possible reservoir for the bacteria. Cho et al. (1975) reported that ornamental plants are also the carriers of the green fluorescent bacteria.

McCooy and Donselman (1980) isolated the bacteria from inflorescence tissues of different coconut plant, but it had no pathogenicity to the plants. Anderson (1984) reported that the bacterium is present on healthy plant surfaces and infect roots and shoots of bean (Phaseolus vulgaris).

Kudela et al. (1982) isolated nonpathogenic green fluorescent bacteria from bean seeds, leaves and pods from the main Czechoslovakia production centre. Jacob et al. (1985) identified the location of the green fluorescent bacteria in the secondary root emergence zone tissue of the sugar beet. They also observed that the

population is high in this zone compared to the peripheral zone.

(b) Animals :

Botzenhart et al. (1985) by surveying 500 patients in rural area concluded that 4.4 % of the patients are the carrier of these bacteria in their oral cavity. Hazlett et al. (1985) reported that Pseudomonas aeruginosa induced ocular infection.

Krynski et al. (1980) periodically surveyed the new born baby in the delivery-ward in Gdansk and Elblag districts (Poland) reported the presence of the bacteria. Carvajal and Flores (1985) observed that the new pyocine type of the Pseudomonas aeruginosa caused children infection.

By sampling the Kenya market fish of different types, it was found that the bacteria infected the fishes and caused loss of the fish hygiene. Burrell and Macdiarmid (1984) isolated the bacteria from the sheep skin and found the disease to be dermatitis of sheep.

Som et al. (1980) isolated and identified the bacteria from infected larvae of Lymantria ampla. Ramas et al. (1986) made a microbiological study on a sample of fly population in healthy area of Marianao Municipality and concluded that the bacteria are responsible for 87.5 % contamination.

2.4 Methods followed in the studies of green fluorescent bacteria and storage of bacterial cultures :

Studies so far made with green fluorescent bacteria have revealed that they require specific media for their isolation, cultivation etc. Due to its ubiquitous nature and opportunism to cause infection in organisms under stress, lot of studies have been made with this bacterium. In the course different specialised methods have been identified.

It has been found that for such bacteria producing fluorescent pigments, an iron free medium is essential for their pigment production and King's medium -B, was the most suitable one. (Schroth et al., 1977) King E.O. et al. (1954) worked out a medium for isolation of the green fluorescent bacteria which he established that after 42 hours incubation at 42 °C could specifically select the fluorescent bacteria through production of the pigment. One tenth of a ml. of each suspension was placed on the medium subsequently designated as King's Medium-B for isolation. The medium was supplemented with 0.03 % cetrinide (KBC) for isolation of Ps.aeruginosa (Schroth et al., 1977). Lowbury and Collins (1955) used a selective media for isolation of the bacteria and they incorporated a new cetrinide product in the medium for better result in selective isolation of the bacterium. Klinge (1960) used different techniques and methods for isolation of this bacteria. Brown and Lowbury (1965) used an improved cetrinide agar medium for isolating the bacteria. They also followed some other culture methods for isolation. Solari

et al. (1962) used a selective enrichment medium for the isolation of the bacteria from faeces.

In 1969 Hedberg recommended the acetamide agar medium for isolation the green fluorescent bacteria. Thom et al. (1971) isolated the bacteria by using the nitrofurantoin - enriched medium. Lambe and Stewart, P. (1972) evaluated the 'pseudosei agar' as an aid in the identification of the bacteria. Using acetamide broth Smith and Dayton (1972) isolated Pseudomonas aeruginosa from rectal swabs. Brodsky and Nixon (1978) developed a rapid method for detection of the Pseudomonas aeruginosa on MacConkey agar under ultra violet light.

Labeyrie et al. (1977) observed that zinc (ZnSO_4) and Cobalt Sulphate (CoSO_4) ion has great influence on Pseudomonas aeruginosa chromogenesis in methyl-1-phosphonate containing medium. Brodsky and Ciebin (1978) isolated the bacteria from water bodies by using an improved medium i.e. 'mPA-medium' and designated it as mPA-C. Ding et al. (1978) used low concentration of maltose in pyocyanin medium for the production of pyocyanin, they also found that high concentration of maltose inhibit the pyocyanin production. Santose and Vieu (1978) isolated 448 species of Pseudomonas aeruginosa from several hospitals of Lisbon, Portugal by using the Gillies and Govan method. Reynolds et al. (1979) isolated the bacteria on King-'A' medium and they confirmed that casein hydrolysis in milk agar was an assured method for distinguishing Pseudomonas aeruginosa from

other fluorescent bacteria. Tydel et al. (1980) used a selective medium with ethonium and penicillin for cultivation of Pseudomonas aeruginosa and it helped to isolate the bacterium from a pathological material contaminated with various types of microbial population. Jensen et al. (1980) described a defined medium capable of supporting growth and exocellular protease production by clinical isolates of Pseudomonas aeruginosa. The control of protease production in this medium was effected by a mixture of 3 amino acids and glucose.

Garcia et al. (1981) isolated 35 strains of denitrifying bacteria including Pseudomonas aeruginosa from the rice field soil of Senegal by anaerobic enrichment in a mineral medium, containing benzoate and nitrate or nitrous oxide. Marlod et al. (1981) recommended a new selective agent for isolation of Pseudomonas aeruginosa and they used 9-chloro-9 (4-diethylamino phenyl) - 10-Phenylacridan (C-390) for isolation of the bacteria. It inhibited the growth of all microorganisms other than this bacterium. Geuenich et al. (1982) made a comparative growth study on different broth culture i.e. asparagine broth, malachite green broth, acetamide broth I and II (1% acetamide & 0.2% acetamide) and concluded that better yield in acetamide broth I & II (85.1 % and 78.0 %) than that of asparagine and malachite green broth (73.8% and 62.9 %). Vandenberg et al. (1983) reported that the bacteria have the ability to grow in presence of iron chelator and ethyl enediamine di - o - hydroxyphenyl acetic acid. Yumul (1983) modified the method of Gillies and Govan's method by minor modification and they got similar results.

Daly et al. (1984) modified the MacConkey agar (MMA) medium and found that this medium could provide very practical, reliable and cost-effective means for early characterization of Pseudomonas aeruginosa.

Fyee et al. (1984) revised the Gillies and Govan's pyocin-typing method and used a separate medium composition which gives better typing within 24 hrs., increased the number of identifiable types and could be used to type mucoid.

Kalina et al. (1984) used the nutrient medium containing 2,3,5, tri-phenyl - tetrazolium chloride milk and a higher concentration of peptone, and observed that the bacteria gave a metallic (golden) luster.

Kalina et al. (1984) isolated the bacteria by using a culture media containing L-arginine and ions of K, Na, Mg, C & P and it has also on overlay of plain agar with 0.6 % of 2,4-5 triphenyl tetrazolium chlorides added. Brown et al. (1986) described the method for isolation of microorganisms from the oil-in-water. Aqueous cream BP has been examined using a variety of solvent systems to disperse the cream prior to membrane filtration or direct inoculation pour plate methods which utilized combination of either peptone-water containing 5 % W/V polysorbate 80 or nutrient broth containing 4% W/V lubrol W). This method provided the most efficient recovery of Pseudomonas aeruginosa but still allowed less than 20 % recovery.

Ziegert and Stelzer (1986) investigated comparative efficiency of different methods using different media for isolation of the bacteria. Such media included Malachite green broth, preuss broth, selenite broth, asparagine broth, Malachite green Acetamide broth. The last one showed the highest isolation frequency after 48 hr incubation time.

Danilova et al. (1980) lyophilized different bacteria cultures including Pseudomonas aeruginosa at 0.7-2.3% and 3.8-5.7 % residual humidity and preserved the cultures for 10 years. They reported that the viability of the culture was much higher in 3.8-5.7 % residual humidity than that in 0.7-2.3 % residual humidity. Arkadeva et al. (1984) reported that the strains of Ps. aeruginosa retained some characteristics features after 20 years even when stored under lyophilized condition. Nadirova et al. (1984) reported that the strains of Ps. aeruginosa and Ps. fluorescence showed no changes in cultural, physiological and bio-chemical characteristics after 12-18 years of storage under vacuum in darkness at 4-6°C temperature after lyophilization.

2.5 Characteristics of the green fluorescent bacteria :

(a) General characters

Different green fluorescent bacteria have so far been recorded in 'The Manual' (1974). They include among other Pseudomonas aeruginosa too. Such bacteria so far isolated have exactly been characterised and summarised in 'The Manual'.

Litovchenko (1978) studied the ultrastructure of Pseudomonas aeruginosa with different methods of preparation and fixation. It has been found that the bacteria contained 1-3 flagella. The cell membrane was surrounded by a capsule. The cytoplasmic membrane was found to be 3-layered and was separated from the outer one by the peri-plasmic space. The 3-layered structure of cell membrane was manifested better at the initial phase of the bacterial lysis. The cell with the Nucleoid was a ribo- and polyribosomal complex. Fine-grained and coarse grained inclusions were found in the cytoplasm. Some spherical structures on the cell wall surface were found for the first time. Faydi and Nabbut (1978) characterised the bacteria and found that the bacteria is stable and grows between pH 5-9. However, it is partially inactivated when kept for 1 hour at 4°C. Freezing and thawing, lyophilization and heating at 65°C for 5 minutes completely destroyed its activity. The addition @ 4 mg/ml of bovine albumin or @ 2 mg/ml of gelatin caused about 60 % increase in its activity. Treatment with 10 mM EDTA caused a 98 % drop in its activity.

Pozmogova (1978) observed that in the above optimal temperature the bacterium was inhibited, development and reproduction were also affected. A decreased protein content in cells of bacteria was also recorded. Kasyanchuk (1979) studied the chemical composition of the cell wall of different bacteria like Ps. aeruginosa, Ps. putida, and Ps. cepacia and found that lipopolysaccharides were present in the cell wall. Suzuki et al. (1980) found that multi

multitrichously polar flagellated mutants were isolated from a monotrichously flagellated strain of Ps.aeruginosa. The electron-micrography (EM) revealed that the number of flagella produced per cell cycle was increased. Litovchenko and Cherno (1981) observed under EM that the presence of bipolar flagella and pili was as common as non-flagellated cells. Electronmicroscopy also revealed the existence of non-capsular and capsular forms. Folkhard et al. (1981) found that the polarpili of Ps.aeruginosa strains of 'K' and 'O' are hollow cylinders with 52 Å outer diameter and 12 Å inner diameter. Similar X-ray diffraction patterns were obtained with fibres of the two types of pili to a resolution of 7 Å in the equatorial direction and 4 Å in the meridional direction. Dzhusupova (1982) reported that the bacteria contained significant quantities of L-methylstyrol. Montie et al. (1982) isolated the flagella from various strains of the bacteria and by different centrifugation obtained typical filaments. He also found that some isolates gave the highest percentage of flagellin with the least amount of protein impurities, when grown on succinate mineral salt medium. The amino acid analysis showed 16 amino acids, but no proline. Watts et al. (1983) described that the polar pili of the bacteria are flexible filaments, 5.2 nm in diameter and 2.5 µm in average length. It contained a single subunit pilin. Motie et al. (1983) characterized the flagellins from several clinically important species of Pseudomonas by sodium dodecyl sulfate polyacrylamide gel-electrophoresis. Iskhakova et al. (1985) isolated and characterized 745 strains

of Ps.aeruginosa of hospital samples and 216 strains of environment and observed that pigment formation took place between 3-10 days. They found the motility rate, growth, glucose assimilation and arginine decarboxylase activity were very high (above 90 %) in Simmons citrate agar. Schmider and Ottow (1986) characterized the morphology and physiology of the Ps.aeruginosa.

Kudela and Trynerova (1986) characterized the phytopathogenic strains of the bacteria which were isolated from sweet and sour cherries.

Lelliott et al. (1966) proposed a determinative scheme for the characterization of plant pathogenic Pseudomonas. Misaghi and Grogan (1969) compared the nutritional biochemical and physiological behaviour of 177 isolates of Plant pathogenic and saprophytic fluorescent Pseudomonas. Hildebrand and Schroth (1971) describe several physiological test for distinguishing phytopathogenic fluorescent Pseudomonad species from other bacteria. Test includes oxidase, levan formation pigments etc. Ramos et al. (1986) during their study on the animal pathogenicity described the bacteria as a gram negative one and found the strains to be positively animal pathogenic.

2.5 (b) Serological characteristics of green fluorescent bacteria:

So far studied determination of green fluorescent bacteria depend much upon the serological characteristics of such bacteria and this tool has been liberally used in case of Ps.aeruginosa.

Homma et al. (1979) proposed an international standard for the intraspecific serological classification of Pseudomonas aeruginosa. Bloch et al. (1982) proposed serological methods in epidemiological studies and clinical classification of Ps.aeruginosa. Yang et al. (1980) isolated 36 strains of Ps.aeruginosa from hospitals and Madhubala and Shriniwas (1980) also isolated 920 strains from clinical materials and in both the occasions they made serological typing. Natoli et al. (1982) also described the standard serological methods for typing of strains of Ps.aeruginosa for epidemiological studies. They isolated the strains from palerms, Italy. Zaidner et al. (1981) described the immuno chemical analysis and established the protective properties of slime components of Ps.aeruginosa having different molecular weights. The slime was seperated in different fractions. They found that the high molecular weight slime components were serologically more active than the low molecular weight components.

Maresz et al. (1980) and Stanislavsky et al. (1985) studied the immunological properties of the extracellular slime produced by Ps.aeruginosa and reported that the slime extracts were strongly immunogenic for animals. Bulava and Ermohn (1985) produced specific antisera to Ps.aeruginosa by using extracts from these microorganisms, destroyed by ultrasonic treatment or by multiple freezing and thawing for the immunization of rabbits against Ps.aeruginosa infection.

2.6 Pathogenic characteristics of green fluorescent bacteria :

Most of the green fluorescent bacteria have so long been known as non-pathogenic saprophytes. Reports of opportunistic infection of both animals and human being under different stresses like injury, fracture, burn etc. are not uncommon. However, in case of plants such reports are relatively scarce.

2.6 (a) Plant pathogenicity of green fluorescent bacteria :

Paine and Branfoot (1924) during their studies on bacteriosis of lettuce plant found the bacteria to be responsible. Clara (1930) also claimed that the bacterium Pseudomonas aeruginosa was the causal agent of a new bacterial leaf disease of tobacco in the Philippines. Desai (1935) reported that the same bacterium is responsible for the stinking rot of sugarcane. Elrod and Braun (1942) in their pathogenicity studies on lettuce plants, described the role of Ps.aeruginosa as a plant pathogen. Kudela et al. (1982) analysed been seeds, leaves and pods from the main Czechoslovak production regions, and observed the presence of pathogenic Ps.aeruginosa.

Kudela and Trynerova (1986) on the otherhand isolated the phytopathogenic bacteria from leaves and bark tissues of sweet and sour cherries. They also obtained the bacterium from leaves showing signs of bacterial canker. Lebeda et al. (1985) recorded the pathogenicity of Ps.aeruginosa on plants. Stanghellini (1972) claimed

that the bacterium is responsible for the diseases like seed-piece decay and blackleg of potato. The findings and claims of the laboratory of Mortimore P Starr one of the most recognised bacterial taxonomist of modern times is very important. Starr and Chatterjee (1972) established and confirmed that the bacterium is responsible for both plant and animal diseases. This is a finding of great consequences and has enhanced the importance of this particular bacterium.

Knoesel et al. (1978) made investigations into this aspect and found that several strains of Ps.aeruginosa have their demonstrable pathogenicity to plant host tissues. Inoculation into the intercellular spaces of leaves of Vicia faba (28°C) and Vinca rosea (36°C) showed negative results however. In leaves of Nicotiana tabacum (32°C) the bacteria multiplied in the tissue and reacted with the host cell to form partial lesions and cause necrosis. In some other studies the bacterium was found to cause rots of carrot root, onion bud and tobacco leaves, as a result of which the phytopathogenicity of the bacterium was characterised through their rotting capacity.

2.6 (b) Animal and human pathogenicity of green fluorescent bacteria :

Several studies and publications have now claimed positive animal pathogenicity of Ps.aeruginosa and recognition by M.P. Starr's Laboratory in this regard is very important. The Manual (1974) has also identified this bacterium in the same way.

Krynski and Becla (1980) investigated into the bacterial flora or biological characteristics of the strains of the bacteria on new born babies delivered in Gdansk and Elblag districts in Poland. This bacterium occurring on babies when checked periodically during the 1st year showed child pathogenicity of the bacterium. Botzenhart et al. (1985) studied 500 patients of rural areas while they were in general practice and proved that 4.4 % of the cases had ^Oropharyngeal infection due to this bacteria. Hazlett et al. (1985) isolated pathogenic strains of Ps.aeruginosa from 70 years old patient from the corneal infection site.

Delia et al. (1985) observed that Pseudomonas aeruginosa was initially considered to be an innocuous saprophytes but with time it is acquiring more reputation as a pathogen. Even though it is responsible for numerous infection cases among human being and animals, the most frequent of them are secondary infectional and are incited in the opportunity created through the complication from the previous-disease or primary infection or injury. Gvozdyak et al. (1986) isolated the bacteria along with other bacteria from urological patients and demonstrated that the same strains are induce diseases of agricultural plants.

Stover et al. (1983) reported the difference of virulence of different Pseudomonas spp. in burnt mouse which included Ps.aeruginosa too. However some detail studies have been made subsequently inoculating a test animal namely mouse and the nature of the animal pathogenicity of the bacteria was understood better.

Colizzi and Falcone (1978) reported that heat killed Pseudomonas aeruginosa depressed contact sensitivity to oxazoline in C 568 L/6 mice. Nishi and Kanji (1978) injected Pseudomonas aeruginosa in the urinary bladder of the animal to study the urinary tract infection. After one day he observed that the number of organisms in the bladder tissue gradually decreased, whereas in the kidneys the level increased. After 3 days the level reached a constancy and remained same upto 2 weeks. Finally the organisms gradually disappeared spontaneously and recovery took place only by keeping a gross lesions on the kidney.

Campa et al. (1982) reported that repeated systemic injection of Pseudomonas aeruginosa viable cells were able to induce a specific delayed type of hypersensitivity (DTH). The symptoms appeared like the increased footpad swelling, decrease of weight and unfractionated of spleen cells. Same infections symptoms developed when the bacteria(Pseudomonas aeruginosa) injected in the hind footpad (Colizzi~~et al.~~, 1982). Maresz et al. (1983) reported that if the slime extract from mucoid Pseudomonas aeruginosa vaccinated to mice, it become resistant to infection with these bacteria. Joo.I.(1983) reported that Ferric ammonium citrate (FAC) enhanced significantly the mouse virulence of all strains.

Burrell and Macdiarmid (1984) isolated the bacteria from diseased sheep and found that it was pathogenic to the sheep. They also found the same bacterium to be associated with fleece and responsible for rotten. Binta et al. (1982) surveyed the market fishes

in Kenya and reported that Ps.aeruginosa was pathogenic to some fishes and spoilage of fishes was caused through involvement of some other microorganisms. Ramos et al. (1986) made a microbiological study in a sample of fly population in a healthy area of Marianao Municipality, isolated the bacterium into pure culture and identify the bacterium as a pathogenic one.

2.7 Green fluorescent bacteria on growth and health of plants :

Green fluorescent bacteria are reported to show different other kinds of biological activity including some antibiotic properties. Inhibitory or suppressive properties of such bacteria has importance. Among such records those against some plant pathogenic microorganisms may have direct bearing on agriculture. Pidoplichko and Garagulya (1979) reported that the genus Pseudomonas has a good antagonistic effect on some fungi like Fusarium spp. which caused the wheat root rot. When a cell suspension of the bacteria was mixed with wheat seeds, the disease could be checked and some times the germination of seeds and development of the seedlings were enhanced.

Brathwaite and Cunningham (1982) reported that the germination and growth of Sclerotium rolfsii a pathogen of several crops were inhibited by Pseudomonas aeruginosa in culture and soil.

Garcia et al. (1983) in the subsequent year reported that the cell suspension from Pseudomonas aeruginosa (Pyocinogen strain)

inhibited the growth of the some bacteria which caused large number of plant and animal diseases. The inhibited bacteria belonged to Nocardiaceae, Micrococcaceae, Neisseriaceae, Streptococcaceae, Vibrionaceae, Enterobacteriaceae and Pseudomonadaceae.

2.8 Manipulation of the population of the green fluorescent bacteria :

Physical methods :

Considering that the green fluorescent bacteria are common contaminants and have been capability to cause infection in animals and plants, some explorations have been made to identify treatment methods to control their population in the environment.

Bainchi and Bensoussan (1977) reported that if the bacteria like Vibrio cholerae, Streptococcus faecalis, S.aureus or Pseudomonas aeruginosa immersed in sea water within dialysis bags containing sterilized water, the bacteria were rapidly killed or inhibited on contact with the marine environment. Szule et al. (1981) observed that the radiation had great influence on the bacterial cell. They reported that radiation doses ranging from 1-100 Gy (Grey) had a greater influence on proteolytic properties and still higher doses waste the bacteria.

Gilpin et al. (1985) claimed that a continuous UV-light treatment in the circulating water system, disinfected the water. Abshire and Dunton (1981) also recorded that the activity of six species including Pseudomonas aeruginosa were inactive or killed by a moderate level of UV-light in a circulating water system.

Wallbank et al. (1985) purified the water by passing germicidal ultraviolet (UV) light through the teflon pipe. They reported that the activity of the polioviruses and Pseudomonas aeruginosa became inactive by these processes. Zukovs et al. (1986) reported that 350,000 μ W.S./cm² doses of UV-light could disinfect such contaminated low quality of waste water. The disinfection efficiency was evaluated over a range of dosages and contact times for faecal Coliforms, Enterococci, Pseudomonas aeruginosa and Salmonella spp.

2.9 Manipulation of the population of green fluorescent bacteria :

(a) General Chemicals :

Use of chemical disinfectants for control of pathogenic organisms in public places, water bodies etc. is a practice having long tradition in human civilization, which form a major sector in public health aspects.

Gelinas, et al. (1984) studied the combined influence of temperature (4°, 20°, 37° and 50°C) and contact time (10, 20 and 30 minutes) on the efficacy of 8 commercial disinfectants, acid-anionic-surfactant, chlorohexidine acetate, quaternary ammonium compound, glutaraldehyde, Sodium hypochlorite, iodophor, aldoquaternary complex, amphoteric surfactant. Temperature and contact time influenced the activity profile of the disinfectants tested with a maximum efficiency near the optimum growth temperature (37°C) of the test organism (Pseudomonas aeruginosa). Chori and Ghoba (1984) reported the bactericidal activity of 10 and 5 % ethanol alone in the presence

of EDTA against Pseudomonas aeruginosa, E.coli, and Staphylococcus aureus was studied using the extinction time and the death curve method. EDTA at various concentration potentiated the bactericidal activity of ethanol. The potentiation of ethanol with increase in the EDTA -concentration from 0.05 to 0.3% against Ps.aeruginosa and S.aureus. Sondossi et al. (1985) observed the resistance and cross-resistance of Ps.aeruginosa to formaldehyde and a formaldehyde condensate biocide. Lisa et al. (1988) reported that the induction of acid phosphatase and cholinesterase activities were controlled by Choline acetyl choline and betanine. The acid phosphatase activity was competitively inhibited by betanine, whereas the inhibition produced by Choline, acetyl choline showed competitive and non-competitive components.

Spicher and Peters (1977) reported killing of following microorganisms with formaldehyde. The organisms included several animal viruses, bacteria like Ps.aeruginosa and some fungi like Aspergillus niger and Candida albicans. The minimum growth inhibitory effects of dissociated and undissociated sorbic acid has been determined by Eklund (1983) for Bacillus subtilis, B.cereus, E.coli, Ps.aeruginosa, Staphylococcus aureus and Candida albicans. He reported the maximum inhibitory effects on undissociated acid. Hallmans and Elmros (1981) found that the third-degree of burn of guinea-pigs leading to their subsequent infection with Ps.aeruginosa was controlled by zinc tape. They were also treated with plastic foil and a gauze sponge but better results were obtained in case of

zinc tape. Litovchenko et al. (1981) studied the sensitivity of Ps. aeruginosa to boric acid, carbenicillin, depasul and suggested exploration of the possibility of their use in treating pyocyanic infection i.e. , due to pyocyanin producing bacteria like Ps. aeruginosa.

Abalaka and Deibel (1980) made a comparative study of the lethal effect of metabisulfite on the viability of Salmonella typhimurium, E.coli and Ps.aeruginosa and observed that all the species tested were susceptible to metabisulfite. Bitonti et al. (1984) reported that the growth of Ps.aeruginosa slowed down by the addition of monofluoromethylornithine, difluoromethyl arginine and dicyclohexyl ammonium sulfate. The growth however could be restored by addition of 0.1 mM putrescine plus 0.1 mM spermidine, or 0.1 mM spermidine or 5mM putrescine by themselves.

2.9 (b) Antibiotics and Ps.aeruginosa :

While reduction of the level of contamination in any 'biotope' has been the objective of the previous studies another series of testing sensitivity of Ps.aeruginosa to different antibiotics have been made and published for reasons of selecting antibiotics that may be useful for elimination or eradication of established infection of the bacterium in host tissues.

Park, Hae-Kyung and Seung-Hoonlee (1977) studied the mixed bacterial infection (Staphylococcus aureus and Ps.aeruginosa) in mice and human being. To control the infection they found a good

result by use of such antibiotics - cloxacillin, gentamicin, Penicillin and tetracyclines. Pseudomonas aeruginosa isolated from different clinical materials (Yim, 1977) were tested for their antibiotic sensitivity by agar-disc method and the inhibitory concentrations recorded for different antibiotics for the bacteria. However 43.8 % of the strains were resistant to Gentamicin (GM 12.5 µg/ml), 46.1% to Colistin (CS, 900 µg/ml), 7.6 to Carbenicillin (CB, 100 µg/ml) and 85.3 % to Kanamycin (KM, 20 µg/ml). This situation of existence of antibiotic resistant strains of the bacterium has made the situation complicated. Zemelman et al. (1978) however, reported a synergistic effects of gentamicin plus carbenicillin upon 170 strains of Ps.aeruginosa . A high proportion of strains were synergistically inhibited (73.5%), but among strains producing pyocin synergy was more frequent among gentamicin resistant strains.

Faggiani et al. (1978) studied the sensitivity of several strains of Ps.aeruginosa, he collected from hospital and several from outside and reported that the hospital-strains are more resistant to neomycin, nalidixic acid, chloromphenicol, demethylchlorotetra cycline and rifampicin. Strains from outside were greater resistant to colimycin and gentamicin. Savitskaya and Levina (1978) reported that the pyocinogenic strains of Ps.aeruginosa are more sensitive to gentamicin and polymycin. Baron and John (1981) reported that the Gram positive are more susceptible as a group to the antibiotic action than that of gram negative bacteria, because it depend upon the outer pyocyanin concentration of the bacteria. Combined effect of antibiotics on Ps.aeruginosa have been studied by

different scientists in different times. Tydel skaya et al. (1979) studied gentamicin with ethonium. Ryff et al. (1983) cefsulodin with ticarcillin, mezlocillin or aminoglycosides. Saad et al. (1983) used gentamicin with carbenicillin, Wooly et al. (1983). EDTA - tromethamine, with 7 antibiotics (namely chloramphenicol, nalidixic acid, oxytetracycline, penicillin, polymyxin B, Streptomycin & triple sulfa) and Martino et al. (1985) N-formimidoylthienamycin with norfloxacin. Ryff (1983) reported the activity of cefulodin combined with ticarcillin, mejlocillin or aminoglycosides against Ps.aeruginosa. Wooly R.E. (1983) found that the EDTA-tromethamine plus penicillin oxytetracycline gave the best results, others did not show any synergistic effect. The combined synergistic effect on Ps.aeruginosa of some such antibiotics is important. Kiprianova and Korniyushenko (1979) reported that Ps. aeruginosa cells are most resistant to dyes and some antibiotics. Cox and Luther (1980) isolated 180 species of Pseudomonas from the clinical site and reported that all are sensitive to Colistin, but resistant to ampicillin, and more than 90 % were sensitive to gentamicin. Matsueda et al. (1980) reported that cercosporin, a fungal product of Cercospora kikuchi-ana has a good antibacterial activity and found that Ps.aeruginosa cells are sensitive to that fungal product.

Jlatai et al. (1980) reported that Furbenicillin (BL-P-1597), a new semi-synthetic broad spectrum penicillin showed its inhibitory

effects on many Gram negative bacilli including Ps.aeruginosa. Prabhakar (1981) reported that Ps.aeruginosa are sensitive to several antibiotics like Gentamicin, Kannamycin, Ampicillin, Streptomycin, Chloramphenicol and tetracycline. Lazou and Vatopoulov (1981) during their study on different cephalosporins like cephalothin, cephaloridine, cephalexin, cefotaxime etc. found that cefotaxime was superior for its antibacterial activity on Ps.aeruginosa. Kongsamran and Leelaporn (1981) studied the bacteriostatic and bactericidal levels of various antibiotics against Ps. aeruginosa. By broth dilution method of 10 isolates of Ps. aeruginosa they found the positive effect of gentamicin, Tobramycin, Dibekacin, Carbenicillin and Colistin. Barry et al. (1981) reported minimum inhibitory concentration (MIC) of amikacin and gentamicin to Ps.aeruginosa. For amikacin were > 16 mg/ml for resistance and ≤ 12 μ g/ml (not ≤ 8 μ g/ml) for susceptibility. For gentamicin 8 μ g/ml for resistance and ≤ 6 μ g/ml (not ≤ 4 μ g/ml for susceptibility). Hancock et al. (1981) reported that the outer membrane of Ps.aeruginosa takes up the antibiotics like gentamicin and streptomycin resulting in its death.

Martin and Beveridge (1986) reported that gentamicin is an aminoglycoside antibiotic which inhibits protein synthesis, and had a detrimental effect on the integrity of the cell wall of Ps.aeruginosa. Kongsamran and Leelaporn (1981) reported the activity of gentamicin, tobramycin, dibekacin, amikacin, colistin and carbenicillin on Ps.aeruginosa. Digrahes et al. (1983) also studied the comparative effect of these antibiotics on Ps.aeruginosa. Woolfrey

et al. (1983) reported that gentamycin breakpoints of resistance (R) ≤ 12 mm and susceptibility (S) ≥ 16 mm produced error rates of 20 % and 6.8 % respectively. Scioli et al. (1984) tested a large number of bacteria on different antibiotics by kirby-Baues method and reported Pseudomonas and Proteus isolates, were 100 % sensitive to amikacin. A much lower percentage of bacteria was sensitive to amikacin, tobramycin, sisomicin and streptomycin. El-nima (1985) studied the effects of 7 different antimicrobial agents on 48 clinical isolates of Ps. aeruginosa on Mueller - Hilton agar. He reported that the isolates are resistant to ampicillin and sensitive to anti Pseudomonas antibiotics like polymixin B., gentamicin, & carbenicillin. Yamashita et al. (1981) studied the synergistic activities of fortimycin A and β - lactam antibiotics against Ps. aeruginosa. Most β - lactam antibiotics tested had synergistic effects on the inhibitory activity of FTM-A against some strain of Ps. aeruginosa. Eftimiadi (1985) showed that sulbenicillin had better antimicrobial activity than carbenicillin in case of Ps. aeruginosa. Landoy et al. (1985) reported that new cephalosporins were more active than the semisynthetic penicillins against E. coli, Ps. aeruginosa and K. pneumoniae. Mezlocillin had better activity than the other semisynthetic penicillins against most of the Gram-negative organisms as well as the enterococci. Fernandes et al. (1985) also reported that Imipenem showed the highest activity on Ps. aeruginosa. Stobbering et al. (1983) made a comparative antibacterial activity of several β - lactam and aminoglycoside compounds against Ps. aeruginosa.

Vurma and Kayser (1986) studied the minimum inhibitory concentration (MIC) of the monobactam carumonam (RO17-2301) and twelve other antimicrobials against 140 non-replicate clinical isolates of Ps.aeruginosa and reported that the most active drugs were diprofloxacin, amikacin, imipenem and ceftazidime. Van et al. (1986) reported that BRL 36650, a new penicillin is most active on Ps.aeruginosa and other Pseudomonas species, and all strains were inhibited by 2 µg /ml or less. Steele et al. (1985) studied the minimum inhibitory concentration (MIC) of BMY 28142, a new amino thiazolyl cephalosporin on Ps.aeruginosa and reported that only 0.25 mg/lit inhibited all Ps.aeruginosa strains. Marin et al. (1985) reported that norfloxacin and Piperacillin were most effective against Ps.aeruginosa.

Barry et al. (1984) reported that palcillin has the similar antibacterial activity like piperacillin on Ps.aeruginosa . Apalcillin and piperacillin were both active against all 13 spp. of enterobacteriaceae that were tested (MIC 50 <8.0 µg/ml) but some strains were resistant to both the penicillins. Apalcillin was active against Ps.aeruginosa and Actinobacter spp. (MIC 50 <2.0 µg/ml).

Klimnyuk (1984) reported that bile acid lowered the antibiotic - resistance in Staphylococcus aureus, Proteus vulgaris E.coli and Ps.aeruginosa. Chiaradia et al. (1983) evaluated along with other characteristics the antibiotic susceptibility characteristics of Ps.aeruginosa. The antibiotics tried were carbenicillin, Chloramphenicol, Colistin, Gentamicin, Neomycin and

Polymixin -B₉. Antibiotic sensitivity did not show any correlation with the other characteristics. Chawla and Mathur (1981) during their study on the sensitivity of Ps.aeruginosa to antibiotics, found that garamycin, carbenicillin and gabbromycin are inhibitory to the bacteria. Neuhaus et al. (1980) reported that Ps.aeruginosa cells sensitive to cefoloxine, mezlocillin, and ticarcillin. Mezlocillin in this regard was more active than cefotaxine and cefulodin as claimed by Perea et al. (1980). Ceddia et al. (1979) after his study on 43 strains of Ps.aeruginosa isolated from hospitalised patients reported that gentamicin, neomycin and streptomycin were the most effective antibiotics.

Huang et al. (1984) reported that Ps.aeruginosa were sensitive to gentamicin, amikacin, kanamycin, minocycline, colistin, chloramphenical, claforan, piperacillin and carbenicillin. Williams et al. (1984) also examined the antibiotic - susceptibility of 1866 consecutive non-replicate clinical isolates of Ps.aeruginosa from 24 British hospitals and reported that the minimal inhibitory concentration (MIC) of some of the antibiotics to be carbenicillin (>128 mg/l) 9.6 %, azlocillin (>32 mg/l) 3.9 %, cefotaxine (>16 mg/l), 19.0 %, cefoperazone (>16 mg/l) 0.3%, amikacin (>4 mg/l) 3.8%, gentamicin (>2mg/l) 5.5%. The antibiotics identified as good inhibitors naturally worth trial for application against infections of Ps.aeruginosa wherever feasible.

2.9 (c) Plant Extracts and Pseudomonas aeruginosa :

Roia and Smith (1977) studied the antibacterial activities of extracts of some common ornamental plants. The freeze dried powders, methanol extracts and the marcs of various parts of 57 ornamental plants were tested against number of bacteria including Pseudomonas aeruginosa. Using tube dilution and plate diffusion methods 19 plants extracts were found to be antibacterial out of 49 plant extracts tested. Dankert et al. (1980) reported that the crude juice of garlic (Allium sativum), onion (A. cepa) and shallots (A. ascalonicum) inhibited the growth of both Gram positive and Gram negative bacteria and yeast species. They tested these extracts against 8 Gram negative, 3 Gram positive and 2 yeast species. All the test organisms including Ps. aeruginosa were inhibited by garlic juice, while the onion and shallot juices showed no effect on Gram negative bacteria. Guevara et al. (1984) reported the extracts of A. sativum inhibited growth of bacteria like Ps. aeruginosa along with some fungi. Chatterjee et al. (1980) studied the effects of plant seed extracts on different isolates of Pseudomonas aeruginosa. The different test seed extracts included were Albizia lucida, Bauhinia acuminata, B. racemosa, Cassia tora, Calliandra haematocephala, Cicer arietinum, Crotalaria striata, Dalbergia cultrata, Delonix regia, Desmodium cephalotes, D. gangeticum, Pongamia glabra, Ricinus communis and Saraca indica.

Ryu et al. (1982) used 1% of tea dust or powder with agar medium. The agar plates were inoculated with a single streak line

of each of 4 dilutions (10^{-2} - 10^{-5}) of 24 h. broth culture of 8 common pathogenic bacteria like Staphylococcus aureus, S. faecalis, Pseudomonas aeruginosa etc. He reported that the tea dust had a great inhibitory effect on the growth inhibition of Ps. aeruginosa.

Aynechi et al. (1982) study the antimicrobial effects of 127 species of plants of Iran and reported that of 8 species have inhibitory effect against Ps. aeruginosa. Kurosaki and Arasuke (1983) reported that when fungi like Ceratocystis fimbriata and Thielaviopsis basicola were cultured on carrots they produced 6-methoxy mellin has a broad antibacterial spectrum. The compound inhibited the growth of several fungi, yeast and bacteria like Ps. aeruginosa.

Ajao et al. (1985) reported that the aqueous and ethanolic extracts from the leaves of Spondias mombin and Alchornea cordifolia had a inhibitory effect on the growth of some Gram positive and the Gram negative bacteria like Ps. aeruginosa. The ethanolic extract was more effective than the aqueous extract.

Emeruwa (1982) reported that the extract from ripe and unripe fruits of Carica papaya have a significant antibacterial activity on many bacteria including Ps. aeruginosa. Extracted oil from the leaves of Elsholtzia polystachya inhibited the growth of some bacteria like Staphylococcus albus, Bacillus anthracis, Pseudomonas aeruginosa etc. , Chaturvedi and Saxena (1985).

Antibacterial activity of the plant extracts were screened by Singh and Shukla (1985). They reported that aqueous garlic extract had a good effect on Pseudomonas spp. and Klebsiella spp. Chen et al. (1985) studied the antibacterial property of some spice plants before and after heat treatment. The samples included the extracts from core and outer layers of onion, the white and green parts of green onion, garlic bulbs, ginger, ginger root, sweet pepper, chilli, pepper, brown pepper and mustard. Out of these the extracts from green onion, sweet pepper and mustard showed significant effect on Ps.aeruginosa.

Boily and Van (1986) screened a series of 100 medicinal plants of Rwanda (central Africa) for its antimicrobial activity against some bacteria including Ps.aeruginosa and reported that 30 % of the plants showed positive inhibitory effect.

Mantilla and Sanbria (1986) reported that the ethanol extract of some columbian higher plants showed a significant antibacterial activity towards Ps.aeruginosa. Miski et al. (1984) reported that the leaf-extract from Salvia palaestina Bentham (Labiatae) and Cirisimaritin have good antibacterial activity on Staphylococcus aureus, S.epidermidis, E.coli, Klebsiella pneumoniae, Proteus vulgaris and Ps.aeruginosa. Musleh and Khalaf (1986) studied the effect of storage temperature and heat treatments on the activity of garlic against Ps.aeruginosa isolated from local meat.

However, all these plant extracts have so far only been tested for their in vitro effects on Ps.aeruginosa and others. Their application in the practical field needs more studies.

2.10 Biological manipulation :

Very few evidences of attempts for control of these bacteria in the environment through biological means are there may be due to the fact that biological control is not acceptable without debate as a public health measure for human disease control. Grafe and Baier (1982) reported that water algae like Hydrodictyon reticulatum , Chlorella vulgaris and Aphanotheae nidulans have some inhibitory effects on the growth of some microorganisms. They cultivated the above mentioned algae in special culture containers under good illumination and ventilation. The test organisms were Staphylococcus aureus, Klebsiella aerogenes, Pseudomonas aeruginosa, Salmonella typhimurium and E.coli. Out of the 3 algae two namely H.reticulum and A.nidulans showed good antibacterial effects. Matsuki, Yoshihiko (1979) studied the antagonistic activity of Ps.aeruginosa toward different microbial species by paper disc method on agar plate culture.

Lambina et al. (1983) isolated a new ectoparasitic bacterial species (Micavibrio aeruginosa-vorus) from the host bacterium Ps.aeruginosa and reported that the isolated one showed some inhibitory effect on Gram-negative bacteria including Ps.aeruginosa.

Chapter III

MATERIALS AND METHODS

MATERIALS AND METHODS

3.0 The materials used and the methods followed in conducting the experiments in the present work are given below :

3.1 Materials used :

Following materials have been used in the present work.

A. Bacterial cultures used :

Experiments were conducted with ten (10) bacterial isolates namely GFB₁, GFB₂, GFB₃, GFB₄, GFB₅, GFB₆, GFB₇, GFB₈, GFB₉ and GFB₁₀, which were isolated from soil, water and plant surfaces of different locations. The bacteria were later identified as Pseudomonas aeruginosa (Schroeter) Migula 1900,884.

B. Fungal cultures used :

To study some aspects, some cultures of plant pathogenic fungi were used. Names of the fungi are given below.

1. Fusarium oxysporum udum Butler.
2. Sclerotium rolfsii Sacc.
3. Macrophomina phaseolina (Tassi)Goid.
4. Rhizopus stolonifer (Ehrenb ex Link)Lind.
5. Penicillium italicum Wehmer.
6. Trichoderma viride Pers.ex.Fr.
7. Sclerotium oryza-sativae. L.
8. Helminthosporium oryzae. Bredade Hann.
9. Alternaria solani (Ea Mart)Jones and Grout.

The cultures were obtained from Department of Plant Pathology, B.C.K.V., West Bengal.

C. Test plants used in the experiments :

The names of the plants which were used in different experiments in the present work have been given below. The plants included both crop and wild plants.

Sl. No.	Name of the plant			Family
	English	Local	Scientific	
1.	Cabbage	Bandha Kopi	<u>Brassica oleracea</u> L. Var. Capitata L.	Cruciferae
2.	Cauliflower	Phul Kopi	<u>B.oleracea</u> L. Var. botrytis L.	"
3.	Radish	Mula	<u>Raphanus sativus</u> L.	"
4.	Brown mustard	Sarisha	<u>Brassica campestris</u> L.	"
5.	Beet	Mitha Beet	<u>Beta vulgaris</u> L.	Chenopodiaceae
6.	Tomato	Bilati Begun	<u>Lycopersicum</u> <u>esculentum</u> Mill	Solanaceae
7.	-	Keshradam	<u>Ludwigia</u> adscendens(L.)Hara.	Onagraceae
8.	-	Jangalidal	<u>Hygrophiza</u> <u>aristata</u> Nees.	Gramineae
9.	-	Kachuripana	<u>Eichhornia</u> <u>crassipes</u> (Mart.) Solms.	Pontedariaceae
10.	-	Ulu	<u>Imperata cylindrica</u> L.	Gramineae

(-) unknown.

D. Vegetables, fruits and roots used :

Different types of vegetables, fruits and roots of different plants have been used in the survey and experiment works. Names are given below :

Sl. No.	Name of the plant			Family	Portions used (Veg./Fruit/Root)
	English	Local	Scientific		
1. Potato		Alu	<u>Solanum tuberosum</u> L.	Solanaceae	Stem
2. Sweet potato		Misthi alu	<u>Ipomeea batatas</u> L.	Convolvulaceae	Root
3. Cabbage		Bandha-Kapi	<u>Brassica oleracea</u> L. Var. Capitata	Cruciferae	Leaves & Roots
4. Spinach		Palak	<u>Spinacia oleracea</u> L.	Chenopodiaceae	Leaves & Roots
5. Lablab bean		Shim	<u>Dolichos lablab</u> L.	Leguminosae	Green pods
6. Rice bean		Bean	<u>Phaseolus calcaratus</u> Roxb.	"	Seed
7. Bottle gourd		Lau	<u>Lagenaria siceraria</u> Standl.	Cucurbitaceae	Leaves
8. Onion		Piaj	<u>Allium cepa</u> L.	Lilaceae	Bulb
9. Tomato		Bilati Begun	<u>Lycopersicum esculentum</u> Mill.	Solanaceae	Fruits/leaves
10. Grapevine		Angur	<u>Vitis vinifera</u> L.	Vitaceae	Fruits
11. Orange		Kamala-lebu	<u>Citrus reticulata</u> L.	Rutaceae	Fruits
12. Water chestnut		Panifal	<u>Trapa bispinosa</u> Roxb.	Trapaceae	Fruits
13. Radish		Mula	<u>Raphanus sativus</u> L.	Cruciferae	Root
14. Carrot		Gajar	<u>Daucus carota</u> L.	Umbelliferae	Root

E. Plant parts used :

Different plant parts have been used in different experimental works. The names of the plants and parts are given in the table.

Sl. No.	Name of the plants			Family	Parts used
	English	Local	Scientific		
1.	Paddy	Dhan	<u>Oryza sativa</u> L.	Gramineae	Leaf root & stubbles
2.	Wheat	Gam	<u>Triticum aestivum</u> L.	"	Seed
3.	Maize	Bhutta	<u>Zea mays</u> L.	"	Seed
4.	Gram	Chola	<u>Cicer arietinum</u> L.	Leguminosae	Seed
5.	Brown mustard	Sarisha	<u>Brassica campestris</u> L.	Cruciferae	Leaf and root
6.	Jute	Pat	<u>Corchorus</u> sp.	Tiliaceae	Stem/dead roots
7.	Mango	Aam	<u>Mangifera indica</u> L.	Anacardiaceae	Leaves
8.	Guava	Peyara	<u>Psidium guajava</u> L.	Myrtaceae	Leaves
9.	Rose	Golap	<u>Rosa centifolia</u> L.	Rosaceae	Leaves
10.	-	Sonajhuri	<u>Acacia auriculiformis</u>	Mimosae	Leaves
11.	Sal	Sal	<u>Shorea robusta</u> Gaertn.f.	Dipterocarpaceae	Bark/leaves

(-) unknown

F. Plant extracts and materials :

a) Some plants - extracts were used which were prepared from mentioned plants:

1. Tephrosia candida
2. Coriaria sp.
3. Rungia sp.
4. Phaseolus sp.
5. Azadirachta indica

These extracts were supplied from the Department of Soil Chemistry laboratory, B.C.K.V.

b) Some plant materials were used which were prepared from plants are given below :

1. Neem cake - from seeds of Azadirachta indica
2. Mustard cake - from seeds of Brassica campestris
3. Mahua cake - from seeds of Madhuca indica
4. Neem-leaf extract - from leaves of A.indica
5. Rice-straw extract - from stems of oryza sativa

G. Fishes and animals used :

The fishes and different animals used in the survey are listed below. The names of the fishes and animals are given below.

Sl. No.	Name of the fishes and animals			Order
	English	Local	Scientific	
1.	Katla	Katla	<u>Catla catla</u>	Cypriniformes or ostariophysi
2.	Tangra	Tangra	<u>Mystus seenghala</u>	Cypriniformes or Ostariophysi

(Contd..)

<u>English</u>	<u>Local</u>	<u>Scientific</u>	<u>Order</u>
3. Swiss Mouse	Sada Indoor	<u>Mus musculus</u>	Rodentia
4. Rabbit	Khargosh	<u>Oryctolagus</u> sp.	Lagomerpha

H. Animal product and excreta used :

In some experiments products of some animals and excreta material of some animals were used. They are given below :

Sl. No.	Name of the animals		Order	Products used
	English	Scientific		
1.	Cow	<u>Bos indicus</u>	Artiodactyla	Dung
2.	Goat	<u>Capra</u> spp.	Artiodactyla	Dung/Droppings
3.	Man	<u>Homo sapiens</u>	Primates	Hair
4.	Hen (Fowl)	<u>Gallus</u> spp.	Galliformes	Feather/egg shell

I. Media used in the work for different purposes are as follows :

Routine cultural work like maintenance of cultures and multiplication of bacteria were usually done on Kings Medium B, enriched with cetrinide. A few media were used for different studies with fungi and bacteria, composition of which are given below :

Media used : (a) General Media

1) King's Broth B : (KB)

Proteose peptone	- 20 gm
Glycerol	- 10 gm (8 ml)
K ₂ HPO ₄	- 1.5 gm
MgSO ₄ (7 H ₂ O)	- 1.5 gm

Distilled water to make	- 1000 ml
pH	- 7.2

ii) King's Broth B enrichment with cetrinide :

Proteose peptone	- 20 gm
Glycerol	- 10 gm (8 ml)
K_2HPO_4	- 1.5 gm
$MgSO_4(7H_2O)$	- 1.5 mg
Cetrinide	- 300 mg
Distilled water to make	- 1000 ml
pH	- 7.2

iii) King's Medium B enriched with cetrinide (KBC) :

Same as broth, only 15 gm agar was added.

iv) Pyocyanin broth (Tuite) :

Bacto, peptone	- 20 gm
Glycerol	- 10 gm
K_2SO_4 (anhydrous)	- 10 gm
$MgCl_2$	- 14 gm
Distilled water to make	- 1000 ml
pH	- 7.2

v) Pyocyanin agar medium (Tuite) :

Same as Pyocyanin broth; only solidified with 15 gm agar agar.

vi) MacConkey's Broth composed of -

Sodium tauroglycocholate	- 5 gm
Peptone	- 20 gm
Sodium chloride	- 5 gm

The ingredients were dissolved in water and the following ingredients were further added.

Lactose	- 20 gm
1% Neutral red	- 10 ml
(Sterilized at 5 lb for 10 minutes)	
Water	- 1000 ml
pH	- 7.6

vii) MacConkey's Agar Medium :

Same as MacConkey's broth only solidified with 15 gm agar agar.

viii) Nutrient broth composed of :

Beef extract	- 3 gm
Peptone	- 5 gm
Distilled water to make	- 1000 ml

ix) Nutrient Agar Medium :

Same as Nutrient broth, solidified with 15 gm agar agar.

x) Potato Dextrose Agar medium (PDA) :

Peeled potato	- 200 gm
Dextrose	- 20 gm
Agar Agar	- 20 gm
Distilled water to make	- 1000 ml.

xi) Potato Dextrose Broth :

Same as FDA medium only agar agar was excluded.

xii) Sucrose medium :

Peptone	- 10 gm
Beef extract	- 5 gm
Sucrose	- 50 gm
Agar Agar	- 20 gm
Distilled water	- 1000 ml

(b) Plant tissue Media used :

Leaves of the three hydrophytic plants viz. Ludwigia adscendens (L.) Hara, Hygrophiza aristata Nees., and Eichhornia crassipes (Mart.) Solms.; and one terrestrial grass Imperata cylindrica L. were taken, chopped into small pieces and used as media for the study of growth and saprophytic ability of the bacterium.

- i) J.A.M. - Ludwigia adscendens leaf - 25 gm
Distilled water - 10 ml
- ii) H.C.M. - Hygrophiza aristata leaf - 25 gm
Distilled water - 10 ml
- iii) E.C.M. - Eichhornia crassipes leaf - 25 gm
Distilled water - 10 ml
- iv) I.C.M. - Imperata cylindrica leaf - 25 gm
Distilled water - 10 ml

(c) Grain Dust Media used :

For the purpose of experimentation graindusts of wheat and maize were collected separately from local market, and media were prepared as follows :

- i) W.D.M - Wheat dust - 6 gm
 Distilled water - 15 ml
- ii) M.D.M.- Maize dust - 6 gm
 Distilled water - 15 ml
- iii) G.D.M.- Gram dust - 6 gm
 Distilled water - 15 ml

(d) Grain meal media used :

Two types of grain meals were used for the preparation of Grain meal media namely (i) Wheat and (ii) maize.

- i) W.M.M. - Wheat meal - 6 gm
 Distilled water - 10 ml
- ii) M.M.M. - Maize meal - 6 gm
 Distilled water - 10 ml
- iii) G.M.M. - Gram Meal - 6 gm
 Distilled water - 10 ml

J. Staining solutions and Reagents used :

(a) Cleaning Solution

Chromic acid solution :

- Potassium dichromate - 60 gm
 ($K_2Cr_2O_7$)
- Sulfuric acid (Conc.) - 500 ml
- Distilled water - 500 ml

(b) Nigrosin solution

- Nigrosin crystals - 10 gm
- Distilled water - 100 ml

(c) Ammonium oxalate - Crystal violet solution

Solution A -	Crystal violet	- 2 gm
	Ethyl alcohol (95 %)	- 2.0 ml
Solution B -	Ammonium oxalate	- 0.8 gm
	Distilled water	-80.0 ml

Mixture of Solution A and B.

(d) Crystal violet solution

Crystal violet (85 % dye content)	- 1.0 gm
Distilled water	- 100.0 ml

(e) Gram's iodine solution

Iodine	- 1.0 gm
Potassium iodine ^d (KI)	- 2.0 gm
Distilled water	- 300.0 ml

(f) Safranin solution

Safranin (2.5% solution in 95 % ethyl alcohol)	- 10.0 ml
Distilled water	- 100.0 ml

(g) Safranin solution (0.5 % - aqueous)

Safranin O	- 0.5 gm
Distilled water	- 100.0 ml

(h) Malachite green solution (5%)

Malachite green	- 5.0 gm
Distilled water	- 100.0 ml

(i) Neutral red solution

Neutral red	- 1.0 gm
Water or ethanol	- 100.0 ml

(j) Copper sulfate solution (20%)

Copper sulfate - 200.0 gm
 Distilled water to make - 1000.0 ml

(k) Staining solutions of flagellaSolution A :

Mercuric chloride C.P. - 2 ml
 (saturated aqueous solution)
 Tannic acid (20% aqueous solution) - 2 ml
 Aluminium potassium sulphate (Saturated aqueous solution) - 5 ml

Solution B :

Basic fuchsin - 0.4 ml
 (Saturated alcoholic solution)

Solution C : (Ziehl-Neelson's Carbol Fuchsin)

Basic fuchsin - 0.3 gm
 Alcohol (95 %) - 70.0 ml
 Phenol Crystals C.P. - 5.0 gm
 Distilled water - 95 ml

K. Detergents Used :

The following detergents were used during the study :

<u>Name</u>	<u>Chemical composition</u>	<u>Source</u>
1. Tween 80 -	Polyoxyethylene sorbitan monooleate	- Sigma chemicals Co.Ltd.
2. Teepol -	Alcohol; fattyacid; or oil and wax.	- NOCIL
3. Triton 'X' -	Alkylated aryl polyether	- Bharat Pulverising Co. Ltd.
4. Sunlight Soap -	Olive-oil Castile, alcohol Caustic Soda	- Hindusthan Lever Pvt.Ltd.

L. Organic matter used :

Different types of organic matters were used in different experiments namely, Oil cake, rotten leaf, FYM (Farm Yard Manure), different types of water soluble organic matter from ponds.

M. Organic Chemicals :

1. Formaldehyde - HCHO *capitol*
2. Phenol - $\text{C}_6\text{H}_5\text{OH}$

N. Inorganic chemical :

1. Potassium sulfate - K_2SO_4
2. Zinc sulfate - ZnSO_4
3. Boric acid - H_2BO_3
4. Hydrochloric acid - HCl
5. Sodium hydroxyde - NaOH
6. Lime - CaO
7. Bleaching Powder - $\text{CaO}(\text{CL})\text{Cl}$.

O. Antiseptics used :

1. Dettol - A mixture of chloroxylenol; Benzyltol and SO_2Cl_2 .
2. Phenyl - A mixture of Creosote and soap.

P. Antibiotics Used :

Some of the antibiotics namely neomycin, gentamycin, chloramphenical, Streptomycin and Penicillin were obtained from Span diagnostics, Surat, India in paper-disc form.

<u>Antibiotics</u>	<u>Company</u>
1) Bacterionol	Merlin laboratories
2) Norfloxin	Cipla Ltd.
3) Ledermycin	Cynamid India Ltd.
4) Cifran	Ranbaxy

Methods followed :

3.2 Following methods were followed to conduct different experiments. The methods are described below briefly.

3.2.1 Cleaning and sterilization of glass-ware

Before use all the glasswares like petridishes, pipettes, sample collecting bottles, culture tubes etc. were washed with prepared chromic acid solution, rinsed and washed in tap water and finally in distilled water. After proper drying and packing the glass-ware were sterilized at 160°C for 2 hrs in a hot air sterilizer. All materials requiring and withstanding dry-sterilization were sterilized similarly.

3.2.2 Preparation and sterilization of media

Different types of media namely King's Medium B, PDA, plant tissue media, Grain dust and meal media and others were prepared for conducting different types of experiments. Methods of preparation of media and their sterilization are described below :

a) General aspects of media sterilization : Ingredients of media or broth like NA, King's medium B etc. were dissolved in distilled water following standard procedures. The ingredients were weighed separately, dissolved, mixed, dispensed in required volumes in respective containers which were corked or plugged properly and sterilized generally by autoclaving at 121°C for 15 minutes. Necessary adjustments of pH was always made before pouring of the medium in containers and their sterilization. Dilute solutions of alkali or acid and a disc pH-paper (Merck) were used for the purpose.

b) Potato Dextrose Agar medium (PDA) :

Requisite quantity of peeled potato was boiled in 500 ml of distilled water and the decoction was filtered through a cheese-cloth. Agar-agar was then dissolved in the hot potato decoction. Dextrose was also added and the rest quantity of water was added to make the volume upto 1 litre while the mixture was boiling. The medium was poured @ 5-6 ml/tube, plugged with non-absorbent cotton and autoclaved for 15 minutes at 121°C. The tubes containing the media were taken out and laid in slant positions after partial cooling. The slants prepared were normally stored in a refrigerator at 5°C for future use.

c) Preparation of plant tissue media :

Plant tissues were cut into small pieces and crushed in an electrical mixer, weighed in balance, poured into culture tubes or conical flasks, and sufficient water was added to moisten. The tubes

or flasks were plugged with non-absorbent cotton and then sterilized in an autoclave at 121°C (15 lb pressure) for 15 minutes ^{on five successive days} successively on 2 subsequent days.

d) Grain-dust and grain-meal media :

Grain dusts and grain meals were weighed separately in a balance and mixed with distilled water. The mixture was poured in 250 ml conical flask, plugged with cotton and sterilized in an autoclave at 121°C temperature (15 lb pressure) for 15 minutes successively on two days.

3.2.3 Survey and Sample Collection :

The survey usually was conducted on a road-route on the east bank of river Ganga touching the sample sites in 7 cases. In one case the composite sample collected from a rivulet locally known as Swaraswati which joins river Ganga on its western bank, was on the other side of the river. After the road journey, country boat was also used wherever necessary for reaching the exact spot desired of a sample site.

(a) Sites for Sample Collection :

To conduct survey for the green fluorescent bacteria, samples were collected from around the drains/canals leading watery discharges from different sources to river Ganga. The samples have been collected from eight selected sample sites. The eight sites normally come under four broad categories and each category representing by 1 to 3 sample sites. The three sites under category I. Industry, were a) Indian Paper Pulp Co., Hazinagar, (b) Jute Mill, Naihati, (c) Rubber Factory

Dunlop ghat, Category II. Agriculture, included sites like (a) Brick field, Shyamnagar, and (b) Bagerkhal; Kanchrapara, Category III. Of Town discharges sites like (a) Dhobighat, Barrackpore, and (b) Khatal (Cattle shed) discharge, Shyamnagar. The category IV having only one site is of Composite nature and is Swaraswati River, Tribeni (Fig.1). From each of the sites three types of samples were collected. They were (1) water of the discharge channel, (2) moist-soil from Channel-bank and (3) soaked leaves of plants near or around the discharges reaching Ganga through the discharge channels. Besides these, waters from the river body from near the above mentioned eight sites were also collected.

Leaf and root portions of three plants like (1) Palak, (2) Cabbage and (3) Rice were also collected from agricultural fields on the eastern bank of the Ganga in different villages for isolation of the green fluorescent bacteria.

Different types of fruits, vegetables, root crops and fishes were also collected from different markets for isolation of the green fluorescent bacteria.

(b) Sample Collection :

For collection of water samples usually blank, sterilized tubes of suitable size were taken. Sample water was collected with these tubes quickly, putting back the plugs/corks immediately. Sometimes 'angling rods with wheels' like mechanisms were employed for collection of water samples. For soil samples 100 ml sterilized volumetric

flasks were used. Plant materials collected were mainly leaves. They were collected in sterilized polypropylene bags. Plating from all the samples were done within 12-18 hrs. of their collection.

3.2.4 pH-measurement :

Paste of soil was prepared in 150 ml beaker and pH was determined with one photovolt pH-meter by adjusting the pH 7.0 with buffer solution.

In case of water samples they are taken in 150 ml beaker and pH was measured with the photovolt pH meter using buffer tablet of pH 7.0.

3.2.5 Measurement of electrical conductivity :

Electrical conductivity of the different water was measured by direct reading of conductivity-meter-'300'.

3.2.6 Moisture determination :

Moisture determination of the soil has been made following standard methods. 10 gm of soil sample was separately put in previously weighed Kin-box. Then the box + soil weight was taken. The soil in the box was dried in an oven at 105°C to 110°C for a period (approximately 48 hr) until a constant weight was reached. The moisture percentage was calculated thereafter.

3.2.7 Isolation of Fluorescent bacteria :

(a) Dilution plating Method :

Bacteria were isolated from the collected samples by dilution plating technique of following standard bacteriological procedures

(Schroth et al., 1977 : Misagi and Grogan, 1969).

Generally 10 gram of soil or plant material (10 ml in case of water) as samples were taken every time at least 5 ^{from} places ^{at more} from each site at a time. The samples were initially diluted into and upto 100 ml. ^{sterile} distilled water to make a suspension of 10^{-1} strength. The suspension was further diluted ^{serially} by transferring aseptically with pipettes 1 ml of suspension to 9 ml of sterile distilled water to go upto 10^{-6} dilution. One ml of each of the ^{Serial dilutions} samples were plated in King's medium-B enriched with cetrinide @ 20 ml/plate. The plates were incubated at $40 \pm 1^{\circ}\text{C}$ in an incubator. In each case necessary replicates were maintained. Colonies developed on the plates were counted according to the objective of the experiment. Single colonies were isolated from them normally after 24-48 hrs. and streaked aseptically in KBC-slants for further work.

(b) Purification of culture : was done by dilution of single colonies in sterile water and drawing double streaks to get pure single colony.

3.2.8 Maintenance and storage of culture :

The bacterial cultures were maintained on KBC (King's medium 'B' enrichment with cetrinide) slants at $5 \pm 1^{\circ}\text{C}$ by keeping them in the refrigerator and transferred at every fifteen days interval to maintain the virulence and growth characteristics. The transferred cultures after incubation at $40-42^{\circ}\text{C}$ for 24-48 hours were always kept in the refrigerator at $5 \pm 1^{\circ}\text{C}$ for storage for different periods.

The slants were labelled with the name of the bacterium and date of inoculation. For long storage cultures were stored as cell suspension in sterilized water or KB-broth.

3.2.9 Subculturing and age of cultures :

Sub-culturing was done either on KBC or KB slants. A loopful of the bacterium was taken and streaks were drawn on sterilized KBC or KB agar slants aseptically. For better bacterial growth slants were incubated at 40-42°C for 24-48 hours.

Generally in course of the present study in most of the experiment the bacterial culture used was 24-48 hour old KB culture.

3.2.10 Preparation of bacterial suspension :

Cells from 24-48 hr old KB or KBC cultures were harvested by putting in the concerned slants requisite volume of sterilized, distilled water under aseptic conditions with sterilized pipettes. The suspension thus prepared by slight shaking of the slant were later standardised for determination of cell number per ml of suspension with the help of McFarland's scale.

3.2.11 Preparation of McFarland's Scale :

(a) A standard turbidity scale (McFarland Scale) was prepared to determine the number of cells of the bacterium in a definite volume of suspension by measuring its turbidity. The advantage of this scale was that once prepared and preserved in screw-capped tubes in a refrigerator it remained usable for years.

The scale was prepared by mixing solutions of BaCl_2 and H_2SO_4 in different proportions to get turbidity from BaSO_4 precipitation in the mixture solution which is equivalent to the turbidity that may be produced by a definite number of bacterial cells. Details of the scale as provided in (Klement et al., 1978), is given in the table.

b) McFarland's Scale :

Table No.	ml. of 1% BaCl_2	ml of 1% H_2SO_4	No.of bacterial cells/ml
1	0.01	9.99	3×10^7
2	0.05	9.95	1.5×10^8
3	0.1	9.9	3×10^8
4	0.15	9.85	4.5×10^8
5	0.2	9.8	6×10^8
6	0.3	9.7	9×10^8
7	0.4	9.6	1.2×10^9
8	0.5	9.5	1.5×10^9
9	0.6	9.4	1.8×10^9
10	0.7	9.3	2.1×10^8
11	0.8	9.2	2.4×10^9
12	0.9	9.1	2.7×10^9
13	1.0	9.0	3.0×10^9

3.2.12 Measurement of growth of Bacteria :

In order to measure growth of the bacteria on any solid media the bacterial growth was slowly brought into suspension in distilled water by putting the water on the colony scraping with a camel hair brush and shaking the container. The suspension was then decanted followed by one or two subsequent washes with distilled water. The total quantity of water added was determined according to the concentration of cells/ml of water volume required. Thus the turbidity maintained at a stage was compared with those in the McFarland's scales. The total cell number was then calculated on the basis of the reading in McFarland's scale and the volume of water used for dilution of the cell suspension.

In cases of grain dust and meal media, it was washed thoroughly with distilled water and strained with ordinary cloth to separate the dust and meal from the cells.

The total number of bacteria/ml was then obtained from the scale. So, the result was multiplied with the total volume of water added to get the measure of the total number of bacteria.

3.2.13 Raising of plants in pots :

Seedlings of different plants of known variety were purchased from Kalyani nursery and then planted in the earthen pots of 9" diameter for experimental purposes. Manuring and irrigation were properly done for sufficient growth of the plants.

3.2.14 Inoculation of plants with bacteria :

(a) Inoculation of plants with the bacteria was done following different methods :

(a) Rubbing inoculation : For pathogenicity test the leaves of the selected plants were inoculated with the bacteria both following injury and without injury. The plants injured by rubbing with the carborandum powder (300 mesh) on the leaf surface. Small amount of powder was placed on the surface of the cotton soaked in bacterial suspension of suitable concentration. Then the leaf surfaces were smoothly rubbed with the cotton with bacterial inoculation alone and with carborandum powder. The inoculated plants were incubated on laboratory benches with a cover for first 12 hrs.

(b) Spraying inoculation with bacterial suspension : From the suspension of 24 hr. bacterial culture different dilutions were made with the help of McFarland scale and the suspensions were sprayed by Holms-Sprayer. Spraying was done on test plant surfaces.

(c) Root dipping method : One month old seedlings of host plants were dipped for 30 mts in the bacterial suspension of suitable strength before planting.

(d) Injection method : Bacterial suspension of a suitable strength was injected into the stem and leaf of the test plant by small injection syringe with a 23 gauze needle.

3.2.15 Animal pathogenicity test :

Animal pathogenicity of the isolates was tested on albino mouse of months age.

a) Rearing of the animals in cage : White Swiss variety mice were purchased from Calcutta market and properly maintained in the cage. To keep them healthy, food grains, dust of wheat with vitamins powder were mixed properly with water, and plane water were supplied.

b) Method of inoculation : 24 hour ^{-old} bacterial cultures were injected to the 60-70 days aged mice. Before injection niddle and syringe were properly sterilized and then the bacterial suspension were injected in the peritoneal portion of the mice.

c) Maintenance and observation after inoculation : After injection the mice were kept in the cage and observations were recorded by days intervals. The symptoms appeared for the injection were recorded. After the death of the mice sterilized needle were injected through urinogenital apertures to detect the presence of the bacterin within the kidney and passage.

3.2.16 Preparation of Stains and other chemical solution :

(a) Preparation of Chromic acid or cleaning Solution :

In 500 ml distilled water 60 gm potassium dichromate ($K_2Cr_2O_7$) was dissolved gently. Then in 500 ml distilled water concentrated sulfuric acid (H_2SO_4) were added very slowly stirring with a glass rod. They were mixed together and chromic acid solution was prepared.

(b) Preparation of Nigrosin solution :

Ten gm of Nigrosin were boiled for 30 minutes. 5 ml formaldehyde was added as preservative. The solution was filtered twice through double filter paper and stored under aseptic conditions. The solution used for relief staining.

(c) Preparation of 1% neutral red :

One gm of neutral red was mixed with 100 ml of ethanol or dist. water and sterilized under 5 lb pressure for 15 minutes.

(d) Preparation of crystal violet solution :

Requisite quantities of crystal violet dye was taken and dissolved in distilled water. The stain thus prepared and kept in a glass bottle, which were used for capsule staining.

(e) Preparation of ammonium oxalate crystal violet stain :

Crystal violet dye was taken in requisite quantity and dissolved in ethyl alcohol (Soln.A). In another container ammonium oxalate was dissolved in water (Soln.B). Solutions A and B thus prepared, were mixed together to get the ammonium oxalate crystal violet stain which were used for Gram staining.

(f) Preparation of Gram's Iodine/solution :

Iodine & KI were taken in requisite quantities and dissolved in distilled water. The stain thus prepared was kept in a glass bottle.

(g) Preparation of safranin solution :

Safranin was taken in requisite quantities and dissolved in 95 % alcohol. The solution was diluted with water and kept in a bottle for future use. The stain was used for Gram staining.

(h) Preparation of Malachite green solution (5%):

5 gm of fresh malachite green crystals was taken and dissolved in 100 ml sterile distilled water for spore staining.

(i) Preparation of Safranin Solution (0.5%) :

•5 gm of Safranin O was taken and dissolved in 100 ml sterile distilled water which were used for spore staining.

(j) Preparation of Copper Sulfate solution :

Requisite quantity of copper sulfate was dissolved in distilled water and stored in glass bottle properly. The solution was used in capsule staining.

(k) Preparation of Mordant solution :

Solution A - 2 ml. saturated aqueous solution of Mercuric chloride was mixed with 2 ml of 20 % aqueous solution of Tannic acid and then mixed with 5 ml of saturated aqueous solution of Aluminium potassium sulphate. Thus solution A was prepared.

Solution B - Saturated alcoholic solution of Basic Fuchsin was prepared.

Then 9 ml solution A & 4 ml solution B was added to get the Mordant solution. They were mixed within 24 hrs. of use, for flagella staining.

(l) Preparation of Ziehl Neelson's Carbol fuchsin stain :

Requisite quantity of Basic fuchsin was dissolved in alcohol and 5 gm of phenol crystals were dissolved in water. Then the two solutions were mixed which was used for flagella staining.

3.2.17 Determination of the bacterial isolates :

The purified cultures of the isolates of green fluorescent bacteria were studied for their characteristics of different categories like (i) cell morphological (ii) colony characteristics and (iii) physiological and biochemical characteristics.

(a) Cell morphological characteristics were studied following different methods of staining including Gram staining, Relief staining and Flagella staining following standard methods, (Klement et al., 1974).

(i) Relief staining : A loopful of 24 hr culture of the bacterium was taken on a grease-free slide. A drop of freshly prepared Nigrosin solution was added. The two were mixed and smear was prepared with another slide.

(ii) Gram staining : A smeare of the bacterium was drawn on a slide by placing a loop of culture on it and spreading the culture in thin film on the slide. Air drying and fixing in a flame was done subsequently. The smear was stained with CV solution for 2-3 minutes and slide was then flooded with Iodine solution for 30 secs. The slide was rinsed with water. The stained slide was decolorized with 95 % alcohol by dripping alcohol for 10-30 secs. The slide was again rinsed with water. The slide was then counterstained with safranin for about 10 secs. The slide was finally blotted dry and examined under oil immersion objective of the microscope.

(iii) Spore staining : Bacterial smears from a 72 hours cultures were prepared on degreased slides. Then the smears were air dried and fixed with spirit lamp flame heat. The smears were then flooded with malachite green solution (5% aqueous) for 2-3 minutes and heated to steaming for 30 sec. The slide were washed in tap water and covered with safranin solutions (0.5 % aqueous) for 30 sec. The slides were washed in tap water, blotted, air-dried. The preparation were examined under oil immersion objective.

(iv) Studies on presence of capsule of the green fluorescent bacteria :

For capsule staining a smear from 48 hours culture and a drop of sterile distilled water is prepared on a slides, on cleaned slides. The smear is dried and fixed quickly by flaming 5-6 times. Crystal violet was applied for 30 seconds and washed with copper sulphate solutions blotted dry. Capsules were seen as light blue against the dark purple blue cells. Observation was recorded.

(v) Studies on the flagella of the green fluorescent bacteria :

For flagella staining the bacteria were grown in sugar-free Nutrient medium. The cultures of the bacteria were handled carefully since the flagella are very delicate and fragile. The flagella were stained by the following procedure.

a) Cleaning of slide : Paste of vim powder was prepared and coated the slides for few seconds. The slides were cleaned repeatedly by cotton swabs to become grease-free.

b) Smear preparation : Sterilized distilled water was poured to the bacterial slants to cover the entire bacterial growth and allowed for few minutes for coming out the bacteria to the water. Then a sterilized pipette was entered to the bacterial suspension. A small amount of bacterial suspension was enters the pipette by the cappillary action. Then a drop of bacterial suspension were put on the upper end of the slide and kept in slanting position. The slides were air dried at room temperature. The smear area was mearked with glass pencil.

c) Staining of the smear : The smear was covered with mordant solution and allowed for 8-10 minutes. Then the slide was washed with distilled water. Ziehl Neelson's solution was filtered on the slide to cover the smear and allowed for 5 minutes. Then washed with tap water and air dried. The slide were examined under oil-immersion objective.

vi) Studies the motility of the green fluorescent bacteria :

In order to observe unstained living bacteria and their motility the hanging drop preparation were made only from young cultures.

Young slant cultures of the bacterium were taken and sterilized distilled water were poured very gently on the slant with the help of a micropipette along the inner wall of the culture tube. The tubes were allowed to stand for 5 minutes for allowing the bacterial cells to ooze out in water. Then the edges of the coverslip was first

greased and then drop of the bacterial suspension was placed at the centre. The cavity slide was placed over the coverslip in such a way that the depression on the slide remains over the drop and pressed lightly. The slide was then turned quickly such a manner that the drop does not touch the hollow part of the slide. Then the slide observed under microscope.

vii) Studies on the oxygen relations of the green fluorescent bacteria :

The study was made in the KBC broth while sterilising the KBC broth medium, 20 ml of liquid paraffin was also sterilised in a plugged tube. After inoculating the media liquid paraffin @ 2 ml/tube was poured over the medium, thus separating the medium from the contact of air. Equal number of inoculated tubes were left without liquid paraffin covering. In this case also five sets were made of which one was left as control. The observations were made after incubating the tubes at $40 \pm 1^\circ\text{C}$ for 72 hours and results were taken.

viii) Studies on the production of levan by the green fluorescent bacteria :

To test the levan production sucrose medium were used and sterilized by steaming for 30 minutes on 3 successive days, in flasks. The medium were poured in sterile petriplates and solidified. Dilute suspension of the test culture were streaked on the solidified agar medium. Then the plates were incubated at $41 \pm 1^\circ\text{C}$ for 48 hours and observation were made.

3.2.18 Serological studies :

For serological testing of the isolated eight (Ganga) green fluorescent bacteria and two pond isolated green fluorescent bacteria GFB₉ and GFB₁₀ were tested against the antisera of three green fluorescent isolates namely GFB₅ , GFB₁ , GFB₆. Preparation of antisera of the isolates were made following the methods are described below :

i) Preparation of antigen : Single colony isolates of the bacterium were freshly inoculated to a definida medium (KBC) and incubated for five days. The slopes were then washed separately with sterile normal saline solution (0.85 %).

The bacterial suspension in saline solution was filtered through cotton wool to remove the bacterial clumps if there were any. A suspension of antigen containing 5×10^7 bacterial cells/ml (approx) was used for injection into a rabbit.

ii) Preparation of anti-serum : Healthy well developed, white male rabbits were selected for immunization. Antigenic suspension (5ml), as prepared above was mixed with 2-5 ml of fraund adjuvent (Difco) to get a homogenous emulsion. This emulsion was injected intramuscularly into the hind limb muscle of the rabbit with a 18 gauge needle. Four successive intramuscular injections were applied for 7 days interval @ 0.5 ml, 1.0 ml, 1.5 ml and 1.5 ml respectively. A week later, four successive doses of intervenous injections were also applied at 5 days interval with saline suspension of the antigen

without mixing adjuvant to the large margin of ear-vein with a 22 gauge needle. The doses of suspensions administered were at the rate of 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5 and 3 ml respectively. Seven days after the last injection .5 ml of blood was drawn from the ear-vein. The blood was incubated at 37°C for 1 hr and then kept at 4°C over night. The serum was then drawn off and centrifuged to free it of residual cells. The titre of the serum was checked and after 3 days one booster dose of intravenous injection (3 ml) was applied. After a week a main blood sample (10-15 ml) was collected from the animal and serum was prepared as usual. The serum samples were distributed in amber coloured small vials and were stored at -15°C.

iii) Gel-immune diffusions methods : For immuno-diffusion test 0.75% agarose gel was prepared in 0.85 % NaCl solutions. Sodiumazide was added to the molten agar to a concentration of 0.025 % as a preservative. The molten agar was then poured into petriplates (previously cleaned and dried) to get the desired gels of 4 mm. thickness. After solidification, wells of 4 mm. diameter were cut out following a hexagonal pattern. The bottoms of the wells were then sealed with molten agar to prevent leakage. These plates were then stored in moist chambers at 4°C till they were used.

For sero-diagnosis of the Pseudomonas strains, antigen suspensions of the present strain (control) were placed in the top and bottom wells. In the remaining four lateral wells, four suspensions of unknown strains were put to evaluate their cross-reactivities with the present

strain. Antiserum of definite titre value (determined earlier) was placed in the middle well. The plates were then kept in a moist chamber at 25°C. The precipitin bands were found to appear within 48 hrs. Band formations were recorded by viewing the plates held over a black ^{background} and illuminated obliquely. Photographs of the precipitin patterns were taken by a single lense reflex x 35 mini camera fitted with extension tubes. Orow Panchromatic 50 ASA films were used for the purpose.

3.2.19 Assay of sensitivity of bacteria to chemicals :

(a) Fish spine method :

Plates with KBC were seeded with bacteria similarly and were used for adding the chemicals, plant extracts etc. in the seeded plates. Fish spines were dipped in the solutions taken in watch glass put on the agar surfaces of plates aseptically by sterilized forceps. The plates were incubated at $40 \pm 1^\circ\text{C}$ for 48 hrs, after which observations were made. Chemicals from fish spines diffused through the agar medium and in case of inhibitory effect made clear zones.

(b) Paper disc method :

This method was used only in case of the antibiotics which were available from Span diagnostics, Surat, India in paper disc form. Seeded plates were prepared as usual and the paper discs (6 mm dia.) were put aseptically on the agar surface @ three discs per plates. Plates were incubated at $40 \pm 1^\circ\text{C}$ as before for 48 hrs. Chemicals from paper discs diffused through the agar medium and in case of inhibitory ones clear zones were found.

3.2.20 Statistical Analysis :

Statistical analysis was done following standard methods. They include analysis of variance etc.

a) Analysis of variance :

The basic assumptions underlying the analysis of variance of the experimental data are i) that the observations are normally distributed about their true or expected values, ii) that the observations are independent and iii) that the variance of the observations is constant.

The present observations on disease intensities made in a cumulative scale giving percentage data do not satisfy all the above assumptions. So in order to fulfill the conditions for application of analysis of variance in the present case, the percentage data have been transformed into angular scales by $\sqrt{\sin^{-1} x}$ transformation. The Table X of Fisher and Yates (1963) with necessary interpolation was used for the purpose. The variance analysis was engaged in cases of Tables 1,2,3,4 & 5.

Chapter IV

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Survey of the extent of green fluorescent bacteria around Ganga river :

Considering the importance of some species of green fluorescent Pseudomonas (Schroth et al., 1977), a survey for determination of the extent of occurrence of green fluorescent bacteria was taken up as an important work.

A study was taken up to determine the occurrence of green fluorescent bacteria in or on different surfaces and / or substrates around the river Ganga, the most important river of the zone. Such bacteria are reported elsewhere, occurring in good number in different types of watery effluents coming out from different industries, agricultural fields (Green et al., 1974) urban drainages and sewages (Schroth et al., 1977). So it was thought that the bacteria might have natural hideouts in water and moist-soils of such ecological situations or even on surfaces of plants growing in such ecological context.

The present survey was aimed to understand the extent of green fluorescent Pseudomonas particularly a species, Ps.aeruginosa in and around the Ganga water with particular interest of estimating their pathogenic properties. Some members of this group of bacteria like Ps.aeruginosa are very serious, opportunistic pathogen which may occur in such moist or wet situations and at times cause serious infections in animals or even human system.

The total survey work is being presented in seven sections (4.1.1 to 4.1.7) according to the nature of the habitats that have been surveyed.

In the first section general extent of occurrence and distribution of green fluorescent bacteria around the river Ganga has been covered. This mostly assessed the occurrence of these bacteria in surroundings of different channels or canals which are carrying effluents or discharges from different industries, villages, towns etc.

In the subsequent six sections extent of such bacteria in the river water itself, on growing vegetable crops in the fields, on vegetables and fruits in the markets, on the fishes in the market, on the plant litters and on the dead plant parts have been assessed separately.

4.1.1 Extent of green fluorescent bacteria in waste water bodies, the vegetation and soil around :

Green fluorescent bacteria have been found to occur in the surroundings of different water bodies elsewhere (Schroth et al., 1977). They are reported to occur in such waters, soil, near the water bodies and also on nearby plant surfaces.

In the present work a similar study was intended with the objective of assessing the extent and distribution of green fluorescent Pseudomonas around a part of the lower stretch of river the Ganga from Tribeni to Barrackpore (Fig.1). The study was aimed to make an estimate of the population of the green fluorescent bacteria

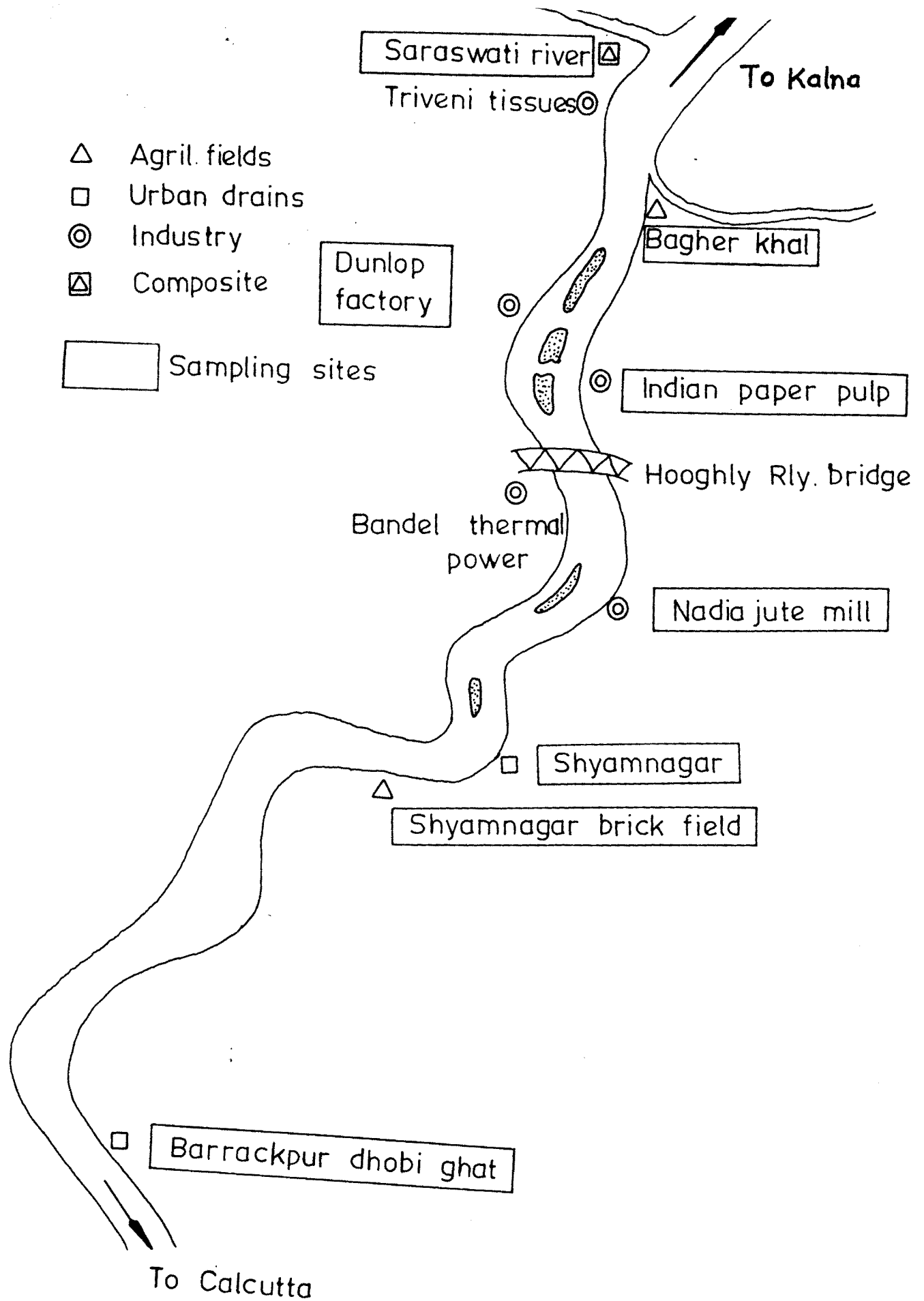


Figure 1. THE GANGA : BARRACKPUR TO KALYANI
 from a sketch map of CPT, 1967-68.

as against total bacteria in the effluent channels carrying water from industries, town, villages etc. and the soil and plants lying and growing around these water bodies. The eight selected sample sites are shown in Fig.1.

Collection of samples were made from 8 sites along the river Ganga. They included 3-Industrial effluents, 2-Urban sewerage or drainage discharges, 2-Village discharges and 1-Composite discharge through a tributary of the Ganga. From each of the sites, collection of (i) Water, (ii) moist soil and (iii) soaked leaves of plants from around the effluents and discharges reaching river Ganga was made. The samples were collected twice in the year namely during monsoon and pre-monsoon seasons. The sample sites selected are situated in the districts of Nadia, Hooghli and 24-Parganas.

(a) The total survey work is being presented in seven sectors.

(4.1.1 to 4.1.7) according to the nature of habitats that have been surveyed.

In this first section general extent of occurrence and distribution of green fluorescent bacteria around the river Ganga has been covered. This mostly assessed the occurrence of these bacteria in a surroundings of different channels or canals which are carrying effluents on discharges from different industries, villages, towns etc.

In the subsequent six sections extent of such bacteria in the river water itself, on growing vegetables in the fields, on vegetables and fruits in the markets, on the fishes in the market, on the plant litters and dead roots, debris of the plant have been assessed separately.

(b) While water and soil samples were dilution-plated in different dilutions in the selective medium for fluorescent bacteria namely King's Medium B enriched with cetrimide (KBC) the plant samples were dilution plated in different dilutions after taking their washates in sterilized water under aseptic conditions and plating them in the same KBC medium.

The plates in necessary replicates and control plates were incubated at $40 \pm 1^\circ\text{C}$ temperature in an incubator for 24-48 hrs. The plates were thereafter examined and number of total bacterial colonies and green fluorescent colonies were counted and the data on number of colony forming units (CFU) for green fluorescent bacteria (GFB) to total bacteria was converted into percentage of the former to later and presented in Table-1. While the statistical three factor analysis data is presented in Table 1(a) the corresponding Anova table is given in Table, 1(b).

(c) The data clearly established occurrence of a significant number of green fluorescent bacteria (2.62 - 10.85 %) of total bacteria in different substrates around the lower stretch of the river Ganga. When analysed between seasons the percentage of such bacteria ranged

Table 1. Extent of green fluorescent bacteria in soil, water and plant surfaces of different bodies carrying waste water to a river.

(average of three replicates)

Sample collection sites	Percentage of green fluorescent bacteria in seasons					
	Premonsoon			Monsoon		
	Soil	Water	Plant surfaces	Soil	Water	Plant surfaces
I. Industry						
1. Paper pulp factory (Hazinagar)	9.26 (17.69)	9.43 (17.86)	6.36 (14.64)	10.66 (19.00)	7.08 (15.33)	4.03 (11.55)
2. Jute Mill (Naihati)	7.58 (15.85)	8.67 (17.08)	5.98 (14.16)	10.58 (18.99)	5.42 (13.33)	4.47 (12.52)
3. Rubber factory (Sahagang)	6.92 (15.13)	8.29 (16.71)	5.17 (13.13)	9.40 (17.70)	6.23 (14.31)	3.72 (11.32)
Av. of Industries	7.92	8.80	5.84	10.21	6.24	4.07
II. Town						
1. Dhobighat (Barrackpore)	6.52 (14.67)	8.33 (16.73)	7.51 (15.91)	9.38 (17.78)	6.77 (14.93)	5.14 (13.09)
2. Khatal discharge (Shyamnagar)	6.39 (14.37)	8.65 (17.04)	5.65 (13.78)	10.20 (18.48)	5.16 (13.07)	2.79 (9.64)
Av. of Towns	6.46	8.49	6.58	9.79	5.96	3.96
III. Village						
1. Bagerkhal (Kanchrapara)	6.74 (14.95)	8.70 (17.90)	7.34 (15.74)	8.85 (17.24)	5.75 (13.84)	5.89 (14.38)
2. Brick field (Shyamnagar)	6.35 (14.54)	7.94 (16.35)	7.02 (15.32)	9.89 (18.32)	6.58 (14.66)	5.25 (13.24)
Av. of Villages	6.54	7.18	7.18	9.37	6.16	5.57
Iv. Tributary						
Swaraswati river (Tribeni)	8.81 (17.53)	9.07 (17.49)	5.06 (13.04)	10.85 (19.27)	7.48 (15.82)	2.62 (9.46)

C.D. of source at 1% level = 1.0280

5% level = 0.7761

C.D. of season at 1% level = 0.6338

5% level = 0.8395

C.D. of site and source at 5 % level = 2.1958

C.D. of source x season at 1 % level = 1.4540

5 % level = 1.0979

(In the parenthesis p represent the average angular value of the table value).

Table 1 (a). Analysis of three Factor experiment.

<u>Solution</u>	
Grand Total	2205.1900
Correction Factor	33769.8785
Total sum of squares	1136.4162
Sum of squares for site	33.8924
Sum of squares for source	359.7609
Sum of squares for season	25.7865
Treatment sum of squares for site and source	492.4228
Treatment sum of squares for site and season	67.1640
Treatment sum of squares for source and season	619.6459
Total treatment sum of squares for all factors	783.9267
Sum of squares for site and source	98.7696
Sum of squares for site and season	7.4851
Sum of squares for source and season	234.0986
Sum of squares for site, source and season	24.1336
Error sum of squares	352.4896

Table 1(b). Analysis of variance table for the three factors.

Source of variation	D.F.	S. S.	M. S. S.	Variance Ratio F	't' values	
					1%	5%
Site	7	33.8924	4.8418	1.3186	2.854	2.122
Source	2	359.7609	179.8804	48.9902	4.866*	3.102*
Season	1	25.7865	25.7865	7.0229	6.942*	3.952*
Site x Source	14	98.7696	7.0550	1.9214	2.34	1.829*
Site x Season	7	7.4851	1.0693	0.2912	2.854	2.122
Source x Season	2	234.0986	117.0493	31.8782	4.866*	3.102*
Site x Source x Season	14	24.1336	1.7238	0.4695	2.34	1.829
Error	96	352.4896	3.6718			
TOTAL	143					

* Significant.

between 5.06 to 9.43 % in pre-monsoon and 2.62 to 10.85 % in monsoon seasons. Between the substrates like soil, water and plant surfaces the range was 10.85 to 6.39 % in soil, 9.43 to 5.16 % in water and 7.51 to 2.61 % on plant surfaces indicating a higher percentage of such bacteria in soil and water and slightly lower percentage on plant surfaces.

Not much of difference between the percentages of GFB between the samples collected from around the discharges from industries and towns was discernable if the range of percentages was considered. They were 10.21 to 4.07 % in case of industries and 9.79 to 3.96 % in case of towns. It may be considered as a matter of concern that the same for villages was also not much low. It was 5.54 to 9.37 %, the lower side of the range itself being actually high.

The overall situation with regard to occurrence of green fluorescent bacteria seemed to be very serious.

The statistical analysis of the data of the total survey showed that the differences in occurrence of green fluorescent bacteria between source, season and interactions of source x season were significant in 1% and 5% level but interactions between site x source was significant at 5% level, but the site and interactions between site x season and site x source x seasons were not significant at any level.

Evidently the population of the green fluorescent bacteria to other bacteria ranged between 0.11 to 1.66 in cases of industrial

effluents which is not very high a level. In village washings also it was low i.e. 0.09 to 1.75 % but with a wider range. The town waste water showed the extent of green fluorescent bacteria between 0.04 to 2.82 %. The swaraswati water which seemed to carry waste-water of a large number of villages and urban conglomerations, produced 0.19 to 3.25 % green fluorescent bacteria. As expected the town and Swaraswati samples recorded significantly high (2.82 and 3.25 % respectively) percentage of the bacteria being studied.

Detection of green fluorescent bacteria in such water itself has established the concern to be correct.

4.1.2 Extent of green fluorescent bacteria in water of the river Ganga :

Ganga is a perennial river of 1550 metre length passing through large number of cities, villages and industrial complexes in northern India with a good portion of the lower Ganga passing through West Bengal. Considering that great concerns that have been expressed about its pollution over last 3-4 decades, different investigations have been taken up on the extent of such pollution. Present programme has been unique in its character since investigation, for this group of bacteria has not so far been taken up and reported in the country.

In continuation of the previous study (4.1.1), another study was taken up for assessment of the extent of green fluorescent bacteria in the water of river Ganga itself to understand the fate of green fluorescent bacteria reaching Ganga water through different waste water discharges discussed in the previous work.

Samples of the Ganga water was collected in both premonsoon and monsoon seasons from eight sample-locations in the Ganga which are near the 8 discharges mentioned earlier. Three locations inside industrial areas, 2 near villages, 2 near towns and 1 near the confluence of Ganga with Swaraswati, a tributary of the Ganga meeting the later near Triveni, Hooghly were included.

Samples were collected from about 3 metre off from the respective discharges from a boat following procedure for collection of water samples, described earlier.

The water samples were dilution-plated in the same way as before in KBC-medium in necessary replications along with control plates. The plates were incubated at $40 \pm 1^{\circ}\text{C}$ for 48 hr. The number of colonies of green fluorescent bacteria and total number of bacteria were counted and the results are presented in Table 2. Parallel samples were collected as per method described and pH and electrical conductivity of the water samples were recorded following methods described earlier. The results obtained are presented in the same table.

Green fluorescent bacteria have been found to occur in the Ganga river water in similar extent as in waste discharges. Percentage of green fluorescent bacteria in relation to other bacteria has shown generally a higher figure in case of pre-monsoon samples than monsoon samples.

Table 2: Extent of green fluorescent bacteria in the water of river, Ganga (Average of three replicates)

Sample Sites	Percentage of green fluorescent bacteria					
	Pre-monsoon			Monsoon		
	Actual	pH	E.C. m.m.hos/cm	Actual	pH	E.C. m.m.hos/cm
<u>I. Near Industries</u>						
1. Hajinagar (Near paper pulp factory)	7.27 (15.66)	8.0	1.38	5.60 (13.63)	6.5	0.46
2. Naihati (Near Jute Mills)	6.98 (15.30)	8.1	1.01	5.39 (13.46)	7.2	0.38
3. Sahagung (Near Rubber factory)	6.51 (14.69)	7.5	0.41	4.78 (12.60)	7.0	0.40
<u>II. Near Villages</u>						
1. Shyamnagar (Near Brick field)	6.34 (14.50)	7.8	1.00	5.01 (12.88)	7.5	0.38
2. Kanchrapara (Near Bager-khal)	6.50 (14.76)	7.6	1.01	4.53 (12.23)	8.0	0.40
<u>III. Near Towns</u>						
1. Barrackpore (Near Dhobighat)	7.06 (15.36)	8.6	0.50	5.16 (13.07)	8.0	0.42
2. Shyamnagar (Near Cattleshed)	5.93 (14.08)	8.0	0.45	4.26 (11.88)	8.5	0.35
<u>IV. Ganga Tributary</u> (Near Tribeni)	7.40 (15.74)	9.1	0.62	5.68 (13.72)	8.3	0.25

C.D. of site at 5 % level = 0.5853

C.D. of season at 5% level = 0.2926
1% level = 0.3934

(In the parenthesis, represent the average angular value of the table value)

The survey data from 8 sample locations under 4 groups have generally indicated occurrence of significant number of fluorescent bacteria in Ganga water. The situation improved, but not much even when the waste discharges reached the Ganga. The pH and electrical conductivity data as obtained showed that the condition of water i.e. alkalinity of the water in most of the samples as expected was more in pre-monsoon than in monsoon season. The results indicated that these bacteria could survive in a pH range between 6.5 to 9.1 and occurred mostly around 7.5 to 8.5. The electrical conductivity range of 0.25 to 1.38 in mmhos/cm was tolerable to the bacteria concerned.

The two factorial statistical analysis of the data showed that sites and seasons were significant at 5 % level. The study clearly revealed that the higher salt concentration in the Ganga water was of no avail to cause a decline in the population of green fluorescent bacteria and that the present bacterial isolates are capable of tolerating a good salt concentration in the medium of their living.

Table 2(a): Analysis of two factor experiment

Solution	
Grand total	670.8600
Correction factor	9376.1068
Total sum of squares	91.5057
Sum of Squares for site	15.9209
Sum of Squares for season	51.8333
Treatment sum of squares	68.5689
Sum of squares for site and season	0.8147
Error sum of squares	23.7514

Table 2(b): Analysis of variance Table for two factors

Source of variation	D.F.	S. S.	M.S.S.	Variance ratio F	't' Values	
					5%	1%
Site	7	15.9209	2.2744	3.0643	2.314*	3.264
Season	1	51.8333	51.8333	69.8343	4.152*	7.51*
Site x Season	7	0.8147	0.1164	0.1568	2.314	3.264
Error	32	23.7514	0.7422			
Total	47					

* = significant

4.1.3 Extent of green fluorescent bacteria on the surfaces of crops in the fields in villages :

As the next step in the survey programme it was considered necessary to examine the surfaces of number of field and vegetable crops in three selected villages namely Shantipur, Shaguna and Madanpur - all in the areas of Nadia district adjoining Ganga. Leaf and root samples of a number of crops like Palak, Cabbage and Rice were collected in sterilized polythene bags in sufficient quantities and separately. The plant materials taken fresh in definite quantities by weight (10 gms) were washed with sterile, distilled water for dilution plating in KBC following procedure described earlier. The plants in necessary replicates along with control plates were incubated at $40 \pm 1^\circ\text{C}$ for 48 hrs. Observations were made after 48 hrs with regard to number of green fluorescent colonies per plate. The data after necessary computation are presented in Table 3. The plants included in the study were Palak, Cabbage and Rice.

Table 3: Extent of green fluorescent bacteria on leaf and root surfaces of crops from different villages (average of three replicates)

Name of the villages	Number of green fluorescent bacteria in different washes ($\times 10^3$) per ml)					
	Leaf wash			Root wash		
	Palak	Cabbage	Rice	Palak	Cabbage	Rice
1. Shantipur	2 (1.55)	0.66 (1.04)	0.33 (0.87)	2 (1.55)	1 (1.16)	0.66 (1.04)
2. Shaguna	0.33 (0.87)	0.33 (0.87)	1 (1.22)	1.33 (1.43)	0 (0.70)	0.66 (1.04)
3. Madanpur	1.33 (1.34)	1.33 (1.34)	2 (1.55)	2.33 (1.64)	0.66 (1.04)	0.33 (0.87)

C.D. of site at 5% level = 0.2056

C.D. of vegetable at 1% level = 0.2760

C.D. of wash x vegetables
at 5 % level = 0.2909

(In the parenthesis, represent the average angular value of the table value)

The data in Table 3 showed that green fluorescent bacteria were present on all the root and leaf surfaces of Palak, Cabbage and Rice. Subject to identification the detection of green fluorescent bacteria on vegetable surfaces seemed to be of enormous importance. The mean of the total bacteria for each plant and their square-root values have been presented in the Table 3.

Statistical analysis of the data showed that the differences in population between sites, vegetables and interactions like wash x vegetables were significant at 5 %, 1% and 5% levels and respectively. The details of the statistical analysis have been presented in Table 3(a) and Table 3(b).

Table 3(a): Three factor analysis .

Grand total	63.5800
Correction factor	74.8596
Total sum of squares	7.6396
Sum of squares for site	0.7028
Sum of squares for Wash	0.0046
Sum of squares for Veg.	1.3936
Treatment sum of squares for site and wash	0.9982
Treatment sum of squares for site and veg.	2.7037
Treatment sum of squares for wash and veg.	2.0598
Sum of squares for Treatment	4.3010
Sum of squares for site and wash	0.2908
Sum of squares for site and Veg.	0.6073
Sum of squares for wash and veg.	0.6616
Sum of squares for site, veg. and wash	0.6403
Error sum of squares	3.3347

Table 3(b) : Three factor analysis of variance table.

Source	D.F.	S.S.	M.S.S.	F.value
Site	2	0.7028	0.3514	3.7948
Wash	1	0.0046	0.0046	0.0497
Veg.	2	1.3936	0.6968	7.5248
Site x Wash	2	0.2908	0.1454	1.5702
Site x Veg.	4	0.6073	0.1516	1.6393
Wash x Veg.	2	0.6616	0.3308	3.5723
Site x Wash x Veg.	4	0.6403	0.1601	1.7289
Error	36	3.3347	0.0926	
Total	53			

4.1.4 Extent of green fluorescent bacteria on vegetables and fruits in some markets :

The occurrence of green fluorescent bacteria in different water bodies, soils and plant leaf and root surfaces of some selected crop plants has been established. The results promoted for taking up further studies. In the next phase it was considered necessary to assess the extent of occurrence of green fluorescent bacteria on some vegetables and fruits that have been brought and are sold in the market. Survey was conducted in different markets of the locality around the University namely Block 'A' Market and Kalyani B-Block No.2 Market under Nadia district and Kanchrapara Mondal Bazar Market under North 24-Parganas.

Among the vegetables, fruits and root crops, Palak (Spinacia oleracea), Sweet potato (Ipomea batatas), Radish (Raphanus sativus), Seem (Dolichos lablab), Carrot (Daucus carota), Tomato (Lycopersicon esculentum), Grapes (Vitis vinifera), Water-chestnut (Trapa bispinosa) and Orange (Citrus reticulata) were selected for the study. Samples of the produces have been collected from the above markets. The samples were generally collected twice in the early and late part of the time of their arrival in markets.

The washings in sterile, distilled water were plated in two dilutions namely 10^{-2} and 10^{-3} in KBC - medium maintaining 3 replications and necessary control plates. The plates were incubated at $40 \pm 1^{\circ}\text{C}$ for 48 hrs. after which number of colonies of green fluorescent bacteria were counted per plate. The average number of the green fluorescent bacteria in two dilutions (10^{-2} and 10^{-3}) and two samples on KBC-medium and their square root values were obtained after analysis. The data have been presented in the table 4. Among the vegetables two green fluorescent bacteria could not be detected on Sweet potato and Seem. Fruits like grapes did not show presence of the bacteria.

Results showed that the green fluorescent bacteria were present on the surfaces of marketed vegetables like Palak, Radish, Tomato and Carrot, which were detected in the washings of the vegetables in sterile distilled water.

Table 4 : Extent of green fluorescent bacteria on different vegetables and fruits on sale in three markets
(average of three replicates)

Vegetables and fruits	Number of green fluorescent bacteria(CFU/ml) on market vegetables and fruits in			Average position %
	Kalyani Block 'A' Market	Kalyani Block 'B' Market	Kanchrapara Mondal Bazar Market	
I. <u>Vegetables</u>				
1. Radish	1.33 (1.13)	1 (1)	1.33 (1.13)	1.22
2. Carrot	1.66 (1.27)	1.33 (1.84)	1.66 (1.24)	1.24
3. Palak	3.33 (1.82)	2.66 (1.62)	2.66 (1.57)	2.88
4. Tomato	3.33 (1.75)	3.66 (1.86)	2.66 (1.54)	3.21
MARKET AV.	2.41	2.16	2.07	2.13
II. <u>Fruits</u>				
1. Orange	1.33 (1.13)	1 (1)	1.66 (1.24)	1.33
2. Water chestnut	3 (1.71)	3 (1.68)	3.66 (1.86)	3.22
MARKET AV.	2.16	2.00	2.66	2.27

C.D. of Agricultural product at 5% level = 0.3750.

(Parenthesis indicate average angular value)

The two factorial analysis of the data in table 4 showed that amongs the differences examined between different samples, were significant at 1 and 5% respectively.

The details of analysis have been presented in Table 4(a) and 4(b). Evidently the extent of green fluorescent bacteria was quite significant on the marketed vegetables (2.4) and much less in Kalyani A Block market on fruits in B Block Market. Among the market however the situation in No.2 Market (B) at Kalyani appeared to be better, the reason for which was not clear.

Table 4(a) : Analysis of two factor experiment

Solution	
Grand Total	77.2300
Correction Factor	110.4532
Total sum of squares	10.1039
Sum of squares for Agricultural products	4.4913
Sum of squares for markets	0.0707
Treatment sum of squares	4.9548
Sum of squares for Agricultural Products and Markets	0.3928
Error sum of squares	5.5419

Table 4(b): Analysis of variance Table for two Factors

Source of variation	D.F.	S.S.	M.S.,S.	Variance Ratio F	t-value	
					1%	5%
Agricultural products	5	4.4913	0.8983	5.8350	3.586*	2.482*
Markets	2	0.0707	0.0353	0.2296	5.264	3.266
Agri products x Markets	10	0.3928	0.0393	0.2552	2.885	2.116
Error	36	5.5419	0.1539			
TOTAL	53					

4.1.5 Extent of green fluorescent bacteria on different fishes in some markets :

In continuation of the survey programme, another study was taken up to assess the extent of green fluorescent bacteria on fishes being marketed in local markets.

To study the presence of the green fluorescent bacteria on fishes a survey was conducted on two fishes living in two different situations in the water in a pond. The test-fishes were (i) Katla (Catla catla) and (ii) Tangra (Mystus seenghala). Usually small sized (100-200g) fishes mentioned were collected from different markets in this area like (1) Kalyani Block 'A' Market (2) Kalyani Block-B No.2 Market and (3) Kalyani Central Park Market. These fish samples were collected twice at intervals of one month during monsoon numbering not less than 5 at a time. The fishes were washed under

aseptic conditions with sterile, distilled water @ 10 ml /fish. The washates were pulled together into per fish samples for each market and plated in KBC medium. The plates with necessary replicates and control were incubated at $40 \pm 1^\circ\text{C}$ for 48 hr. and data in terms of number of green fluorescent bacteria were counted.

The number of the green fluorescent bacterial colony are presented in table 5. The data revealed that in all the washings of the fishes collected from three local markets the green fluorescent bacteria were present. The market average ranged from 1.99 to 2.66 per ml. which was quite low. But the detection undoubtedly established the occurrence of the bacteria on such substrates like fishes. Among the fishes however, a higher number was recorded consistently on the fishes living near mud layer of the water body i.e. Tangra.

Table 5: Extent of green fluorescent bacteria in different market fishes
(average of three replicates)

Markets	Number of green fluorescent bacteria on fishes in market		
	Katla	Tangra	Average
1)Kalyani Block A (Market I)	3 (1.71)	1.33 (1.13)	2.16
2)Kalyani Block B (Market-II)	2.33 (1.48)	1.66 (1.27)	1.99
3)Kalyani Central Park (Market-III)	3 (1.71)	2.33 (1.51)	2.66
Avg. of position	3.01	1.77	

C.D. of fish at .5% level = 0.3159

() gives the average of $\sqrt{\quad}$ value of the table value.

The statistical two factorial analysis of the data in table 5 showed that population differences between the samples from different sites, on fishes was highly significant at 5% level.

It seemed that the green fluorescent bacteria are present on many substrates including fishes being marketed in different markets. Although the population is not very high it is significant and it varied between market to market and types of fishes.

Table 5(a): Analysis of two factor experiment
Solution

Grand Total	26.5200
Correction Factor	39.0728
Total sum of squares	1.8084
Sum of squares for site	0.1867
Sum of squares for Fish	0.4868
Treatment sum of squares	0.8118
Sum of squares for site and Fish	0.1383
Error sum of squares	1.1350

Table 5(b): Analysis of variance table for factors

Source of variation	D.F.	S.S.	M.S.S.	Variation Ratio 'F'	't' value	
					1%	5%
Site	2	0.1867	0.0933	0.9870	6.93	3.89
Fish	1	0.4868	0.4868	5.1465	9.33	4.75*
Site x Fish	2	0.1383	0.0692	0.7314	6.93	3.89
Error	12	1.1350	0.0946			
TOTAL	17					

* Significant

4.1.6 Occurrence of green fluorescent bacteria on plant litters :

Plant litters are good habitats or host of bacteria in nature and the likelihood of their providing habitat for green fluorescent bacteria in nature was considered very important to be examined.

It was then decided to examine the litters from orchards, forest trees and ornamental gardens for any presence of the green fluorescent bacteria.

One each of orchards of mango and guava in the University Horticulture Farm, Mondouri were selected for the study. For the forest tree the road side social forest plants Sonajhuri and a true forest plant Sal were selected in JNM Hospital premises. A rose garden was selected in a house hold garden in Kalyani town.

Freshly fallen leaves were collected in sterilized cellulose acetate bags, their openings were sealed and taken to the laboratory.

Following the same methods of isolation of bacteria as described earlier the surfact bacteria were isolated by dilution method and number of total bacteria and green bacteria isolated were computed as number pergram of litter sample.

The results obtained are presented in Table 6. The data as presented in Table 6 showed that the percentage of green fluorescent bacteria was nearly 30-50 % of the total bacterial population on all such samples examined. While the total bacterial population on such litters ranged from $1.4-2.5 \times 10^4$ per g of sample it was $0.5-1.4 \times 10^4$ per g of sample for green fluorescent bacteria.

Table 6: Occurrence of green fluorescent bacteria on plant litters
(Average of 4 replicates)

Location	Vegetation	Number of bacteria per gm of litter $\times 10^4$	
		Green bacteria	Total bacteria
I. Fruit orchards	Mango	0.5	1.4
	Guava	1.0	2.0
II. Forest trees	Sonajhuri	1.0	2.5
	Sal	0.6	1.4
III. Ornamental Garden	Rose	1.4	2.0

Such high percentage of occurrence of green fluorescent bacteria on litters in forests, orchards or even ornamental gardens not only established their wide distribution but also indicated that they occur on surfaces having no contact with flowing water or polluted water even.

4.1.7 Occurrence of green fluorescent bacteria on woody debries, dead roots and stubbles :

Surfaces of stems of big trees or crop plants, in forest or cultivated fields respectively which are very close to the ground were also thought to be likely substrates where such bacteria might occur.

After detection of green fluorescent bacteria on litters in forests, road sides or ornamental gardens it was considered worthy to examine their occurrence on barks of shal (Shorea robusta), dead roots of Jute(Corchorus olitorius) plants and stubbles of rice(Oryza sativa) plants growing in forests and fields.

Following the same method of isolation etc. described earlier, total and green fluorescent bacteria were isolated and their number per gram of samples was determined following dilution method. The number of colony forming unit (CFU) obtained per sample has been presented in Table 7.

The results obtained showed that the total bacterial population per gram of stem surface samples ranged between $0.7-0.8 \times 10^4$ numbers. The number of green fluorescent bacteria however ranged

between $0.2-0.5 \times 10^4$ per gram. This was about 18 % in the lowest and 63 % in the highest of the total bacterial population. This also established the occurrence of green fluorescent bacteria on plant surfaces in both forests and crop fields which are very close to soil.

Table 7: Distribution of green fluorescent bacteria
on woody debris, dead roots and stubbles
(Average of 3 replicates)

Substrate	Plant	No. of bacteria CFU $\times 10^4$ per g of sample	
		Green bacteria	Total bacteria
I. Woody Debris	Shal	0.2	0.8
II. Dead Roots	Jute	0.5	0.7
III. Stubbles	Rice	0.4	0.8

4.2 Identification of the isolates of the green fluorescent bacteria :

Isolated cultures of the green fluorescent bacteria have been brought into pure culture by dilution and re-isolation method described earlier. The isolated and purified cultures were studied with regard to their key determinative characteristics following standard procedures (Schroth et al. 1977, Misagi and Grogan, 1969). The characteristics studied included cell morphology like shape,

size, Gram-reaction, motility etc. Colony morphological characteristics like shape, smoothness, margin etc. of the colonies on solid media and growth and turbidity in broth and physiological characteristics like O_2 -relation Levan production etc. Serological properties of the isolates have also been studied in this programme following procedure described earlier. The results obtained are presented in Table 8. The results may be summarised as below :

4.2.1 (a). Cell morphology of green fluorescent bacteria

- i) Shape - The isolated bacteria from all eight sites were found to be rod shaped,
- ii) Size - The rod shaped cells of the bacteria were generally within the size range of $0.3-0.8 \times 1.5-2.5$ micrometer.
- iii) Motility - All the isolates of the bacteria have been found to be motile with flagella which was separately determined by staining. Their motility was also confirmed by water-drop method.
- iv) Gram reaction - The Gram reaction in all the cases have been negative for their inability to retain the 1st stain against alcohol wash
- v) Spore formation - All the isolates of the bacteria have been found to be non-spore former because spore which expected to be green and remainder of the cell, a light red in color.

- vi) Capsule staining - By capsule staining, all the isolates of the bacteria appeared to be non capsulated as capsules were stained light blue against the dark purple blue cells.
- vii) Flagella staining - By flagella staining it have been confirmed that all the isolates of the bacteria having 1-3 polar flagella.

(b) Colony morphology

- i) Growth on Solid media - All the isolates formed colonies with production of water-soluble light to deep greenish pigment of fluorescent characteristics, Fig.3.
- ii) Growth on liquid media - Growth was commonly as pellicle with membranous to non-membranous nature, fragments suspended in brownish sediments with common greenish to yellowish pigment remaining dissolved in the liquid base of the medium.

(c) Physiological reactions

Among the different physiological characteristics, some important ones studied, produced results which are valuable for determination the bacteria.

(i) Pigment production - Isolates differed in their capability of production of the pigments in different degrees. Some of the isolates produced deep green while others light green or even light yellowish

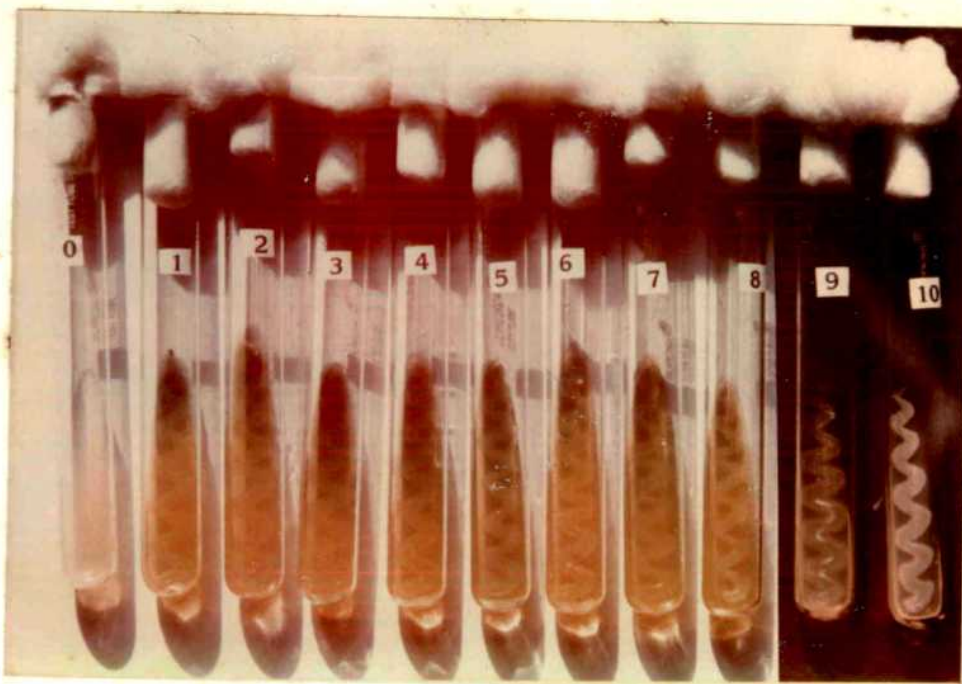


Fig.2 24 hours slant cultures showing colonies of Ps.aeruginosa.

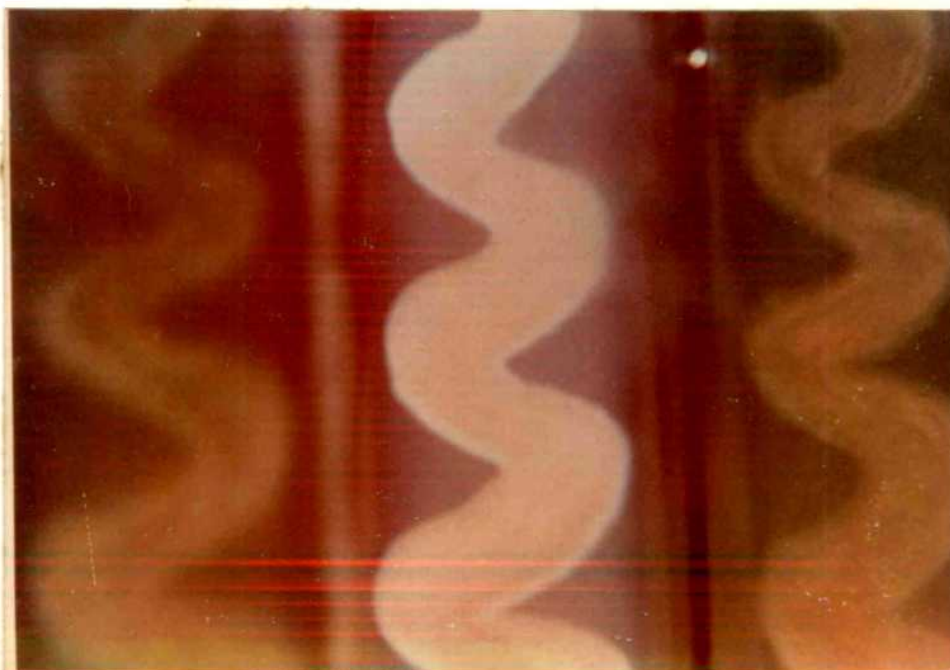


Fig.3 Close-up of Ps.aeruginosa colonies after 24 hours.

green water soluble pigments in both KBC-slant and broth within 24 hours of incubation at 40-42°C temperature, Fig.2.

The bacteria were quite fast growing and produced the pigment only in an iron-free medium, the one used in this work being King's Medium B, Fig.4.

The pigmentation pattern changed with time, mainly becoming brownish-black after 6-7 days of growth. Possibly this was due to production of a second pigment of pyocyanin type by some of the strains, Fig.5.

(ii) O₂-relation - All isolates of the bacteria grow aerobically. The isolates were definitely unable to grow under anaerobic or even partially anaerobic conditions. This clearly established the bacteria as aerobic.

(iii) Levan production - Levan was not produced by all the isolates of the bacteria as large, white, domed, mucoid colonies not appeared in the sucrose medium.

The isolates from their revealed characteristics were determined to be those of Pseudomonas aeruginosa. The identity have been confirmed by CMI. Bact.II-M dt. 26.10.87

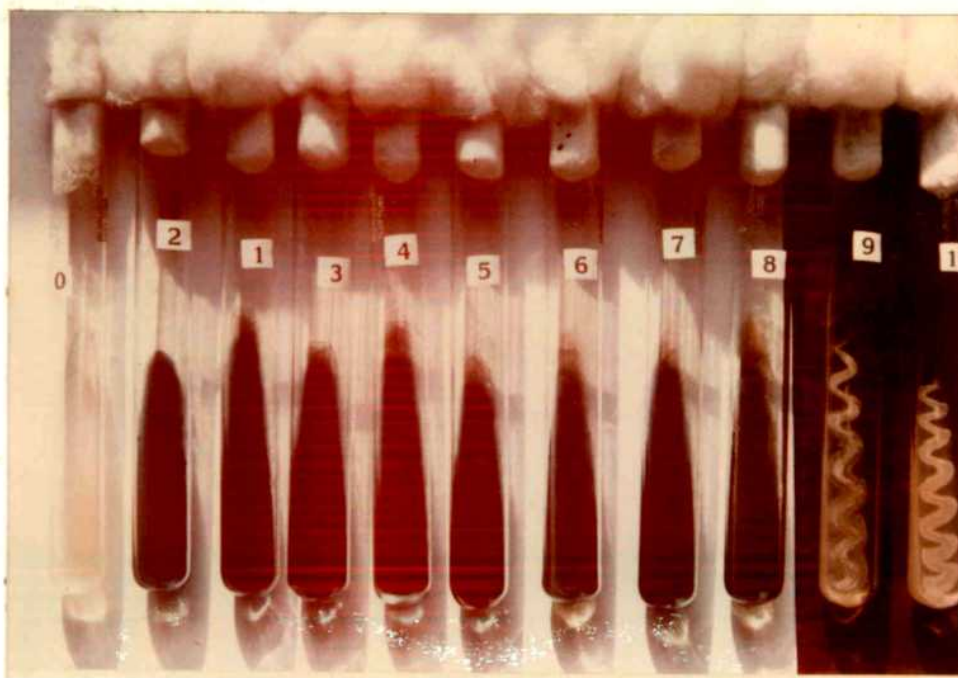


Fig.4 Green pigment production by the isolates of Ps.aeruginosa after 48 hours.

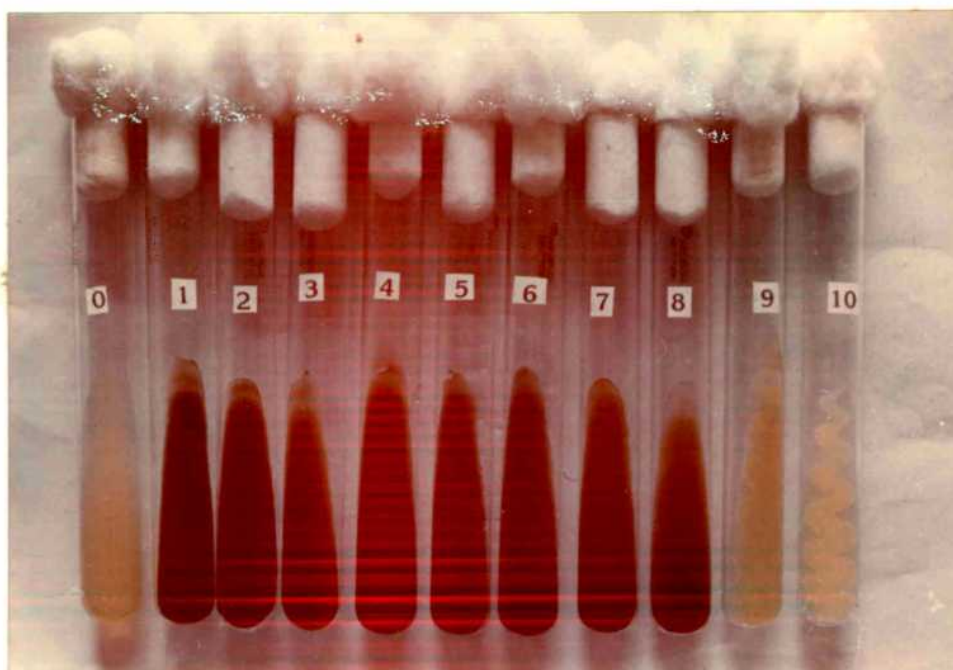


Fig.5 Pyocyanin pigment produced by the aged isolates of Ps.aeruginosa.

Table 8. Characteristics of green fluorescent bacteria.
(average of 4 replicates)

Characters	Selected isolates							
	GFB ₁	GFB ₂	GFB ₃	GFB ₄	GFB ₅	GFB ₆	GFB ₇	GFB ₈
1. Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
2. Size								
Length (μ)	1.5-2.2	1.8-2.5	1.5-2.25	1.6-2.2	1.8-2.4	1.5-2	1.8-2.5	1.6-2.4
Breadth (μ)	.4-.7	.5-.8	.4-.8	.3-.6	.5-.8	.3-.5	.4-.8	.3-.6
3. Gram reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
4. Capsule	NC	NC	NC	NC	NC	NC	NC	NC
5. Spore	NS	NS	NS	NS	NS	NS	NS	NS
6. Motility	M	M	M	M	M	M	M	M
7. Flagella	1-3, Polar	1-3, Polar	1-3, Polar	1-3, Polar	1-3, Polar	1-3, Polar	1-3, Polar	1-3, Polar
8. Colony Growth on solid KBC media	Abn. fluo. flu.	Abn. fluo. vis.	Abn. fluo. flu.	Mod. fluo. flu.	Abn. fluo. flu.	Mod. fluo. vis.	Abn. fluo. flu.	Mod. fluo. flu.
9. Growth on liquid KBC media	Pe.mem. Pin-W Sed.	Pe.mem. Bla-W. Sed.	Pe.mem. Pin-W. Sed.	Pe.mem. Brow-W. Sed.	Pe.mem. Brow, Sed.	Non.mem. W-Sed.	Pe.mem. Pin-W. Sed.	Pe.mem. Bla-W. Sed.
10. Pigmen- tation	Deep green	Light yellow- ish gr- een	Deep green	Yellow- wish green	Deep green	Light Yellow- ish gr- een	Deep green	Green
11. Oxygen relation	Ae.	Ae.	Ae.	Ae.	Ae.	Ae.	Ae.	Ae.
12. Levan produc- tion	-ve	-vê	-ve	-ve	-ve	-ve	-ve	-ve

-ve = negative

Abn = Abundant

Pe = Pellicle

Pin= Pinkish

NC = noncapsulated

Mod = Moderate

mem= Membranous

Bla= Blakish

NS = non-spore
 former

fluo= fluorescent

non-mem=non-memb-
 ranous

Brow=Brownish

M = Motile

vis = Viscid

w = white

Ae = Aerobic

4.2.2 Determination of relationship of green fluorescent bacteria through serological techniques :

The green fluorescent bacteria isolated and characterised through number of bacteriological studies were more or less similar having some differences in their characteristics. In order to determine the extent of their similarity and establish their mutual natural relationship serological study was undertaken.

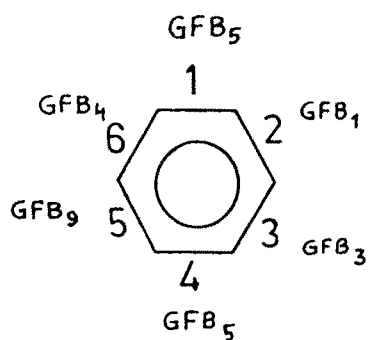
Altogether ten isolates of the green fluorescent bacteria including the four already studied in greater detail, were included in the present programme.

Out of these 10 isolates three were selected to be used in preparation of antisera in white rabbit (Cryptolagus sp.) .

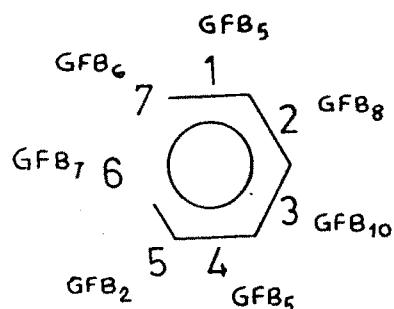
These three isolates were GFB₁, GFB₅ and GFB₆. They were injected in rabbits of 6 months age thrice at intervals with suspension of cultures as described earlier.

The antisera prepared were collected by bleeding the rabbits as and when required. The antisera taken in drops on a cleaned glass-slide was allowed to react with antigens prepared with the 10 isolates in 'agar-gel system'.

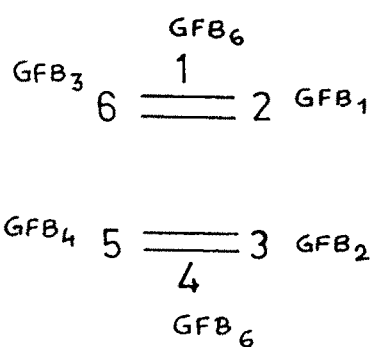
The results obtained are presented in Table 9 and Fig.6. Results obtained showed that in case of GFB₅ - antisera no precipitin-band developed or was formed against GFB₆ and GFB₇ antigen. In case of GFB₁ - antisera no precipitin band were found in GFB₂, GFB₆ and



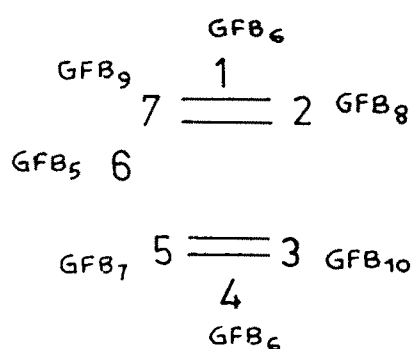
A



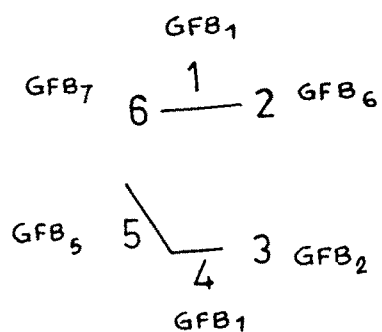
B



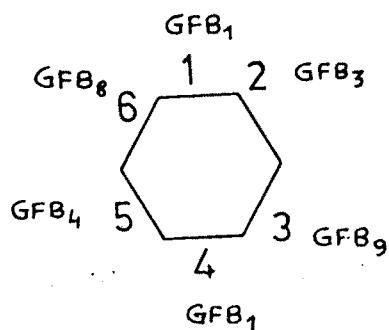
C



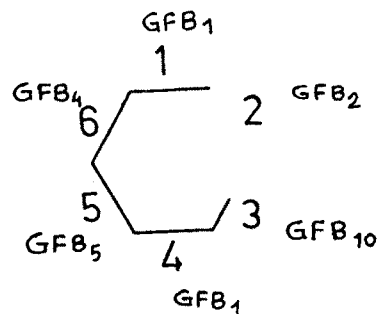
D



E



F



G

Figure 6. Three antisera showing reaction with ten antigens.

A,B - GFB₅ Antisera : C,D - GFB₆ Antisera :

E,F,G - GFB₁ Antisera

GFB₇, but feable bands were found in GFB₄, Fig.7 a and b.

Table 9: Presence of Common antigens among Pseudomonas spp.

Antigens	Antisera		
	GFB ₁	GFB ₅	GFB ₆
GFB ₁	+	+	-
GFB ₂	-	+	-
GFB ₃	+	+	-
GFB ₄	+	+	-
GFB ₅	+	+	-
GFB ₆	-	-	+
GFB ₇	-	-	-
GFB ₈	+	+	-
GFB ₉	+	+	-
GFB ₁₀	+	+	-

+ = Positive precipitin band

- = Negative precipitin band

± = Feeble Precipitin band

The antisera GFB₆ showed no cross-reactive reaction with other antigen. Results of analysis revealed following facts. A total of ten isolates of the fluorescent Pseudomonas were tested against three antisera. It was also found that the antisera of GFB₅ showed precipitin bands with GFB₁, GFB₂, GFB₃, GFB₄, GFB₅, GFB₈, GFB₉, GFB₁₀ but no precipitin band was found with GFB₆ and GFB₇. The antisera

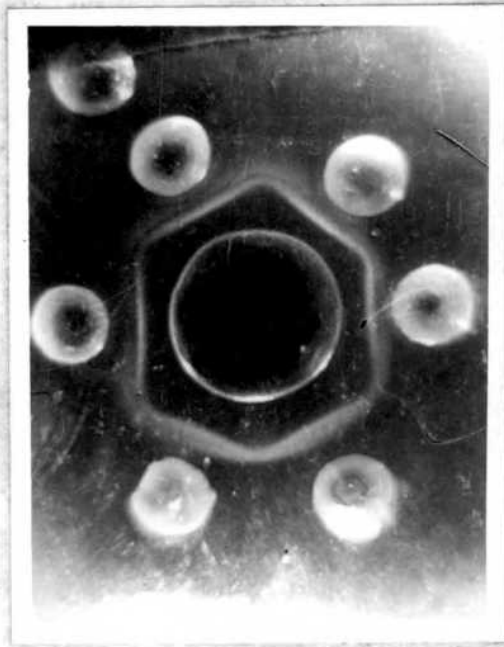
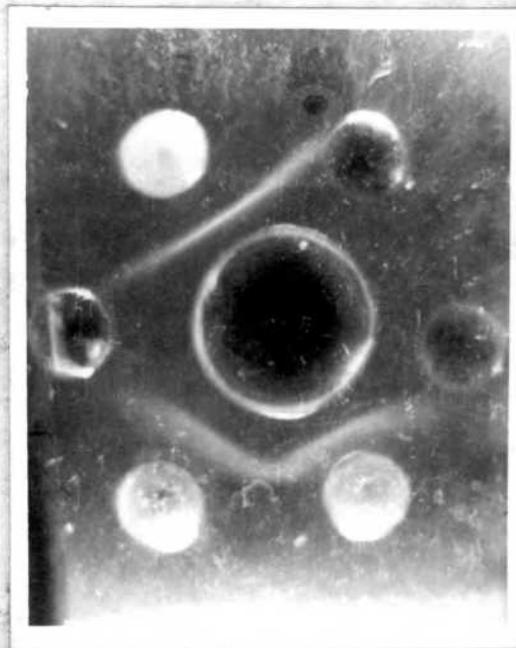


Fig.7 a) Antisera showing precipitin bands with some antigens.



b) Antisera showing no precipitin bands with some antigens.

of GFB₁ also showed precipitin bands with the antigens of GFB₃, GFB₅, GFB₈, GFB₉ and GFB₁₀ and feeble bands were found with GFB₄ but no bands were found with GFB₂, GFB₆ and GFB₇. The antisera of GFB₆ showed no cross reactive reaction with other antigens (Table-9).

The reaction between three antisera and 10 antigens demonstrated that the isolates have mostly a close relationship excepting the isolates GFB₆, it appears that serologically a large number of isolates are similar.

4.3 Pathogenicity of green fluorescent bacteria towards plant and animal hosts :

Some of the green fluorescent bacteria are reported to invade plants under stress. They occur very close to the host tissues and invade host tissues under some opportunities only. The present work was aimed at examining this opportunistic faculty of the presently isolated green fluorescent bacteria, even though it was known that such bacteria were not normal pathogens.

Plant pathogenicity of the four isolated bacteria were studied on three plants by different methods like (a) foliar spraying and (b) by root-dipping method. Detail methods followed and results obtained have been described below :

4.3.1 Plant pathogenicity of green fluorescent species of Pseudomonas on inoculation by foliar spraying :

Plant pathogenicity of the 4 isolates of the green fluorescent bacteria namely (a) GFB₁ (2) GFB₂ (3) GFB₃ and (4) GFB₄ were studied

by spraying bacterial suspensions on the vegetative parts or foliages of 3 plants. The test plants were Tomato, Beet and Cabbage. One month old seedlings of varieties of Pusa Ruby, local and Pride of India of the crops respectively were collected from Kalyani Market and grown on the earthen pots (9" diameter). The plants were maintained with proper nutrition, irrigation and care for another 2 months to get good vegetative growth of the plants. Then from 24 hour old cultures of the selected isolates of the green fluorescent bacteria on KB-slants bacterial suspensions were prepared with sterile, distilled water using the standard scale (McFarland's Scale) for measurement of concentration of bacterial cells in the suspensions, two different dilution namely 3×10^7 and 3×10^8 concentrations of the bacterial suspensions were prepared. Approximately ten ml. of cell suspension of each of the isolates were sprayed with a Holm's Sprayer (Throat Sprayer) on each group of plants in a way so that surfaces of the entire plant was covered. The whole experiment was conducted on two sets of plants namely injured and uninjured. Before spraying one sets of plants were injured by mildly rubbing with a cotton swab containing fine powder (200 mesh) of carborandum. Replicates and unsprayed control were maintained. All the plants were incubated under moist incubation for 12 hrs. after which they were transferred to laboratory benches at room temperature ($28-30 \pm 1^\circ\text{C}$). Observations on infection of the leaves, if any and their extent was made on the plants, starting from after 72 hrs. of incubation. Results obtained are presented in Table 10.

Table 10 : Plant pathogenicity of the green fluorescent bacteria by foliar spraying
by days intervals
(average of 3 replicates)

Isola- tes	Percent infection inoculation at different population											
	Tomato						Beet					
	Injured host			Uninjured host			Injured host			Uninjured host		
	Lower 15 30	Higher 15 30	Higher 15 30	Lower 15 30	Higher 15 30	Higher 15 30	Lower 15 30	Higher 15 30	Higher 15 30	Lower 15 30	Higher 15 30	Higher 15 30
GFB ₁	100 100	100 100	0 0	0 0	0 0	0 0	100 100	100 100	0 0	100 100	100 100	100 100
GFB ₂	100 100	100 100	0 0	0 0	0 0	0 0	100 100	100 100	0 0	100 100	100 100	100 100
GFB ₃	100 100	100 100	0 0	0 0	0 0	0 0	100 100	100 100	0 0	100 100	100 100	100 100
GFB ₄	100 100	100 100	0 0	0 0	0 0	0 0	100 100	100 100	0 0	100 100	100 100	100 100
Unino- culated control	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0	0 0

Fig. 8 Symptom of infection on Cabbage.



Treated

Control

Results obtained clearly showed that in case of cabbage plants positive infection was caused by the bacteria and the symptoms appeared both on injured and uninjured plants. All the isolates used for inoculation could cause infection and produce symptoms on cabbage Fig.8. In case of Beet and Tomato the same isolates could not cause any infection or symptom production particularly in uninjured hosts.

In case of injured hosts namely beet and tomato however the plants showed some infection and produced some symptoms like yellowing of the leaves and plants with poor vegetative growth but the plants survived for the period of experimentation.

The success of infection and symptom production on uninjured leaves definitely established the pathogenicity of the bacteria on cabbage plants. Their failure to produce infection and symptoms to some extent disproved the so far known fact that such bacteria may be weak and opportunistic pathogens, infecting plants under stress.

The two concentrations of inoculum used, did not show much difference in their pathogenicity which also proved that these strains of the bacterium atleast are far above the saprophytic isolates of the bacterium with regard to their ability to invade plants. Success in recovery of the bacteria from infected tissues also established the plant pathogenicity of the bacteria.

4.3.2 Plant pathogenicity of the green fluorescent
Pseudomonas species on inoculation by root
dipping :

Spray inoculation usually leads to invasion of the host plant by entry through stomata which may allow the bacteria to reach the parenchyma alone. In order to understand and examine the nature of plant pathogenicity the isolates of green fluorescent Pseudomonas three plants were inoculated with bacterial suspension by root-dipping with the expectation that they will reach vascular tissues. The object was to see wheather they can invade vascular tissues too.

The plant pathogenicity of the 4 isolates of the bacteria namely (1) GFB₁, (2) GFB₂ (3) GFB₃ and (4) GFB₄ were studied on three vegetable plants like tomato, beet and cabbage by root-dipping method. Seedlings of 1 month age of varieties like Pusa Ruby, Local and Pride of India of the crops respectively were collected from Kalyani Market. Bacterial suspensions of the isolates were prepared in sterile, distilled water from 24-hour slant cultures of the bacteria on KB-medium at two dilutions namely 3×10^7 and 3×10^8 with the help of the turbidity scale (McFarland). This was done by adding sterile distilled water in slants containing 24 hr cultures. The root portion of the freshly collected 1 month old seedlings after careful washing, were submerged in the bacterial suspension for 15 minutes in the day time. After 15 minutes the plants were taken out and carefully planted on the earthen pots (6" diameter) prepared for growing of the plants. Replicates and uninoculated control were

maintained. The root-inoculated plants were incubated on laboratory benches under humid conditions for 12 hrs and thereafter in an open space in the laboratory. Results were taken on 10, 15 and 30 days after inoculation. Infection, if any and its extent and symptoms developed were recorded. Results obtained are presented in Table 11. It showed that the cabbage plants treated by root dipping with all the isolates of the bacteria were possibly infected and none of the plants were able to survive after 10 days. They actually showed curling and withering symptoms. The tomato and beet seedlings however were able to survive upto 30 days inspite of root-dip inoculation with the green fluorescent bacteria at both high and low bacterial inoculum levels. Symptoms appeared on the seedlings of cabbage plants. Failure in appearance of any symptoms on the inoculated tomato or beet plants actually demonstrated a kind of selectivity in the pathogenicity of the isolates. There were no variation in the symptoms or survivability of host plants in case of different dilutions. Positive reisolation of the bacterium was done from the stems and roots of dead cabbage seedlings was possible.

The revelation of the fact that the green fluorescent bacteria occurring on different surfaces do have or have acquired the capability of causing infection in plant tissues of cabbage plants and not beet or tomato is new and interesting.

Table 11: Pathogenicity of the green fluorescent bacteria on root-dip inoculation of different seedlings
(average of three replicates)

Isolates	% infection of plant on inoculation with different population of bacteria											
	Tomato						Beet					
	Higher			Lower			Higher			Lower		
	10	15	30	10	15	30	10	15	30	10	15	30
GFB ₁	0	0	0	0	0	0	0	0	0	0	0	0
GFB ₂	0	0	0	0	0	0	0	0	0	0	0	0
GFB ₃	0	0	0	0	0	0	0	0	0	0	0	0
GFB ₄	0	0	0	0	0	0	0	0	0	0	0	0
Uninoculated control	0	0	0	0	0	0	0	0	0	0	0	0

4.3.3 Pathogenicity of green fluorescent Pseudomonas toward different members of Cruciferae :

While analysing the results of the pathogenicity tests on different plant species it has been found that isolates of the green fluorescent bacteria were capable of causing infection injured and non-injured tissues of cabbage (Cruciferae) plants which recorded maximum susceptibility.

At this stage it was considered necessary to examine whether other crop plants belonging to the family Cruceferae were similarly susceptible to these bacterial isolates.

An experiment was taken up to examine the pathogenicity of the four isolates of the green fluorescent bacteria namely GFB₁, GFB₂, GFB₃, GFB₄, toward four cruciferous crop plants including cabbage. The other three plants included are Cauliflower (Brassica oleracea), Mustard (Brassica campestris) and Radish (Raphanus sativus) .

The plants were raised in 9" dia. earthen pots from seeds of varieties namely Early Snow ball, Benoy and Pusha Deshi respectively. In case of Cabbage, Pusa Early Drum Head was included. At two months age of the plants they were inoculated by spraying in two sets namely spraying with (i) a high inoculum pressure (suspension containing 5×10^9 cells/ml) and (ii) low inoculum pressure (suspension containing 3×10^7 cells/ml) with a throat-sprayer duly sterilised.

The sprayed plants were covered with polythene bags for 24 hrs after spraying and uncovered thereafter. Necessary replicates and uninoculated controls were maintained.

Observations on disease development was made on the basis of a score-card developed on the basis of leaf blotches (in previous experiment) and given in Chapter III and percentage infection has been recorded in Table 12 and Fig. 9.

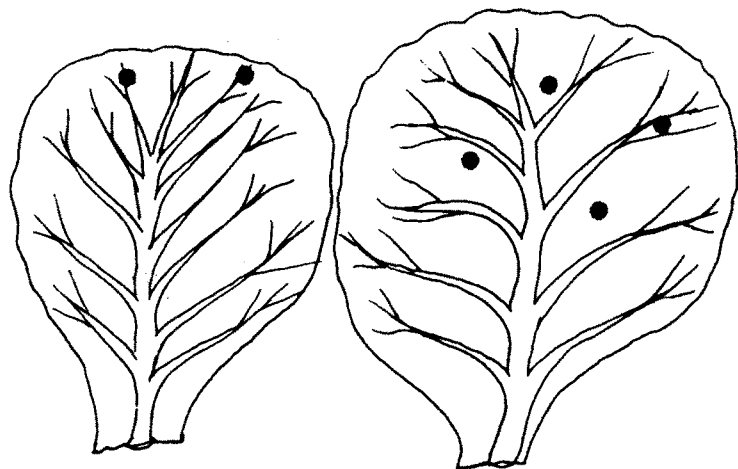
Table 12 : Pathogenicity of green fluorescent Pseudomonas on different members of Cruciferae (average of three replicates)

Isolates	% infection and disease development on							
	Cabbage		Radish		Mustard		Cauliflower	
	High	Low	High	Low	High	Low	High	Low
GFB ₁	100	100	100	50	50	0	70	70
GFB ₂	100	100	70	0	0	0	100	50
GFB ₃	100	100	50	0	0	0	100	100
GFB ₄	100	100	70	50	50	0	100	50

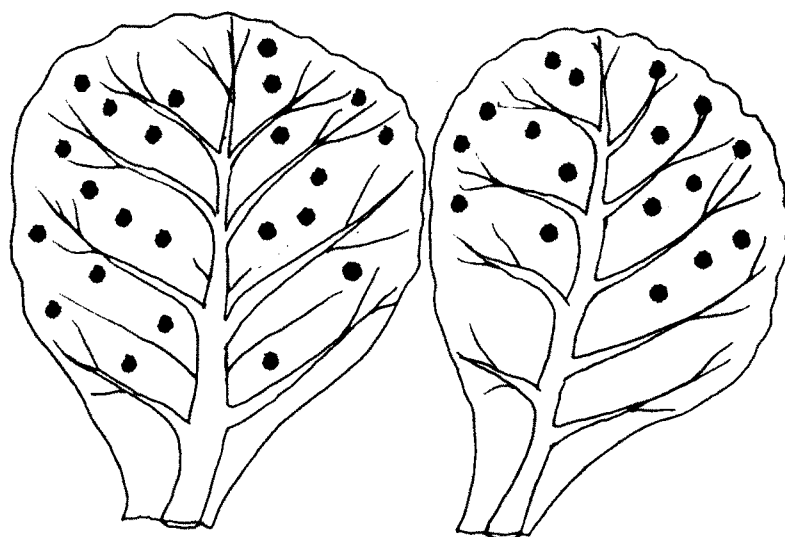
High inoculum pressure = 5×10^9 cells/ml

Low inoculum pressure = 3×10^7 cells/ml

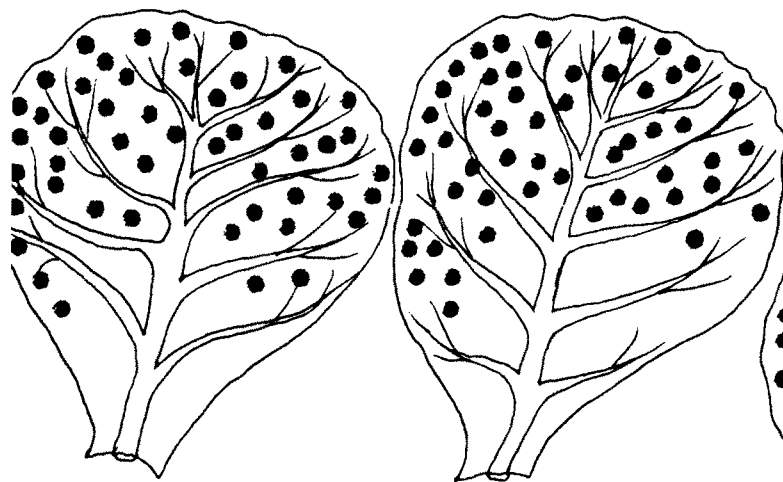
Evidently a higher inoculum load led to successful infection in most of the cases which is characteristic of any opportunistic pathogen. Variations between the isolates with regard to their pathogenicity is also evident from the data.



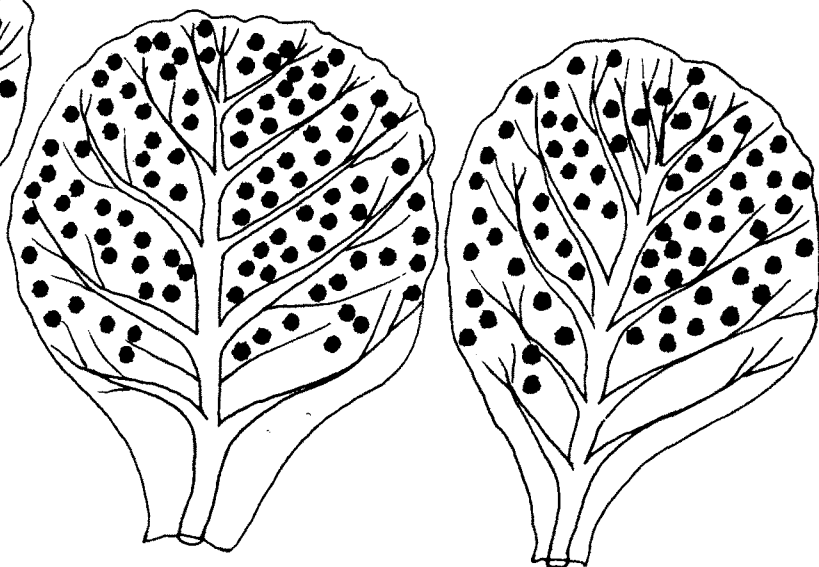
I 1 - 6% (Mid value 3 %)



II 6 - 34% (Mid value 20 %)



III 35 - 65% (Mid value 50 %)



IV 66 - 100% (Mid value 83 %)

Figure 9 . SCORE CARD FOR CABBAGE LEAF BLOTCH BY P. aeruginosa

4.3.4 Plant tissue rotting capability of green fluorescent bacteria :

Green fluorescent bacteria isolated from different surfaces in this programme appeared to be living in nature in close association with plant life - aquatic or terrestrial. Studies in the field of evolution of plant pathogenic bacteria has revealed that long and closer association of this kind generally helped to develop the faculty of using the plant materials, products, residues and wastes in such bacteria. In another subsequent long spell of time some such bacteria reached by natural selection the status of plant tissue retting bacteria or sometimes opportunistic plant pathogens. Plant tissue retting capability of the four isolates of green fluorescent bacteria were studied on different plant tissues namely (1) potato tuber, (2) bean seeds, (3) Bottle gourd leaf (4) Onion bulb and (5) Jute stems.

Small potato tubers, bean seeds, 2" long pieces of jute stems and onion bulbs were placed in sterilized petriplates and 150 ml beaker respectively after surface sterilization with 70% ethyl alcohol. A thin layer of sterile, distilled water was maintained in the petriplates and beakers in cases of potato and jute stems. Bean seeds and onion bulbs were kept in moist water-soaked blotting paper inside the respective containers. In cases of injured tissues they were rubbed on one surface with carborandum powder of fine mesh (200) with a cotton swab. The tissues were inoculated by

dropping bacterial suspensions with a sterile micropipette in three places on each tissue surface. The plates/beakers containing inoculated tissues were incubated in an incubator at $30 \pm 1^{\circ}\text{C}$ for 24-48 hrs. In case of ~~dicotyledonous~~ leaves total petiole of the leaves were kept submerged in sterilized water in 250 ml conical flasks while the lamina remained outside. The leaves were inoculated by spraying on normal and carborandum injured leaves with bacterial suspensions containing 3×10^7 cells/ml. The cucurbit leaves were incubated under room temperature at 16 to $31 \pm 2^{\circ}\text{C}$ temperature prevailing at that time under bell-jar.cover. Necessary replications and uninoculated controls were maintained in all the cases.

Results obtained are presented in Table 13 and Fig. 10. Results showed that most of the isolates of the green fluorescent bacteria were successful soft-rotters under given inoculum-load and incubation conditions. The time required for retting in various plant materials are clearly depicted in the data. Interestingly no rotting symptom appeared in case of bean seeds. Only the germination of the seeds was delayed in comparison to seeds under control conditions. The germination-delay might be due to the heavy deposition of slime on the seed surfaces due faster bacterial growth in seed exudates.

In case of onion, the bulb became rotten and the rate of rotting was very fast in cases of injured bulb with all the isolates. Upto the 2nd and 4th day of incubation, germination of the bulbs was not hampered. However, in later phases or finally the tip of the

Table 13: Plant tissue rotting capability of green fluorescent bacteria

Plant Materials	Success of plant tissues rotting by isolates			
	(Hours required for 100 % rotting)			
	GFB ₁	GFB ₂	GFB ₃	GFB ₄
1) <u>Potato tuber</u>				
a) injured	13	13	13	13
b) uninjured	22	22	22	22
2) <u>Bean seeds</u>				
a) injured	Nil	Nil	Nil	Nil
b) uninjured	Nil	Nil	Nil	Nil
3) <u>Leaves (B.G.)</u>				
a) injured	72	72	72	72
b) uninjured	96	96	96	96
4) <u>Onion bulb</u>				
a) injured	72	72	72	72
b) uninjured	144	144	144	144
5) <u>Jute stems</u>				
a) injured	20	20	21	22
b) uninjured	31	33	29	28

emerging tube became blackish brown and rotten. More or less a similar result was obtained in case of leaves.

The present results confirmed similar findings of Desai (1935), Kawamoto and Lorbeer (1974) and Cho et al. (1975).

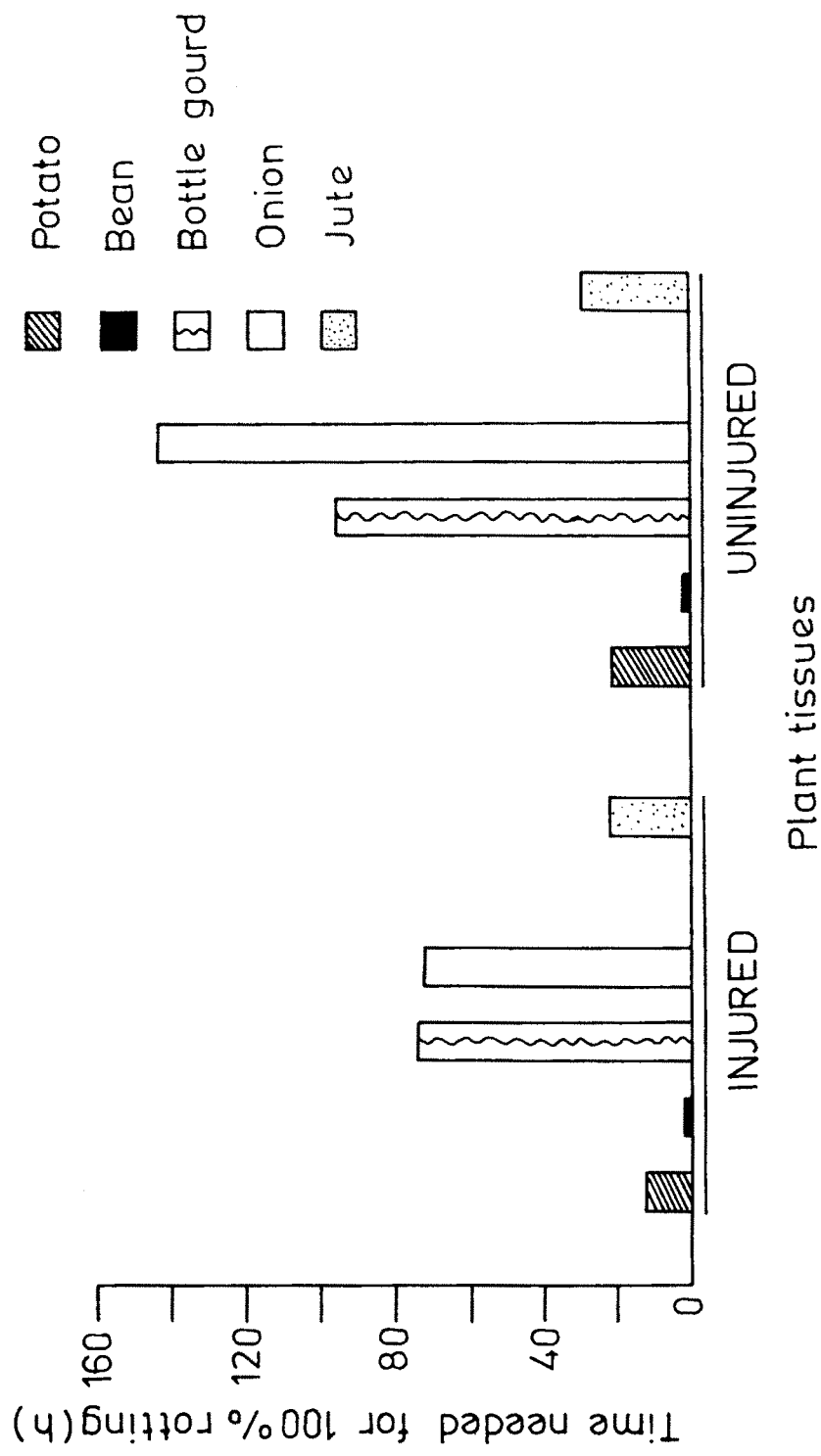


Figure 10. Time required by Green Fluorescent Bacteria for plant tissue rotting (average of 4 isolates)

4.3.5 Animal pathogenicity of green fluorescent bacteria :

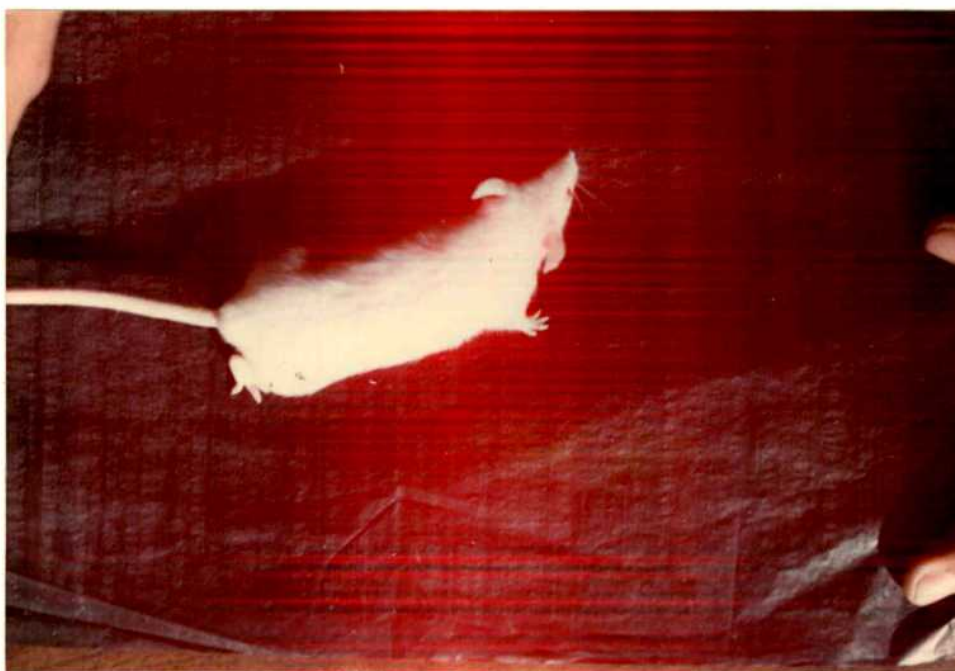
Some species of green fluorescent bacteria are earlier reported to cause of animals infection. The main point in this regard is that such bacteria are normally found around the animal world, in their stool, polluted water bodies and diverse other surfaces (Schroth et al. 1977). However, these bacteria are essentially opportunistic in causing infection in animal or human bodies or tissues, particularly under stress.

It was thus considered necessary to test the pathogenicity of the isolates on a mammal test animal namely mouse (Mus musculus). Thus animal pathogenicity of the green fluorescent bacteria was studied on 'Mouse Model' on white "Swiss variety" estimating the success of pathogenicity in terms of percent mortality of the animals and also the time (hrs) required to kill the animals.

4.3.5 (a) Inoculum dose and mortality :

Initially animal pathogenicity was determined on 'Mouse Model' by using suspension of the bacteria at an inoculum density of 3×10^3 cells/ml prepared following standard method described earlier and they were injected on white mice Fig.11 (Swiss variety) at different dosages namely 1.0, 0.5, 0.2, and 0.1 ml/animal of approximately 50-70 gms body wt. Four isolates of the green fluorescent bacteria namely GFB₁ , GFB₂ , GFB₃ , and GFB₄ taken from their 24 hour fresh cultures and the suspensions were prepared. The animals of 2 month age were injected in the inter-peritoneal zone with a

Fig.11 Experimental animal for animal pathogenicity test :



Swiss mouse

24 gauge needles. Beforehand the syringe and the needles were properly sterilized in boiling water. Necessary replicates and uninoculated controls were maintained.

After injection the mice were kept under normal conditions in cages in a room. Observations were made on any abnormal signs or the mortality caused.

Results obtained are presented in Table 14. The results clearly indicated that all the injected mice with 1 ml, 0.5 ml and 0.2 ml doses died within 72 hours, but the animals which were inoculated with only 0.1 ml suspension survived a period longer than 20 days. After injection, within 24 hours several symptoms started appearing on all the animals except those under control. Symptoms included refusal of food, excessive thirst and slow or no movement. The back legs also appeared swollen with hairs becoming erect. Fall of hairs was another symptom. In the later stages red patches appeared on the tail and lower abdomen. After death of the animal secretion of green juicy fluid from the portions showing sores was common. Green-puss was also evident. The bacteria could be re-isolated from the infected portion of the animal body by needle streaking. All the four isolates were equally successful in causing infection in the mouse on injection-inoculation.

Table 14 : Animal pathogenicity of green fluorescent
bacteria : percent mortality

(average of 3 replicates)

Isolates	% Death with ml. of inoculum (10^3 cell/ml) injected after 72 hours			
	1.0	0.5	0.2	0.1
GFB ₁	100	100	100	0
GFB ₂	100	100	100	0
GFB ₃	100	100	100	0
GFB ₄	100	100	100	0
Uninoculated control	0	0	0	0

4.3.5 (b) Determination of the time to kill
the mouse on inoculation :

After the establishment of the animal pathogenicity of the four isolates of green fluorescent Pseudomonas it was considered necessary to check the time required to cause mortality of the mice at a definite inoculum dose. Animal pathogenicity of the four isolates of the bacteria was studied on white mice (Swiss variety) following the procedure described earlier. The isolates of the test bacterium

included in the study were GFB₁, GFB₂, GFB₃ and GFB₄.

From 24 hour fresh bacterial cultures on KBC bacterial suspensions were prepared, following the procedure described earlier and the experimental mice were injected in the inter-peritoneal portion @ 0.2 ml /animal with a 24 gauge needle. The number of bacterial cells per ml. of suspension was 3×10^8 according to the standard scale. The time required to kill the animals by the bacteria as determined have been presented in the table 15. Before injection the needle and the syringe were sterilized by boiling in water. At the time of injection the abdomen portion of the mouse was cleared with rectified spirit. Before and after injection the mice were kept in mice-cage under normal conditions of management and food and water supply. Necessary replicates and control were maintained. The animals in cage were maintained in usual way in the laboratory and observations on the extent of infection and mortality were made from 1 hours after inoculation onward at 24, 48, 72 hours after inoculation.

Table 15: Animal pathogenicity of green fluorescent bacteria : time to kill (average of 3 replicates)

Isolates	% death after hours				
	12	24	48	62	72
GFB ₁	0	0	0	0	100
GFB ₂	0	0	0	100	100
GFB ₃	0	0	100	100	100
GFB ₄	0	50	100	100	100
Uninoculated control	0	0	0	0	0

Results showed that 100 % death of the animals with all the isolates of the bacteria could be achieved only after 72 hours. Two of the four isolates namely GFB₃ and GFB₄ could kill the animals (cent percent) by 48 hrs, whereas isolates GFB₁ and GFB₂ could not cause mortality before 72 hours and 62 hrs respectively. The isolates did show some amount of differences in their virulence towards the animals.

4.4 Growth of green fluorescent bacteria under different atmospheric conditions :

4.4.1 (a) Growth at different temperatures : Cell number

Growth of a bacterium is influenced to a great extent by temperature, although each type of bacterium shall have a specific temperature for their growth. Their position in a situation is largely determined by temperature. Hence it was considered necessary to study the effects of temperatures on growth of the green fluorescent bacteria.

Growth of the four isolates of the bacteria (1) GFB₁, (2) GFB₂, (3) GFB₃, and (4) GFB₄ were tested at 20,°C, 30°C, 40°C and 45°C temperatures. From 24-hour old bacterial cultures, loopful of bacteria was taken and transferred to sterilized KBC-slants, and KBC broth in tubes. Each slant and broth containing 5 ml of medium, were then placed in different incubators adjusted at four different temperatures. Necessary replicates and control were maintained. Results on growth of the bacteria were recorded following turbidity scale after 24 hours of incubation at respective temperatures. In-cases of slants the bacterial colony was washed by 10 ml of sterile,

Table 16. Growth of the green fluorescent bacteria under different temperatures
(average of 3 replicates)

Iso- lates	Extent of growth (cell No./ml) at temperature °C					
	20		30		40	
	Slant culture	Broth	Slant culture	Broth	Slant culture	Broth
GFB ₁	1.5×10^8	1.5×10^8	3×10^8	3×10^8	6×10^8	4.5×10^8
GFB ₂	3×10^8	3×10^8	6×10^8	4.5×10^8	9×10^8	7.5×10^8
GFB ₃	1.5×10^8	1.5×10^8	4.5×10^8	4.5×10^8	7.5×10^8	6×10^8
GFB ₄	3×10^7	3×10^7	1.5×10^8	1.5×10^8	4.5×10^8	3×10^8

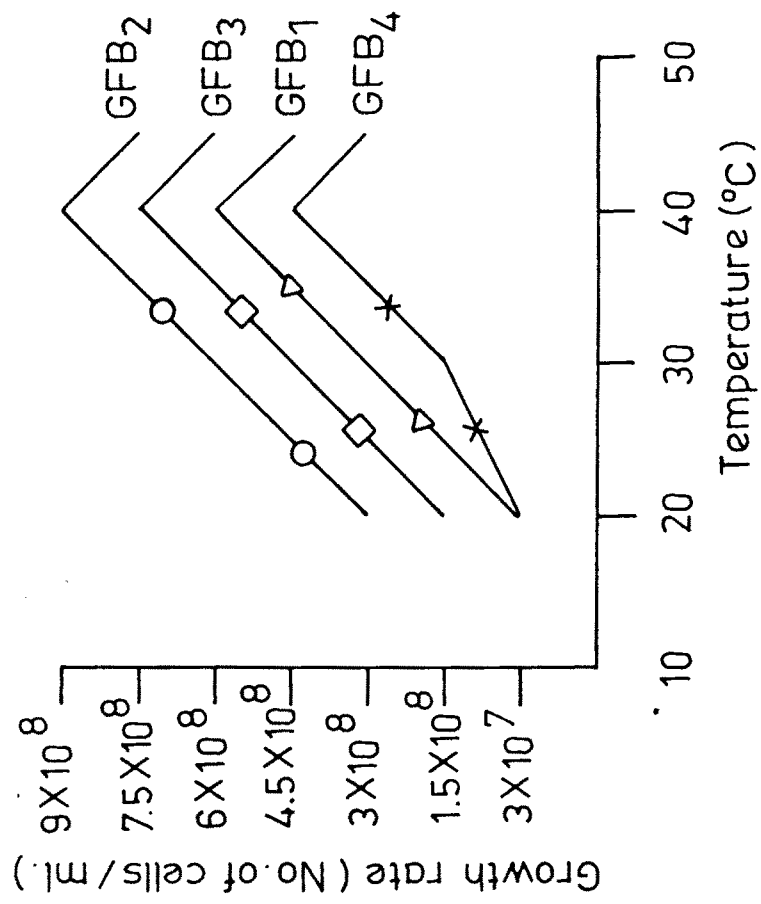


Figure 12. Growth of the green fluorescent bacteria under different temperatures on solid cultures.

distilled water and then from that suspension an aliquot of 1 ml was taken. The suspension was diluted with sterile, distilled water. The prepared bacterial suspension was then matched with turbidities of McFarland's scale, the population of the bacteria was recorded and presented in the table 16 and Fig. 12. In case of the broths, the suspensions were centrifuged at 3000 rpm for half an hour and after decanting down the supernatant bacterial cells were collected and mixed with 10 ml of sterile distilled water. One ml of that suspension was added to 9 ml of sterile distilled water, to prepare 10^{-1} dilution. Results obtained were recorded and also presented in Table 16 & Fig.12. Results showed that the growth of the bacteria in both the broth and plant was very high at 40°C and very low in 20°C. In control tubes there were no growth. The increasing trend with increase in temperature recorded a decline after 40°C.

4.4.1 (b) Pigment production of green fluorescent bacteria at different temperatures :

Growth of a bacterium is dependent much upon the temperature of the growing environment. Occurrence of these bacteria have been reported extensively in temperate areas as compiled by Schroth et al. 1977. But with regard to their occurrence in tropics also, the temperature response of such bacteria was very important. Whether the temperature effect could affect the pigment production was tested in an experiment. The experiment was conducted to study the effect of different temperatures on growth of these bacteria and production of pigment by them. The study was made with 4 isolates of the bacteria namely

Table 17 : Pigment production by green fluorescent bacteria at different temperatures
(Average of 3 replicates)

Isolates	Broth				Slant culture			
	20	30	40	45	20	30	40	45
GFB ₁	2	3	4	3	2	3	5	4
GFB ₂	1	1	2	1	1	1	2	1
GFB ₃	2	3	4	3	2	4	6	5
GFB ₄	1	2	3	2	1	2	4	3

High pigmentation

Very deep = 6
Deep = 5
Moderately deep = 4

Low pigmentation

Light = 3
Moderately light = 2
Very light = 1

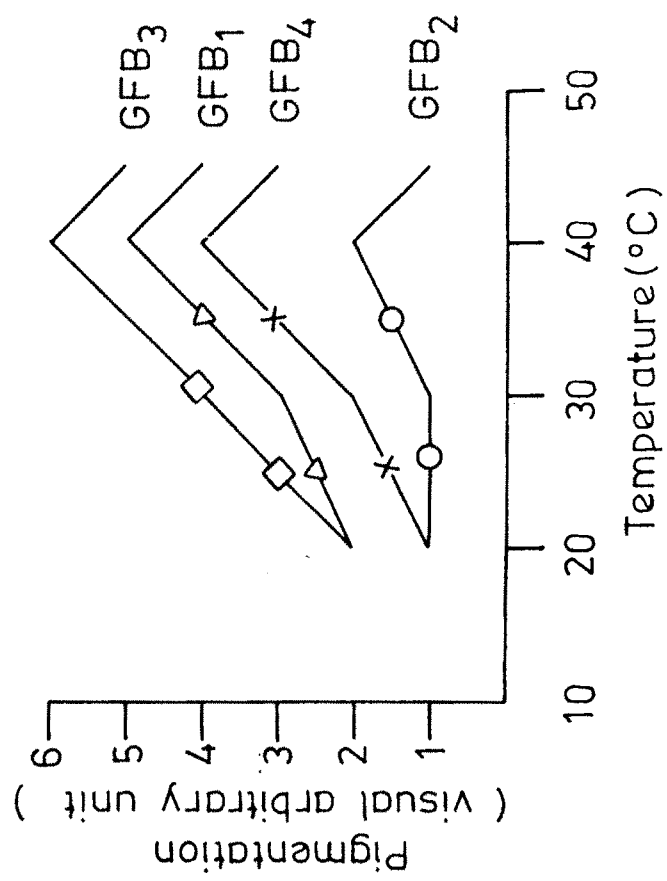


Figure 13. Pigment intensity in relation to temperature on 24 hours solid culture.

(1) GFB₁ (2) GFB₂, (3) GFB₃ and (4) GFB₄. The temperatures at which the study was made were 20, 30, 40 and 45°C which were arranged using BOD and ordinary incubators. The pigmentation study was made both on solid and liquid media.

From 24 hour fresh growing bacterial culture a loopful of bacteria were transferred in the sterilized 'KBC'-slant and broth cultures. Each contained 5 ml of KBC medium.

After proper inoculation the slant and broth were kept at above mentioned incubators. After 24 hours incubation, results were taken by visual recording of the intensity of pigmentation. Uninoculated control and necessary replicates were maintained.

The produced pigment were water-soluble and light to deep greenish in colour and fluorescent in nature.

Evidently the isolates produce very deep to very light green fluorescent pigment under similar conditions of temperature and growth Table.17 & Fig.13. Isolates demonstrated consistent and clear variation in their extent of pigment production.

4.4.2 Growth of green fluorescent bacteria at different pH :

Just like temperature, pH of the substrate or the growth medium also affects to a great extent the growth of a bacterium in a particular environment. In fact pH of the substrate ultimately becomes determinant as to what extent the bacterium will be capable of growth and multiplication. An experiment has been framed to determine the ideal pH for the green fluorescent bacteria under study.

Table 18. Growth of the green fluorescent bacteria under different pH.

Isolates	Extent of growth (cell no./ml) at pH							
	6		7.0		7.5		8.0	
	Slant culture	Broth	Slant culture	Broth	Slant culture	Broth	Slant culture	Broth
GFB ₁	1.5×10^8	1.5×10^8	6×10^6	6×10^8	7.5×10^8	6×10^8	6×10^8	4.5×10^8
GFB ₂	3×10^8	3×10^8	7.5×10^8	7.5×10^8	9×10^8	9×10^8	7.5×10^8	6×10^8
GFB ₃	3×10^8	3×10^8	7.5×10^8	6×10^8	7.5×10^8	6×10^8	6×10^8	4.5×10^8
GFB ₄	3×10^7	3×10^7	4.5×10^8	4.5×10^8	6×10^8	4.5×10^8	4.5×10^8	3×10^8

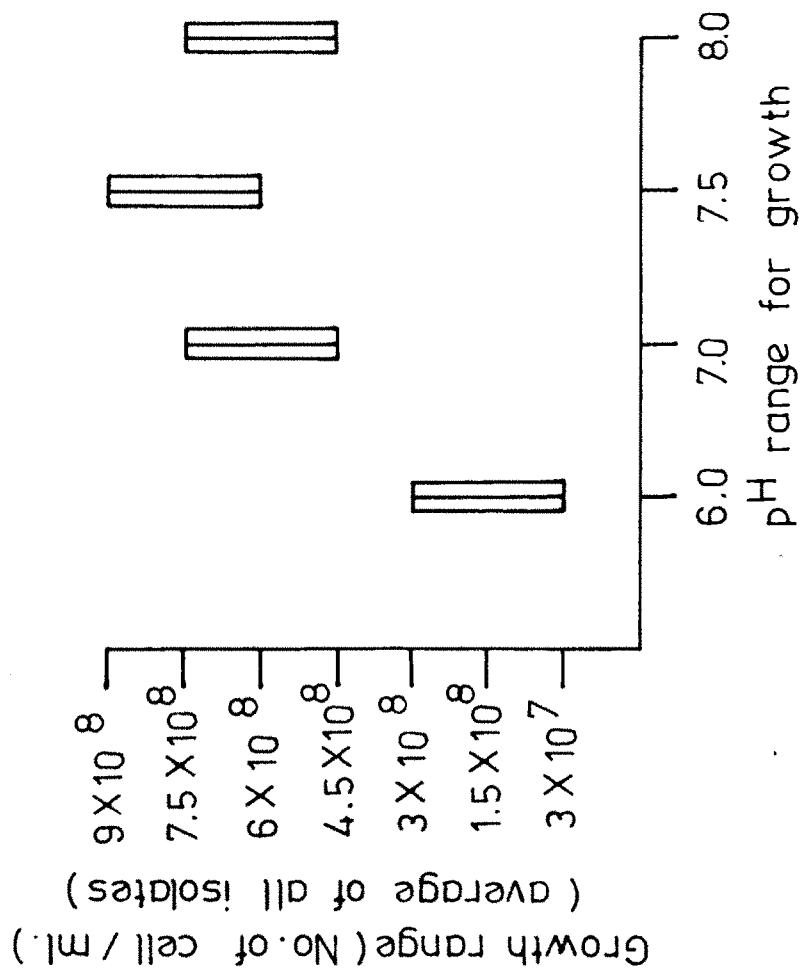


Figure 14. Growth of the green fluorescent bacteria at different pH on slant culture.

The growth of the four isolates of the bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ were studied at 6, 7, 7.5 and 8 pH in KBC-slants and KBC-broths. The KBC-broth and KBC-slants were taken in culture tubes @ 5 ml/tube and sterilized at 121°C for 15 minutes. With a loopful of 24 hour old fresh bacterial cultures both the slants and the broths were inoculated and incubated at 42°C for 24 hours. Results were taken following same procedure as in case of temperature. Necessary replications and control were maintained. Results on growth of the bacterium at different pH were recorded as before and presented in Table 18 and Fig.14.

Results showed that the growth of the bacteria at pH 7, 7.5, 8 were higher in comparison to that at pH 6. Maximum growth was obtained at 7.5 and minimum at 6 both in slant and broth.

4.4.3 Tolerance of green fluorescent bacteria to desiccation :

The capability of the isolates of the green fluorescent Pseudomonas to continue in environment depended much upon its success in withstanding different natural forces. Desiccation was considered to be one such important obstacle for a non-spore forming bacterial species to thrive. Hence the present experiment was taken up to assess this faculty of the isolates of the bacteria.

Tolerance to desiccation was studied in case of the four isolates namely GFB₁, GFB₂, GFB₃, GFB₄. The isolates of the bacteria were grown in 100 ml conical flasks containing 25 ml of KBC-medium solidified.

Table 19: Tolerance of green fluorescent bacteria to desiccation.
(Average of 3 replicates)

Isola- tes	Survival in days against desiccation														
	Summer							Winter							
	Large ball			Small ball				Large ball				Small ball			
	10	20	40	60	80	100	100	10	20	40	60	80	100	20	40
GFB ₁	100	100	100	0	0	0	100	100	100	0	0	0	100	100	100
GFB ₂	100	100	100	0	0	0	100	100	100	0	0	0	100	100	100
GFB ₃	100	100	100	0	0	0	100	100	100	0	0	0	100	100	100
GFB ₄	100	100	100	0	0	0	100	100	100	0	0	0	100	100	100

Before inoculating the medium inside the conical flasks they were sterilized at 121°C for 15 minutes. Then the flasks were inoculated with loopful of 24 hour old bacterial cultures. The flasks were kept at 42°C temperature for 48 hours. After 2 days glass marbles of two different sizes namely small (9 mm diameter) and large (14 mm diameter) were dropped carefully inside the inclined flasks and allowed to roll on the surface by gentle shaking. After the surfaces of the marbles are covered with bacteria they were brought out aseptically with long forceps and put inside sterile culture tubes (25 mm diameter). The tubes with the balls were plugged with cotton and stored in laboratory under normal conditions ($20-30 \pm 2^\circ\text{C}$ and $12-26 \pm 2^\circ\text{C}$). Each tube containing 5 marbles formed five replicates. Then at 10, 20, 40, 60 and 80 day intervals the marble of each size were transferred to sterile KBC-slant aseptically to test the survivability of the bacteria on marble surfaces. Tolerance to desiccation by the bacterial isolates were studied in two seasons i.e. summer and winter. Results obtained are presented in the table 19. In summer all the isolates were able to tolerate desiccation upto 40 days on the surfaces of each ball, but in winter GFB₁ and GFB₃ survived upto 60 days on the surfaces of both the size of the balls and GFB₂ and GFB₄ were found to be able to survive upto 60 days on surfaces of both the balls.

4.5 Saprophytic growth of the green fluorescent bacteria on different organic substrates :

4.5.1 Growth on different organic substrates in nature

Considering the nature of the substrates and situations where green fluorescent bacteria were detected in the survey work, the present set of studies were taken up. The growth and/or survival of the

bacteria was examined on some selected substrates which might act as natural habitats for the bacteria. They included organic matter dissolved in different kinds of water also. As checks pure cellulose and glucose + urea were also included as substrates. Thus, the growth of the four selected isolates of the bacteria was studied on different substrates namely (1) FYM (Farm Yard Manure), (2) Oil-cake, (3) Leaf mould (4) Drainage silt, (5) Glucose + urea (3 gm + 3 gm), (6) Crop Field water (7) Pond water and (8) Cellulose.

Six gm of each of the substrate materials were taken in 250 ml conical flasks. In case of pond and field waters 6 ml was taken per flask. Distilled water was added to each of the materials to keep them moist during the study. After proper plugging of the flasks with non-absorbent cotton sterilization was done successively on two days each time at 121°C for 30 minutes.

The sterilized substrates in the flasks were then inoculated with a loopful of 4 isolates of the bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ under aseptic conditions. Necessary replicates and uninoculated control flasks of each of the sets were maintained. The flasks were incubated at 40 ± 1°C in an incubator. Observations on survival and growth were made by streaking from the substrate on KBC-slants and visual observation at intervals of 10 days and upto 20 days.

Results obtained are presented in Table 20. The results were taken after 10 and 20 day intervals from the date of incubation by transferring the growth if any in the KBC-slants.

Table 20: Growth of the green fluorescent bacteria on organic matter.
(average of 3 replicates)

Isola- tes	% of growth at days intervals on substrates											
	Field water			Pond water			Glucose + Urea			Leaf mould		
	10	20	10	10	20	10	10	20	10	10	20	10
	10	20	10	10	20	10	10	20	10	10	20	10
GFB ₁	100	100	100	100	100	0	0	0	0	0	0	100
												100
												0
GFB ₂	100	100	100	100	100	0	0	0	0	0	0	100
												100
												0
GFB ₃	100	100	100	100	100	0	0	0	0	0	0	100
												100
												0
GFB ₄	100	100	100	100	100	0	0	0	0	0	0	100
												100
												0

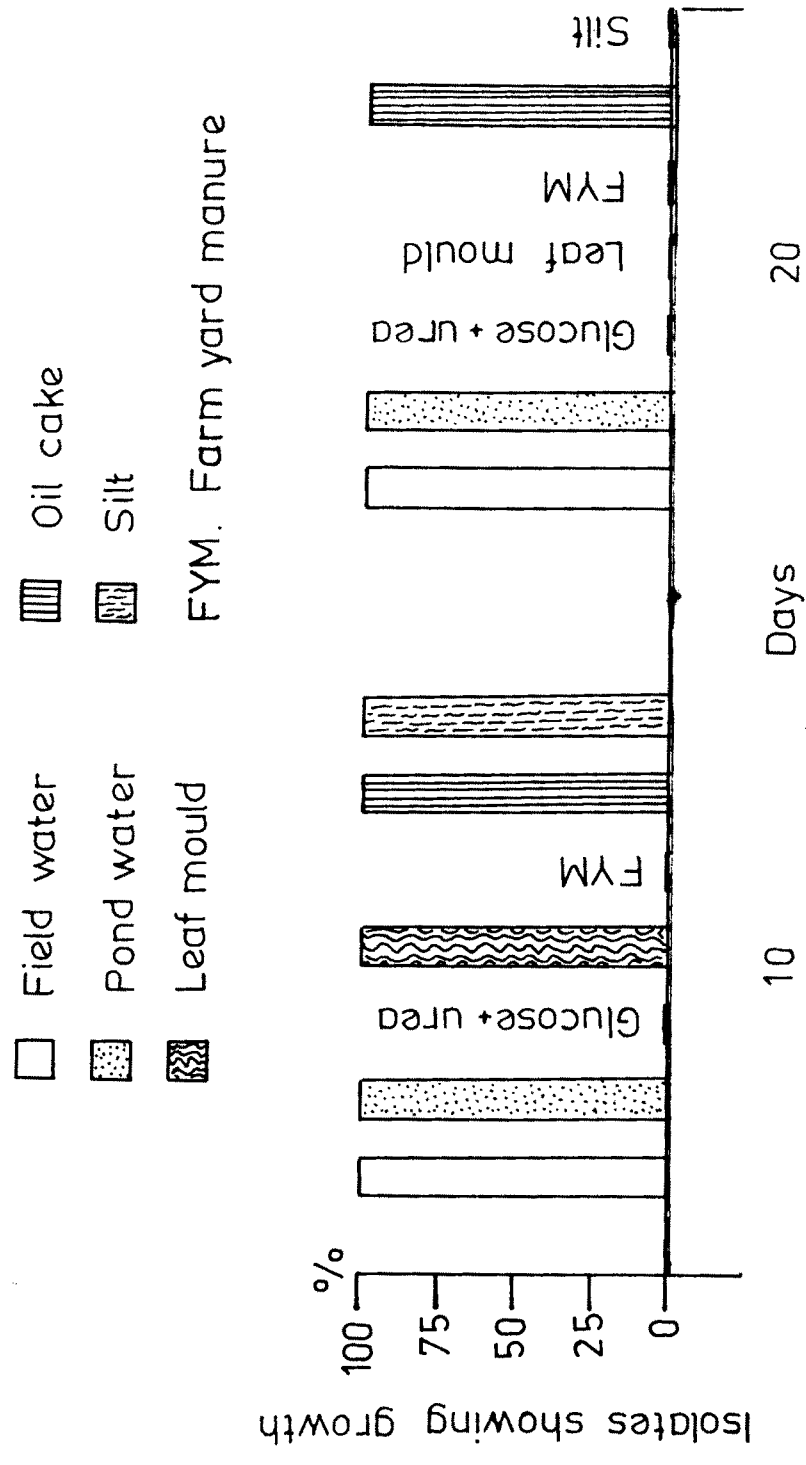


Figure 15 Survival of the green fluorescent bacteria on organic matter.

Results showed that all the isolates recorded 100 % survival upto 20 days in the agricultural field water, pond water and oil cake. But in cases of leaf mould and silt the isolates survived for 10 days only. The bacteria could not be detected after 10 days even in cases of cellulose and glucose plus urea.

However no visible growth was detectable on any of the substrates used. Their survival and growth as appear in Fig.15 also clearly illustrated the nature of their behaviour.

4.5.2 Saprophytic growth of the green fluorescent bacteria on different grain meals and dusts

In continuation of previous studies on the saprophytic growth ability of the green fluorescent bacteria on different grain dusts and grain meals with different nutritional qualities have been examined. Considering that the saprophytic ability depended much upon the quality of substances that are around and coming across such bacteria, growth of the four isolates of the green fluorescent bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ were tested and studies on different grain meals and dusts of Maize (Zea mays), Gram (Cicer arietinum) and wheat (Triticum aestivum). Six gram of each of the grain meals and dusts were taken in large sized (1.5 x 12 cm) culture tubes. The grain dust and meals were soaked with distilled water @ of 15 ml and 10 ml/tube respectively. The tubes were then plugged with cotton and sterilized for two successive days at 121°C for 15 minutes on each day. After proper sterilization the tubes were inoculated with loopfull of bacteria from 24 hour culture of the bacteria. Necessary replicates and

control were maintained. After inoculation the tubes were incubated under room temperature ($19-29 \pm 2^{\circ}\text{C}$).

Observations on growth of the bacteria were made visually at 10 and 20 days intervals and confirmed by transferring them on KBC-slants each time.

Results obtained are presented in the Table 21. The results clearly indicated that the growth of the bacteria was positive and quite slow and on 10th day in all the grain dusts and meals but the growth rate increased in most cases except on meals in cases of GFB₃, GFB₄. However, the same isolates namely GFB₃ and GFB₄ showed highest growth on dusts in comparison to other isolates at the 20th day stage. In control tubes there were no growth. With regard to the growth characteristics of the isolates on grain dusts, slime production by all the isolates was not very high like that on KBC - slants but slime was produced in all the cases. The characteristic pigment also appeared on all the dusts in case of all the isolates except in case of GFB₁ on wheat dust. The colour was also greenish but not exactly the same as on KBC -slant. At the end of the 20 day the colour of the tubes became light to deep blackish possibly showing signs of pyocyanin production.

In case of grain meals the table showed that the growth of the bacteria on all the three meals was positive in cases of all the four isolates. However, the population was low at 10 days in case of all the isolates and all the grain meals, and become higher to medium in GFB₁ and GFB₂ after 10 days. There was however no change in growth after 10 days in case of isolates GFB₃ and GFB₄ .

Table 21: Visible growth of green fluorescent bacteria on different grain meals and dusts.

Isolates	Substrate	Visible growth of green fluorescent bacteria after days					
		Wheat		Gram		Maize	
		10	20	10	20	10	20
GFB ₁	D	Low	Medium	Low	Medium	Low	Medium
	M	Low	Medium	Low	Medium	Low	Medium
GFB ₂	D	Low	Medium	Low	Medium	Low	Medium
	M	Low	Medium	Low	Low	Low	Medium
GFB ₃	D	Low	High	Low	High	Low	High
	M	Low	Low	Low	Low	Low	Low
GFB ₄	D	Low	High	Low	Medium	Low	High
	M	Low	Low	Low	Low	Low	Low

D= Dusts, M= Meals.

4.5.3 Saprophytic growth of the green fluorescent bacteria on different plant tissues :

Besides the plant produces of nutritional value and category, surfaces of some commonly growing plants of weeds category may have some relation with the green fluorescent bacteria. Initially saprophytic ability of the bacteria was studied on the crushed sterilised leaves of such plants taken as medium of growth. Leaves of the four plants namely (i) Ludwigia adscendens, (ii) Imperata cylindrica, (iii) Hygrophiza aristata and (iv) Eichhornia crassipes, commonly growing in and around semi-aquatic environment of the effluent channels leading to river Ganga, were taken. Out of the four plants three were aquatic and the fourth Imperata cylindrica was terrestrial. The plants were collected from water-bodies of Kalyani town and adjoining agricultural fields.

From each of the collected plants leaves and soft stems were taken and crushed in an electrica grinder at a low speed for 5-7 minutes. Twenty gm of each of the crushed plants were taken into 250 ml conical flasks and to each of them water was added @ 10 ml/flask to keep them moist. The conical flasks were then plugged with non-absorbent cotton and sterilized at 121°C for 15 minutes on two successive days to make it free from other microorganisms. Before sterilization pH of the each of the crushed plant materials were noted.

After proper sterilization the plant-tissue media were inoculated with loopfull of 24 hr culture of the four isolates of the bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ under aseptic conditions.

Necessary replicates with control were maintained. The flasks were incubated at $40 \pm 1^\circ\text{C}$ for 20 days and observations on growth of the bacteria were made at different intervals like, 5, 15, 30 and 45 days after incubation. On the above mentioned days from each flask a loopfull of growth from moist, crushed surfaces of plant tissues was transferred into sterilized KBC-slants for confirming growth and /or survival of the bacteria. Visible growth of the bacteria if any were also recorded.

Results obtained are presented in Table 22. The results in the table 22 showed that the isolates were present on the surface of the crushed leaf media even after one and a half month or 45 days. Growth in these occasions were visible with difficulties. There were minor

Table 22: Saprophytic ability of green fluorescent bacteria on different plant tissues (average of 3 replicates)

Tissues	pH	% isolates showing '+' ve growth after days			
		5	15	30	45
<u>Ludwigia</u> <u>adscendens</u>	6.0	100(n)	100(h)	100(a)	100(a)
<u>Imperata</u> <u>cylindrica</u>	7.5	100(n)	100(h)	100(a)	100(b)
<u>Hydrochiza</u> <u>aristata</u>	7.5	100(n)	100(a)	100(b)	100(b)
<u>Eichhornia</u> <u>crassipes</u>	6.0	100(a)	100(a)	100(b)	100(b)

(n) = no visible growth (a) = slight visible growth

(b) good visible growth

differences in the visibility of growth on different plant tissue medium may be due to the colour of the tissues. The pH of the two crushed-plant-tissue media were on high side and that of two others were on low side but the bacteria were able to grow and survive at least 15 days.

4.5.4 Saprophytic ability of green fluorescent bacteria on different animal products :

Saprophytic ability of the green fluorescent bacteria have been examined taking different substrates into consideration. While investigating the ecological status and position of the bacteria in the mentioned environment it was considered necessary to study the saprophytic ability with regard to substrates of animal origin e.g. hairs, feathers, shells of eggs etc. To study the saprophytic ability of the isolated green fluorescent bacteria animal epidermal organs like human hairs, feathers of hens and shells of eggs were collected locally and included. Considering the fact that these materials are quite common in the mentioned environment the selection was made like this. The materials were collected from out-door locations, fields and market places mostly from roadsides. Two gm of each of the samples were taken in the culture tubes (1.5 x 12 cm) and 2 ml of distilled water was added per tube to moisten the substrates in the tubes. After cotton plugging, all the tubes were sterilized in autoclave at 121°C for 30 minutes on two successive days. The pH of the materials in each tube were recorded with Merck Disc pH paper before autoclaving.

After proper sterilization the tubes were inoculated with loopfull of 24 hour fresh culture of the four isolates of bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄. Necessary replicates and uninoculated control were maintained. The tubes after inoculation were incubated at $40 \pm 1^\circ\text{C}$ for 20 days. Observations on the growth of the bacteria were made and are presented in Table 23. Results indicated that while 100 % survival of the isolates of the bacteria was clear, their growth on either of the substrates were not significant. Changes of colour and growth with slimyness have been recorded but they were very insignificant.

Results taken at 10 and 20 day intervals by streaking on KBC - slants established clearly the survival of the bacterial isolates on such substrates. Results also showed that with growing of the bacterial isolates there were slight change in pH generally by 0.5 degrees. This also positively indicated growth of the bacteria.

Table 23: Saprophytic ability of green fluorescent bacteria on animals products
(average of 3 replicates)

Growth after	% isolates showing '+' ve growth on		
	Hair (pH 7.0)	Feather (pH 6.0)	Egg shell (pH 6.5)
10 days	100	100	100
20 days	100	100	100
Growth characteristics	White creamy, slimy substance appeared on the hair surfaces; slowly it became blackish.	White colony appeared on the feather surfaces with heavy slime.	Light yellowish colony appeared on the surfaces: the colour became brownish later.

4.5.5 Saprophytic growth of green fluorescent bacteria on animal excreta :

Considering the versatility of the green fluorescent bacteria it was decided to examine whether these bacteria could grow as saprophyte on substrates like animal excreta which are very common in the surroundings of river Ganga.

Saprophytic growth of the four isolates of the bacteria was studied on some animal excreta material like cowdung and goat-droppings, common in this locality. The excreta were collected fresh from the locality for the experiment.

Two gm each of the samples were taken in the large (dia.1.5 cm) culture tubes and 0.5 ml of distilled water was added in each tube to keep the materials moist. The pH of the materials in the tubes were recorded to be 7.5 and 8.0 respectively. After proper cotton-plugging, the tubes were sterilized at 121°C on two successive days for 15 minutes on each occasion. After sterilization the tubes were inoculated with loopfull of 24 -hour fresh culture of the isolates of bacteria namely (1)GFB₁, (2) GFB₂(3)GFB₃ (4) GFB₄. Uninoculated control sets were maintained. All the sets were replicated thrice. The plates were incubated at 40 ± 1°C for 20 days and data on growth of the bacteria was collected twice after 10 and 20 days by checking any positive visible growth of the bacteria on the surfaces of the materials.

Results obtained clearly showed that the bacterium was unable to grow satisfactorily on such surfaces and no visible growth could be detected. On examination of any survival of the inoculated bacteria it was revealed that the bacteria could survive even on such surfaces at least upto 20 days.

It seemed that even if the isolates could grow on these substrates particularly under sterilized conditions and the growth was not visible but the extent of growth was not at all impressive. However, some white slimy, jelly like growth could be detected on the surface after 20 days on cow-dung while a brownish, slimy, growth after 10 days on goat-droppings. The growth on being transferred to KBC-slants after 1 month could produce the same bacterial colony. However, no growth could be detected on unsterilized surfaces.

4.6 Survival of green fluorescent bacteria in different situations :

4.6.1 Survival in different soils :

To study the survival ability of green fluorescent bacteria in soils, 3 types of soils from the Ganga bed, pond and agricultural field were collected. Twenty gm of each of the soil samples were taken in separate conical flasks (150 ml) and number of such sets have been prepared for the study. One set of such soil which was sterilized for 2 successive days at 121°C for 15 minutes and another set was kept unsterilized. Sterile distilled water was added to the soil from

time to time keeping the weight of the conical flask constant to keep the soil moist. A loopfull of 24 hr old bacterial culture was taken and the soils in each flask were inoculated with the bacteria at 4-5 places on the surface layer of the soil. The bacterial isolates used for inoculation were (1) GFB₁ (2) GFB₂ (3) GFB₃ and (4) GFB₄.

To record the survival of the bacteria in soil small samples from surfaces of the soils in flask were taken out aseptically, suspended in sterile distilled water and streaked on KBC-slants for detection of the bacteria. All the inoculated flasks were kept in normal room temperature (20 to $30^{\circ} \pm 2^{\circ}\text{C}$) condition. Necessary replicates and uninoculated controls were maintained.

Results obtained have been presented in the table 24 and Fig. 16. From the data it has been clear that all the isolates survived upto 220 days on surfaces of different sterilized soils, collected from the Ganga, pond and agricultural field, where as in unsterilized sets they generally survived upto 30 days only. The range of the period of survival amongs different isolates and under different conditions was from 30-180 days. The variation between the isolates in regard to their survival in soil was clearly revealed under unsterilised conditions of soils.

In the Ganga soil all the isolates survived upto 180 days. In case of agricultural field soil isolates lost their survivability between 30-180 days. In case of pond-soil, the isolates quickly lost their survivility between 30-90 days.

Table 24. Survival of fluorescent bacteria in soils collected from different situations.
(average of 3 replicates)

Incubated in	Isola- tes	Percent isolates survived for days					
		10	20	30	90	180	220
A. Sterilized from:							
I. <u>Ganga</u> :	GFB ₁	100	100	100	100	100	100
	GFB ₂	100	100	100	100	100	100
	GFB ₃	100	100	100	100	100	100
	GFB ₄	100	100	100	100	100	100
II. <u>Aq. field</u> :	GFB ₁	100	100	100	100	100	100
	GFB ₂	100	100	100	100	100	100
	GFB ₃	100	100	100	100	100	100
	GFB ₄	100	100	100	100	100	100
III. <u>Pond</u> :	GFB ₁	100	100	100	100	100	100
	GFB ₂	100	100	100	100	100	100
	GFB ₃	100	100	100	100	100	100
	GFB ₄	100	100	100	100	100	100
B. Unsterilized soil from :							
I. <u>Ganga</u> :	GFB ₁	100	100	100	100	50	0
	GFB ₂	100	100	100	100	50	0
	GFB ₃	100	100	100	100	50	0
	GFB ₄	100	100	100	100	50	0
II. <u>Aq. field</u> :	GFB ₁	100	100	75	50	25	0
	GFB ₂	100	100	75	50	25	0
	GFB ₃	100	100	75	50	25	0
	GFB ₄	100	100	75	50	25	0
III. <u>Pond</u> :	GFB ₁	100	100	100	25	0	0
	GFB ₂	100	100	100	25	0	0
	GFB ₃	100	100	100	25	0	0
	GFB ₄	100	100	100	25	0	0

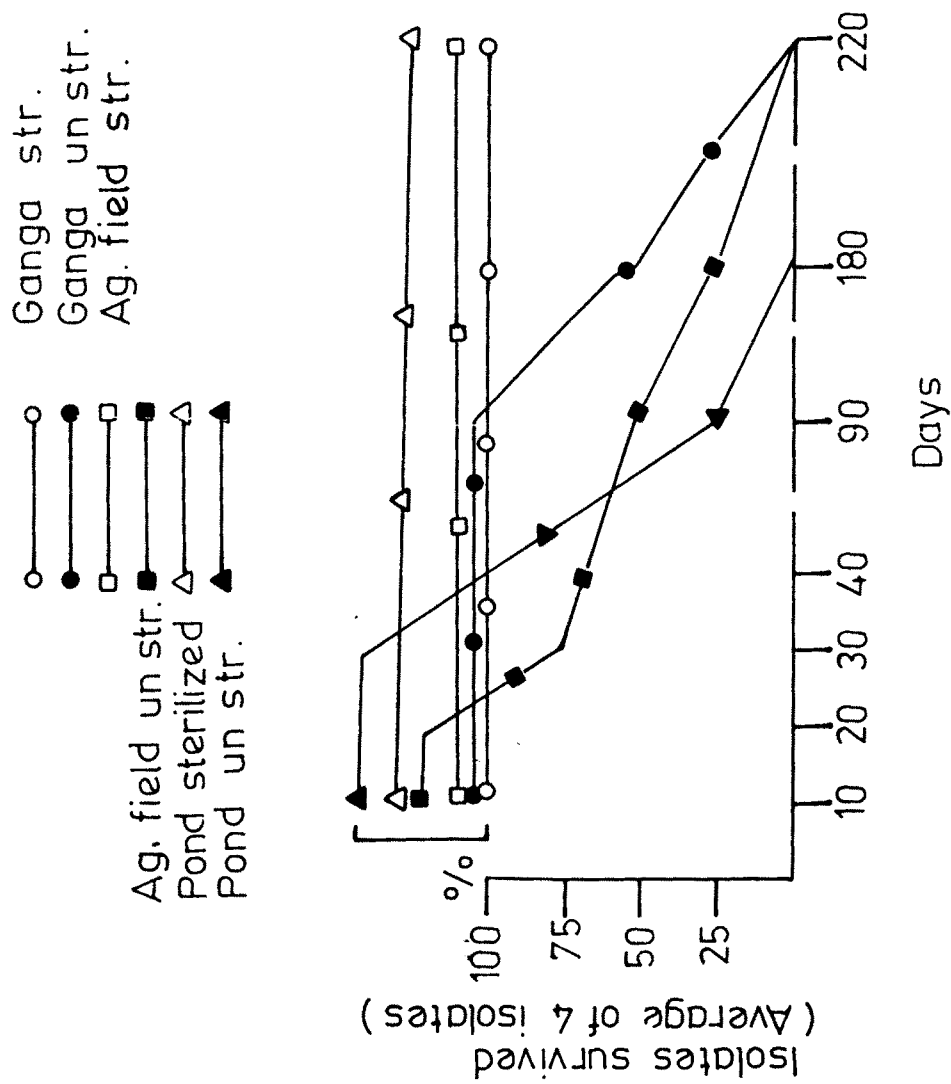


Figure 16. Survival of fluorescent bacteria in soils from different situations.

4.6.2 Survival in different kinds of water :

Green fluorescent bacteria so far isolated, have been common on wet surfaces and water bodies. It was thus considered necessary to examine the survival of a given population of bacteria in definite volumes of water collected from different sources common in the present situation. For such bacteria, production of fluorescent pigment depended on their growing environment. The present study was aimed at exploring the period of survival and extent of pigmentation by such bacteria in different waters

To study the survival ability and pigmentation pattern in waters from different sources 3 types of water namely water from ponds, river Ganga and distilled water were used. Ten ml. of each of these waters were taken in separate large sized (1.2 x 10 cm) culture tubes and all the tubes were plugged with non-absorbent cotton. One set of each were sterilized for two successive days at 121°C for 15 minutes and another set was kept unsterilized. The waters were then inoculated with one loopfull of fresh (24 hr.) culture of the 4 isolates of the bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ in separate tubes under aseptic conditions. The tubes taken in necessary replicates along with uninoculated control were then incubated at room temperature (20 to 30 ± 2°C) in laboratory.

To check the survival of the bacteria in water, a loopfull of water was taken and streaked on KBC-slants under aseptic conditions for detection of the bacteria. The streaked slants were kept at 40 ± 1°C for 24 hours and observations were taken.

Results obtained showed that all the isolates survived in both sterilised and unsterilised waters equally upto 365 days. In fact further checking might have revealed that they could survive in such waters for a still longer period.

The pigmentation pattern by such bacteria was observed, and it has been found that at the beginning, the isolates released very small quantity of pigment so that the water colour was very light greenish. At the middle of the year the water colour was very deep green and it was due to heavy production of pigment primarily of fluorescent type and lastly the water colour became reddish brown to deep brown for production possibly of pyocyanin in addition.

4.7.1 Competitive survival of green fluorescent bacteria in rhizospheres of some vegetable plants :

Rhizospheres of plants are 'spheres' in soil which are under the influence of the root-system of the concerned plants and harbour different microorganisms with a dominance of bacteria among microorganisms. It was considered meaningful to investigate the possibility of the green fluorescent bacteria to find habitat in them.

Competitive survival ability of green fluorescent bacteria in the rhizosphere region of three vegetable plants namely cabbage (Brassica oleracea), Tomato (Lycopersicum esculentum) and Beet (Beta vulgaris) were studied under experimental conditions. One month age of the plants of concerned vars. namely Pusa Early Drum Head, Pusa Ruby and Grimson Globe respectively, were collected from local Kalyani market. The seedlings were planted and the plants were

grown in earthen pots (9" diameter) following method described earlier. At the age of 3 months when the plants showed sufficient vegetative growth the actual experiment was conducted.

From the 24-hour old cultures, of the 4 bacterial isolates namely GFB₁, GFB₂, GFB₃ and GFB₄, suspensions were prepared with sterile distilled water to dilution of 3×10^7 cells/ml following McFarland's Scale and the method described earlier. Twenty ml. of each of the suspensions of the bacterial isolates were taken and poured down at the base of the plants in the pots. After application of the bacterial suspension, soil surface around the growing plants were carefully covered with black polythene sheets for 24 hours to prevent drying of the soil immediately after drenching with bacterial suspension. This also helped to prevent immediate surface soil contamination of any kind. The pots were kept on laboratory benches under normal conditions of growth and management. Proper irrigation was given time to time to keep the soil moist upto *joe*-condition. Replicates in necessary numbers and uninoculated control were maintained. Result were recorded at 10, 15 and 30 days intervals by plating rhizosphere soils of plants pulled up for the purpose following standard procedure described earlier. The plating etc. were done in KBC -plates following procedure described in methods. Absence or presence of the green fluorescent bacteria in number of samples checked have been counted and presented in percentage of successful isolation of them in Table 25. The data in the table showed that the bacteria being introduced by drenching were present in all the isolations in all the rhizospheres. As established through

Table 25 : Extent of competitive survival of green fluorescent bacteria in rhizosphere of plants.

Isolates	Survival ability after days in rhizospheres of								
	Cabbage			Tomato			Beet		
	10	15	30	10	15	30	10	15	30
GFB ₁	100	100	100	100	100	100	100	100	100
GFB ₂	100	100	100	100	100	100	100	100	100
GFB ₃	100	100	100	100	100	100	100	100	100
GFB ₄	100	100	100	100	100	100	100	100	100

this experiment 100 % survival of the bacteria is of great consequence.

Results clearly demonstrated that all isolates were able to survive in the rhizosphere region of the different plants upto 30 days when applied from outside as suspension and by soil-drench.

4.7.2 Competitive survival of green fluorescent bacteria on growing vegetable plant surfaces :

Based upon the informations gathered from the previous studies on survey of green fluorescent bacteria it has been considered important to examine the prospect of these bacteria to survive on plant foliage surfaces competing with other epiphytic microorganisms when applied on such surfaces artificially from pure culture of such bacteria. An experiment was thus planned to assess the competitive

survival ability of four isolates of green fluorescent bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄ as epiphytes on three vegetable plants namely cabbage (Brassica oleracea) Tomato (Lycopersicum esculentum) and Beet (Beta vulgaris) considering that they are commonly grown on the adjoining agricultural fields around river Ganga.

One month old seedlings of local varieties of the test plants were collected from Kalyani market. The seedlings were grown in earthen pots (9" diameter) upto 3 months' age to gain a good growth. The plants were maintained under standard growing conditions. Suspension of the bacterial cells of 24-hr old cultures of the four isolates were prepared in sterile distilled water and the suspension was diluted to 3×10^7 cells/ml following procedure described earlier.

After proper sterilization of a throat sprayer 10 ml of each of the bacteria were taken and sprayed over the plant surfaces. Necessary replicates and unsprayed control were maintained. The treated and control plants were kept under laboratory conditions of temperature (16 to $30 \pm 2^\circ\text{C}$). Data were recorded at 10, 15 and 30 days intervals in terms of number of green fluorescent bacteria present on such surfaces. Usually single leaf samples were collected, washed in sterile distilled water and plated in KBC-plates following dilution plating technique. The plates were incubated at $40 \pm 1^\circ\text{C}$ for 48 hrs. The percentage of green fluorescent colonies to total colonies were estimated and the data are presented in Table 26 and Fig. 17.

Table 26 : Competitive survival as epiphytes on growing crop plants.

Isolates	Crop	% survival on plants after days			
		10	15	30	45
GFB ₁	Cabbage	25	50	75	100
	Beet	25	50	-	-
	Tomato	25	50	-	-
GFB ₂	Cabbage	25	50	75	100
	Beet	25	-	-	-
	Tomato	25	50	-	-
GFB ₃	Cabbage	25	50	75	100
	Beet	25	50	-	-
	Tomato	25	50	-	-
GFB ₄	Cabbage	25	50	75	100
	Beet	25	-	-	-
	Tomato	25	50	-	-

(-) not survived

Results presented in the table showed that on cabbage leaf surfaces all the isolates were present upto 45 days in a dominant way giving 100 % green fluorescent colonies on isolation. On tomato leaves isolates survived upto 15 days in a 50 % dominance stage while on beet they survived upto 10 days only at a 25 % dominance. However,

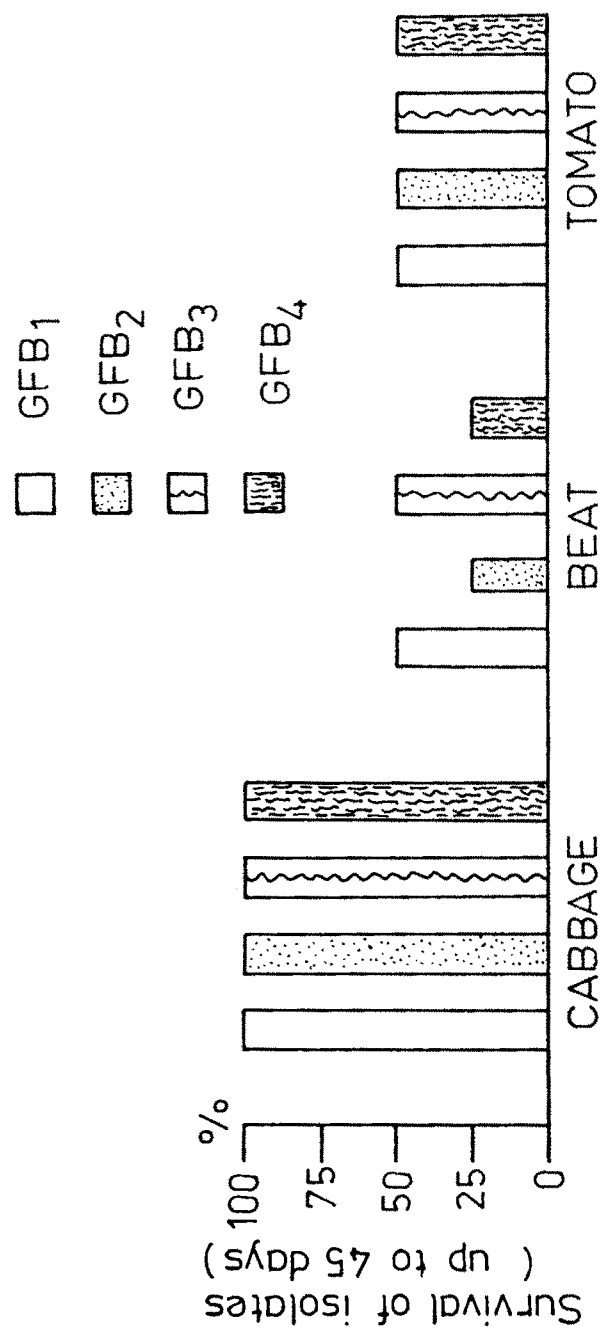


Figure 17. Competitive survival as epiphytes on growing crop plants.

isolates GFB₁ and GFB₃ survived upto 15 days with a 50 % dominance. The overall epiphytotic habitat i.e. the cabbage foliage seemed to be most congenial for these bacteria.

4.7.3 Competitive survival as epiphytes on leaf surface of some aquatic and terrestrial plants :

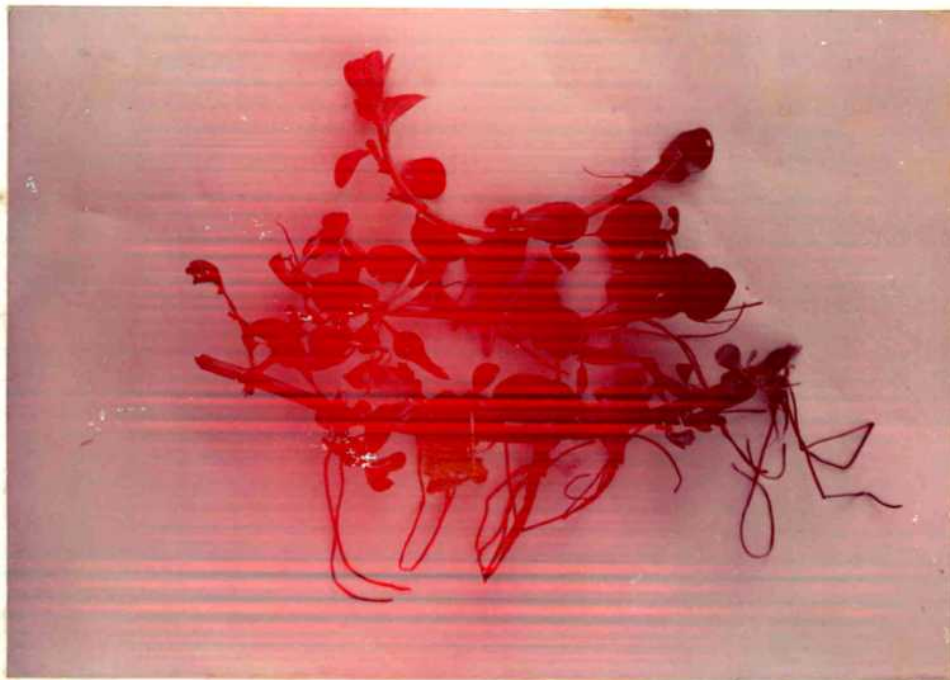
Considering the fact that the green fluorescent bacteria being studied in this programme are fond of moist surfaces and commonly occur in different water bodies it was decided to examine their competitive survival ability as epiphytes on surfaces of aquatic plants.

Hence epiphytic survival of the four selected isolates of the green fluorescent bacteria were studied on three aquatic plants namely Ludwigia adscendens (Onagraceae) Hygrophiza aristata (Gramineae) and Eichhornia crassipes (Pontedariaceae). In addition for comparison and having a non-aquatic standard terrestrial plant Imperata cylindrica (Gramineae) was also included in the study. Another reason for selection of these test plants was that they were common in Kalyani and areas surrounding the Ganga river in ponds, lakes and land areas. The collected plants were grown in pot culture. The aquatic plants were grown in polythene vats of 1'6" diameter and the terrestrial was grown in earthen pots (6" diameter) all upto 15 days from their planting following procedures described. Then suspensions of the four isolates of the bacterium were prepared in sterile water from 24-hour old cultures of the four isolates namely GFB₁, GFB₂, GFB₃ and GFB₄. The dilutions maintained were was 3×10^7 cells/ml in all the cases.

Experimental weeds :



Fig.18 a) Imperata cylindrica



b) Ludwigia adscendens



c) Hydrocharitaceae aristata



d) Eichhornia crassipes

Table 27: Competitive survival as epiphytes on growing plants.

Isolates	Plants	% survival on plant after days			
		10	15	30	45
GFB ₁	<u>L.od.</u>	25	50	75	100
	<u>Hy.ar.</u>	25	50	75	100
	<u>Ei.cra.</u>	25	50	75	100
	<u>Im.cy.</u>	25	-	-	-
GFB ₂	<u>L.od.</u>	25	50	-	-
	<u>Hy.ar.</u>	25	50	75	100
	<u>Ei.cra.</u>	25	50	75	100
	<u>Im.cy.</u>	25	-	-	-
GFB ₃	<u>L.od.</u>	25	50	75	100
	<u>Hy.ar.</u>	25	50	75	100
	<u>Ei.cra.</u>	25	50	75	100
	<u>Im.cy.</u>	25	-	-	-
GFB ₄	<u>L.od.</u>	25	50	75	100
	<u>Hy.ar.</u>	25	50	75	100
	<u>Ei.cra.</u>	25	50	75	100
	<u>Im.cy.</u>	25	-	-	-

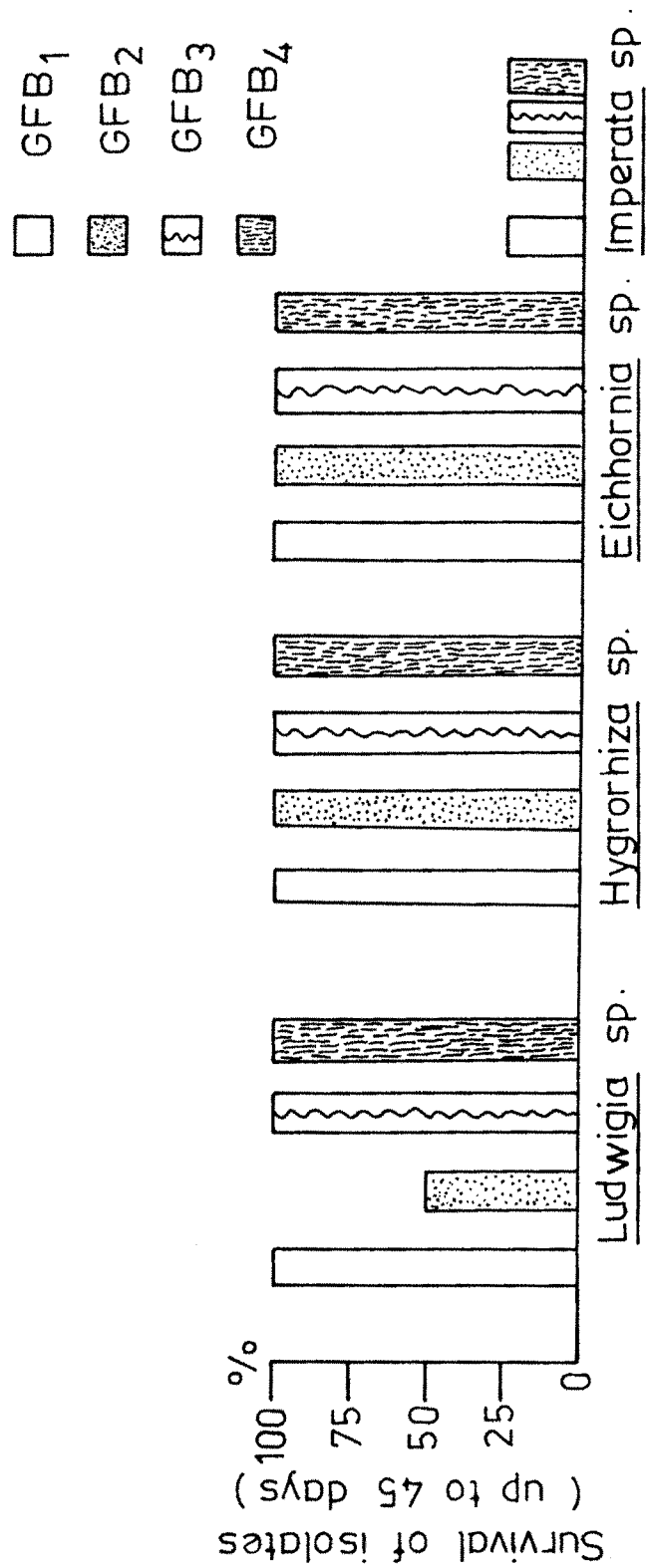


Figure 19. Competitive survival as epiphytes on growing weeds.

Then 10 ml of each of the bacterial suspensions were sprayed on the leaves of the potted plants except those kept as control. The treated and control plants were kept under laboratory conditions. Before spraying the thread sprayer (Holmsprayer) was sterilized suitably in an autoclave. Observations were made at 10, 15, 30 and 40 days after spraying. Small pieces of leaves were cut with sterilized scissors and washed in 1 ml of sterilized distilled water and plated in KBC-plates. The plates were incubated at $42^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 24-48 hours. Results obtained in terms of green fluorescent bacteria as percentage of total bacteria were presented in Table 27, Fig.19 and photographs in Fig.18.

Results showed that all the isolates established successfully on the surfaces of H. aristata, Ei. crassipes and were present upto 45 days at a 100 % dominance level. In case of L. adscendens, except GFB₂ all isolates survived upto 45 days at a 100 % dominance level where as isolate GFB₂ survived upto 15 days at 50 % dominance level. In Im. cylindrica the terrestrial, all isolates survived upto 10th day at 25 % dominance level proving it to be a poorer habitat.

4.8.1 Antibacterial activity of some chemicals toward green fluorescent bacteria :

Green fluorescent bacteria have been found to occur around the river Ganga widely on diverse substrates and surfaces. After having been demonstrated to be opportunistic pathogens of both plants and animals and particularly of mammals it was considered necessary to explore the prospect of control of population of the bacteria in the

environment. Actually the objective has been to find out specific technique to bring down population of this bacterium. A preliminary screening of some chemicals against green fluorescent bacteria have been tested by fish-spine method standardised and presented early. The chemicals have been divided into four groups namely (i) inorganic compounds (ii) organic compounds (iii) antiseptics and (iv) plant products. Under inorganic compounds the chemicals were Hydrochloric acid; Sodium hydroxide, Sodium chloride, Borric acid, Potassium sulphate, Zinc sulphate and Lime. The organic compounds were formaldehyde and phenol while the antiseptics commonly used and available in market as Dettol, Phenyl, and Bleaching powder were included in the study. Four detergents included were Triton, Teepol, Tween-80 and Sunlight soap. All the chemicals were tested on 24-hour culture of four isolates of green fluorescent bacteria namely GFB₁, GFB₂, GFB₃ and GFB₄. By using sterile distilled water the 2 and 5 % solutions of the chemicals were prepared in volumetric flasks. One ml. of the 10^{-1} dilution of the bacterial suspension was taken in sterile petriplate and then malted and cooled KBC-medium was poured in each plate @ 25 ml/plate. After the solidification of the plates, containing KBC, the sterile fish-spines were then dipped in chemical suspension with the help of a sterile forceps under aseptic conditions and then placed on the plate after draining the excess. Three fish-spines were placed on each plate in a triangle. For each of the percentages of suspension and in cases of each of the isolates plating was done following the same procedure. After proper placement of the fish spines containing chemical solutions on the plate, the plates were

Table 28: Some aspects of antibacterial activity of green fluorescent bacteria of some selected inhibitors or chemicals by fish spine method

(average of 6-8 replicates)

Materials	Diameter of zone of inhibition of different percentage in m.m.							
	GFB ₁		GFB ₂		GFB ₃		GFB ₄	
	2	5	2	5	2	5	2	5
A. Inorganic compounds								
1) Hydrochloric acid	10	20	8	21	10	22	12	20
2) Sodium Hydroxide	13	35	16	28	13	25	14	17
3) Sodium chloride	-	-	-	-	-	-	-	-
4) Boric acid	-	-	8	10	10	20	12	12
5) Potassium sulphate	-	-	-	-	-	-	-	-
6) Zinc sulphate	10	12	8	12	12	16	10	15
7) Lime	-	-	-	-	-	-	-	-
B. Organic compound								
8) Formaldehyde	18	27	23	27	15	35	18	21
9) Phenol	40	45	35	33	35	38	30	36
C. Antiseptic Products								
10) Dettol	8	15	-	-	-	-	8	8
11) Phenyl	-	15	-	-	-	-	8	10
12) Bleaching Powder	-	10	-	13	-	-	-	10

(-) No inhibition zone formed.

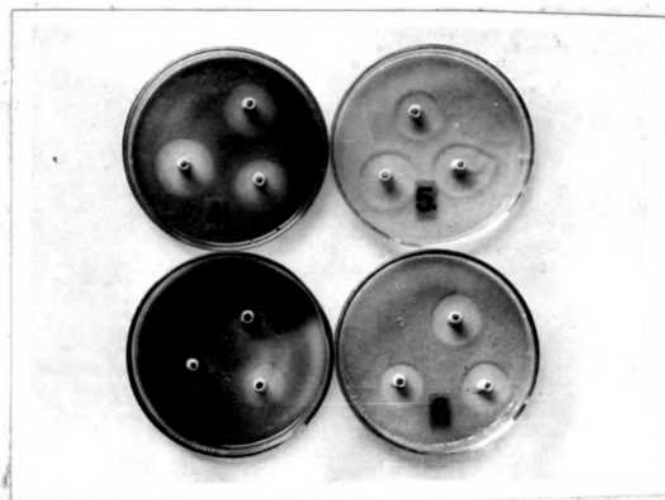
Fig.20 Antibacterial activity of some selected inhibitors at 5 %.



1. HCl

3. Zinc Sulphate

2. NaOH



4. Formaldehyde

5. Phenol

6. Dettol

7. Phenyl

kept in $40^{\circ} \pm 1^{\circ}\text{C}$ for 24-hours. In all the cases necessary replicates and control sets were maintained. Results were taken by measuring the inhibition zone in millimeter. Results are presented in Table 28. The table showed that among the inorganic compounds sodium and Hydrochloric acid were inhibitory to all the isolates of the bacteria at 2-5 % concentration. Zinc sulfate also showed some amount of inhibition. Boric acid however was inhibitory to three of the four isolates of the bacteria. In case of organic compounds both formaldehyde and phenol were good inhibitors to all isolates and it has been found that 5% was more effective than 2%. It was also clear that phenol was much more effective than formaldehyde. Inhibition showed in fig.20.

In case of antiseptics available in the market, none of the three were inhibitory to all the isolates of the bacteria. All the three were inhibitors of two isolates namely GFB₁ and GFB₄ at 5 % concentration. To GFB₄ both Dettol and phenyl were inhibitors at 2% concentration also. None of the detergents were inhibitory towards the bacterial isolates at 2 and 5% concentration. Phenyl and Bleaching powders were thus inhibitory to GFB₁ and GFB₄ but they showed no effects on GFB₂ and GFB₃.

4.8.2 Antibacterial activity of some selected antibiotics toward green fluorescent bacteria :

A large number of antibiotics are known to have good inhibitory effects on green fluorescent bacteria. In order to determine the sensitivity pattern of the present isolates of green fluorescent bacteria

some selected antibiotics namely Neomycin, Gentamycin, Chloromphenicol, streptomycin, penicillin, Bacterionol, Norfloxin, Ledermycin and Ciprafloracin (Cifran) were taken to determine their inhibitory properties toward the isolates of the bacteria. The first five antibiotics were tested by paper-discs. method. The discs were obtained from span.Diagnostics, Surat, India. The strength of the antibiotic discs. were 30 mcg, 10 mcg, 30 mcg, 10 mcg and 10 u respectively. Remaining four antibiotics were tested by fish-spine method described earlier. These antibiotics were obtained from standard Pharmaceutical shops at Kalyani and solutions of them were prepared by mixing them in requisite quantities in sterile distilled water, keeping concentration at 500, 300 and 100 ppm for all these antibiotics. In both the cases of paper-discs and fish-spine methods four isolates of the bacterium namely GFB₁, GFB₂, GFB₃ and GFB₄ were taken for the test. In the first case one ml of bacterial suspension of 10⁻¹ dilution were taken and poured in the sterile petriplates under aseptic conditions followed by addition of melted and cooled, sterile KBC-medium @ 25 ml/plate. After proper solidification of the plates 3 discs were placed on the surface of each plate in a triangular design and the plates kept at 40 ± 1°C for 24 hours. Same procedure and same methods were followed in cases of all the isolates.

In case of fish-spine method sterilised fish-spines were dipped in solutions of antibiotics under aseptic conditions, the excess of solution drained and the fish-spines carrying the solutions were placed on the KBC-plates seeded with green fluorescent bacteria in the same triangular design. The test bacteria or isolates included

were GFB₁, GFB₂, GFB₃ and GFB₄. Necessary replicates and control plates were maintained. The plates were incubated at $40 \pm 1^\circ\text{C}$ in incubation. Results on zone of inhibition were taken after 24 hrs. Results obtained have been presented in Table 30.

Table 29 : Sensitivity of green fluorescent bacteria to different selected antibiotics in vitro by paper-disc method

(Average of 6-8 replicates)

Antibiotics	Diameter of inhibition zone in m.m.				
	Strength	GFB ₁	GFB ₂	GFB ₃	GFB ₄
1) Neomycin	30 mcg	15	14	12	17
2) Gentamycin	10 mcg	23	22	17	19
3) Chloramphenicol	30 mcg	-	-	-	-
4) Streptomycin	10 mcg	-	-	-	-
5) Penicillin	10 u	-	-	-	-

(-) = no inhibition zone formed.

Table data showed that in cases of antibiotics in paper-discs Gentamycin was more effective inhibitor than neomycin. But no inhibitory effects were found with chloramphenicol, streptomycin and penicillin against any of the isolates (Table 29 and fig.21).

In case of antibiotics applied with fish-spines ciprofloxacin (Cifran) was more effective inhibitor than others and more or less similar inhibitory effect was observed in all the three concentrations tried. Bacterionol and norfloxacin were also inhibitory but the effects

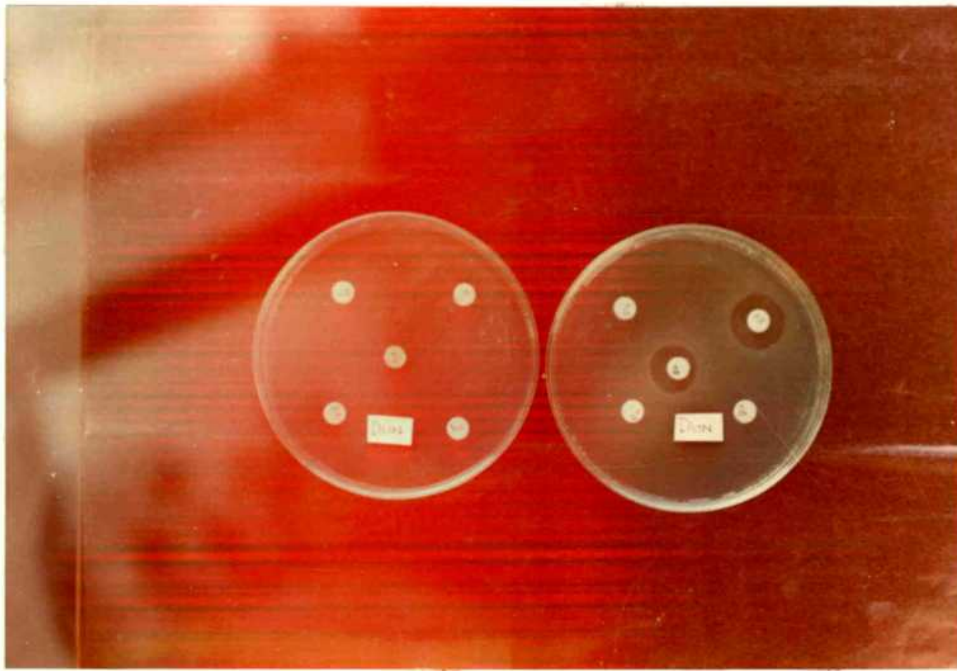


Fig.21 Antibiotic - Assay.

were not as good as ciprofloxacin (Cifran). The diameter of the inhibition zone also varied with isolates in cases of all the antibiotics. Ledermycin was not at all inhibitory.

Table 30: Sensitivity of green fluorescent bacteria to different selected antibiotics in vitro by fish-spine method on different ppm.

(Average of 6-8 replicates)

Antibiotics	Diameter of inhibition zone in m.m.											
	GFB ₁			GFB ₂			GFB ₃			GFB ₄		
	500	300	100	500	300	100	500	300	100	500	300	100
1)Bacterionol	10	8	7	35	30	32	12	12	8	10	7	6
2)Norfloxin	20	15	10	22	16	10	25	20	-	20	12	8
3)Ledermycin	-	-	-	-	-	-	-	-	-	-	-	-
4)Cifran (Ciprofloxacin)	35	32	32	35	30	32	35	30	30	35	32	32

(-) = no inhibition zone formed

4.8.3 Search for inhibitors of green fluorescent bacteria amongs natural resources :

Subsequent to the confirmation of the positive opportunistic pathogenicity of the green fluorescent bacteria and their common occurrence around the river Ganga in its lower stretch which is thickly populated. It was felt necessary to explore the possibility of having some easily available agents for utilization for their control. Besides other chemicals tried so far crude extracts of botanical materials were tested on the one hand. Some plants wastes like straw have also been tested for engaging some other bacteria commonly harboured by

them to check the fluorescent bacteria. Some commonly occurring plant pathogenic fungi have also been tested for their antagonistic effects on the green fluorescent bacteria.

The tests were conducted against 4 selected isolates like GFB₁, GFB₂, GFB₃ and GFB₄.

(a) Plant extracts used were 5% methanol extracts of Phaseolus calcaratus, Tephrosia candida and Coriaria sp., 5% petroleum ether extracts of Rungia sp. & T.candida, 5 % chloroform extracts of Rice Bean and 5 % water extract of Azadirachta indica. The assay was done following fish-spine method described earlier on KBC-plates. Necessary replicates and control sets were maintained.

(b) Dry straws of rice are common habitat of some spore-forming bacteria like Bacillus sp. It is also an easily available crop waste around this area. It was decided to examine whether such wastes may be used as source of some bacilli which in their turn may cause biological inhibition of the green fluorescent bacteria at least by competition. An experiment was undertaken in which small pieces of freshly collected straw were incorporated (1 cm x 0.25 cm piece/5ml) 24 hr. broth culture of green fluorescent bacteria and the tubes were re-incubated at $40 \pm 1^{\circ}\text{C}$ for 48 hr. The growth of green fluorescent or any other bacteria in the reaction-broth was examined thereafter by streaking on KBC-slants.

(c) Some fungi commonly occurring in similar situations in the locality were also tested for their inhibitory property, if any, against

the green fluorescent bacteria. The fungi included were Penicillium sp., Trichoderma viridii, Alternaria sp., Rhizopus sp. and Helminthosporium oryzae, Sclerotium rolfsii, Fusarium oxysporum, Macrophomina phaseolina, and Sclerotium oryzae.

Four detergents namely Tween 80, Teepol, Triton X and Sunlight soap were tested for their inhibitory properties. The inhibition was tested by fish-spine method, described earlier.

The inhibition was tested by cross-streaking method on PDA - plates following method described earlier. The plates inoculated with the fungi and cross-streaked with the four isolates of the bacteria were incubated at $30 \pm 1^{\circ}\text{C}$ for 5 days. Necessary replicates and control plates for both the fungi and bacteria were maintained. Observations on extent of inhibition if any, was made from after 5 days.

Results obtained in all these four experiments (a), (b), (c) and (d) are negative. None of the plant extracts were inhibitory to the isolates of the bacterium. With regard to the attempt for inducing a biological competition or antagonism with straw again there was no positive response when the green fluorescent bacteria was already for allowed a growth of 24 hrs. Thirdly none of the six fungi tested for their any antagonism toward the green fluorescent bacteria were inhibitory to the bacteria. Lastly, none of the detergents were inhibitory to the isolated bacterium.

B. 4.9 Discussion of the results :

(a) Green fluorescent bacteria so far studied in these programme of the research work has been found to be wide spread in its occurrence. In the present study the purified isolates of the bacterium has been identified to be Pseudomonas aeruginosa. Although it is known that this bacterium has the capability to establish in both plant and animal tissues only when the host defences are weakened. Their presence in large numbers around human habitats seemed to be alarming. Considering that the isolates of the bacterium have been obtained from the liquid wastes and discharges of different types of industries, urban-agglomeration of different sizes, villages and agricultural fields on the one hand and surfaces of fruits, vegetables and fishes in the market, litters, in the fruit orchards, forests and ornamental gardens and debries, stubbles and dead roots of crop fields. Their some sort of universal presence thus can not be denied. Actually result from this section raises a question in one's mind has to wheather such bacteria are natural under some such situations. Several publications in these regards as presented in the review in the section 2.1 - 2.5 has clearly indicated that these reports are from recent works published during sixties, seventies and eighties of this century. Evidently even though such bacteria were occurring in natural environments they went unnoticed and uninvestigated. Since different strains of Ps.aeruginosa have been found to cause infection in animals in general and human-being in particular, their large-scale occurrence could not be ignored.

(b) The isolates of Ps.aeruginosa obtained from the extensive survey work, were studied following standard bacteriological methods and identified. There have not been much of difference with regard to characteristics of these bacteria. With those of the animal pathogenic strains of Ps.aeruginosa studied and reported by The Manual, 1974 and bacteriologists like M.P.Starr . Serological studies also established their similarity in natural relationship. Several authors have earlier claimed that strains of Ps.aeruginosa may be plant pathogenic. Stinking rot of sugarcane due to this bacterium was reported by Desai (1935), from lettuce by Elrod and Braun (1942). The present work not only established pathogenicity of this bacterium toward cabbage plants but also indicated a sort of pathogenic specificity in it by establishing its failour to cause infection in some other plants tested. In recent year also further reports of their plant pathogenicity have accumulated (Stanghellini 1972, Starr and Chatterjee 1972, Knoesei 1978). This capability of isolates of Ps.aeruginosa along with their ability to occur as saprophytes on plant surfaces or surfaces of marketed vegetables and fruits have established that this bacterium has a strong and stable attachment with plants and their produces. The bacterium as reported in earlier works, also showed good tissue-rotting capability on different plants tissues. However, their nature of pathogenicity when introduced by cabbage by root-dipping was not very clear excepting that they invaded the plant upto its foliage.

Animal pathogenicity of the isolates of Ps.aeruginosa have also been established in mouse-model. These also have confirmed several earlier claims as provided in the review section 2.6. The review actually claimed these bacteria infecting new-born human babies (Krynski and Beda, 1980), burnt mouse (Stover et al.1983), sheep. (Burrel and Mecdimed, 1984), and fishes (Binta et al., 1982). Thus the results on animal pathogenicity from the present study and supported by earlier reports mentioned has actually made the situation alarming.

Further studies revealed a kind of variation in virulence of their selects in terms of speed of infection and killing of the mouse.

(c) Some preliminary attempts have been made to understand the dependence of these bacteria on different factors in the environment. Atmospheric temperature was considered to be of importance since most of the earlier reports of such bacteria came from temperature areas whereas the optimum temperature requirement for such bacteria was never below 40°C. The present isolates also recorded better growth at a temperature namely 40°C and a definite decline of them was evident at lower temperatures. This fact indicated towards one possibility that the reason for a low rate of reports of plant and animal infection by these bacteria in most of the areas of the world is that the atmospheric temperature is not generally favourable for its activity. These also can explain of the fact that such bacteria are opportunistic and can cause infection of a host under stress. The pH requirement of the growing environment being near neutral, may be very well

satisfied by its prospective host in plants and animals. However, the elaborate survey work under taken in different countries (Schroth et al., 1977) has not so far endeavoured to cover these aspects of the subject. With regard to the tolerance of the desiccation of the bacterium no information was available. A very small experiment conducted under the present programme could indicate the extent of period upto which it could withstand desiccation. The area under study has continuous rains for several months in addition to snap showers round the year. The natural environment of these has large number of water-bodies, chanel, river and rivulets. As such the environment is likely to be favourable for a bacterial species liking moist surfaces even when these areas experience continuous desiccation and dehydration for not less than a period of one to two months. Hence the results clearly revealed the capability of the bacterium in these regards.

(d) Number of experiments have been conducted to examine the growth and survival of the bacterium on different kinds of organic substrates including farm-yard manure, oil-cakes, leaf-molds, different types of water, hairs, feathers, egg shells, animal dungs or droppings, soil, plant rhizosphere and surfaces of vegetable plants and weeds. Generally, the bacterium could survive satisfactorily on most of such surfaces but its growth on them was either unsatisfactory or poor. The findings are very important from the points of views of understanding and identifying the active sources of these bacterium in nature. The studies are first of their kinds also.

While the failure of the bacterium to grow on such surfaces could explain their failure to dominate as pathogen of plants and animals in the geographical situation, their success to survive has been proved to be because of their universal present. This successful growth on different kinds of water from pond and river once again proved their closer relation with water bodies.

(e) Only some initial experimentations have been made to find out an idea or ideas that may help to formulate the experiment for their control as expected. Only a few disinfectants in the class of acids, alkalies, aldehydes and phenolics were identified to be good inhibitors. The inhibitory properties of antibiotics like gentamicin and neomycin as known earlier has been confirmed. A new antibiotics ciprofloxacin has been a good inhibitor. With regard to the object of suppressing the level of contamination of human surroundings with these bacteria only use of some disinfectants, not antibiotics may be considered. However, further detail studies will be required before reaching any conclusion. The search for inhibitors of Ps.aeruginosa among natural inhibitors like extracts of plant, which act as sources of other competitive bacteria like straw and some other micro-organisms has not made with much success. Possibly studies with still broader base may be useful.

(f) Pseudomonas aeruginosa the green fluorescent bacteria occurring on different substrates around the river Ganga has thus been found to have definite pathogenic properties. The study has revealed a very close association of the bacterium with plant surfaces and tissues.

Its ability to cause of rotting of plant tissues under different inoculation conditions has also been established. The work has exposed the ecological conditions that exist and also some preliminary information on epidemiology of plant pathogenic bacterium.

Chapter V

SUMMARY AND CONCLUSION

A. Summary of the Work :

5.0 In recent years some species of green fluorescent bacteria have been found to be important pollutants in water bodies, moist surfaces and on man-made structures in both urban and rural areas. Considering the importance of some species of such bacteria causing opportunistic infection in both plants and animals including human-being, intensive surveys have been conducted elsewhere (Schroth et al , 1977). However, no such survey has been conducted in the country.

Hence, a well-planned survey was taken up in and around the lower Ganga river with the object of assessment of the extent of such bacteria in nature and also understanding of the ecological and epidemiological specification under which they are active.

5.1 Survey of the extent of green fluorescent Bacteria around the lower Ganga :

The survey work conducted over atleast two years on both the eastern and western bank of the river over a stretch of about 2500 km. Spread over three districts was taken up in 1984. For isolation of the target bacteria eight sample-sites in agricultural, industrial and urban areas were selected from each of which three kinds of samples namely, water, soil and plant parts were collected. The isolation was made in a selective medium namely King's medium-B enriched with cetrimide, by dilution plating. A significant number

of green fluorescent bacteria as for 2.82 - 10.85 % of total bacteria could be detected in monsoon and pre-monsoon samplings.

The soil samples contained 10.85 - 6.39 %, water 9.43 - 5.16% and plant surface 7.51 - 2.61 % of green fluorescent bacteria. The extent was 10.21 - 4.07 % in case of industries, 9.79 - 3.76 in case of town, and 5.57 - 9.37 % in case of villages. Evidently, the extent of such bacteria which might include the opportunistic pathogens was definitely a matter of concern for the area.

When the water of the river Ganga itself was examined after collection of samples from near the selected samples sites there was again a significant percentage of green fluorescent bacteria present in such water. The higher salt concentration of such water could not influence much the extent of such bacteria.

When examined the adjoining vegetable crop fields also recorded respectable population on surfaces of such crop plants. Such produces including some fruits, when in market also carried these bacteria. Infact leafy vegetables, root-vegetables, pods like Dolichos or fruits like orange, coming from hills, grapes from far-away places and water-chestnut produced in local ponds, all showed evidence of presence of such bacteria on their surfaces in atleast three local markets. Fishes leaving in different depths in a water-body were also examined. However, a relatively lower percentage of such bacteria could be detected on these surfaces.

Considering the wide occurrence so far revealed of such bacteria it was thought to examine other substrates like plant-litters, wood-debries, dead roots and stubbles occurring under different situations in nature and constituting one of the major organic substrates present in the surroundings. The litters from fruit-orchards, forests, and ornamental-gardens showed presence of green fluorescent bacteria on them at a quite high rate. Whether they formed the natural reserve of such bacteria in the environment needs exploration. Similar high percentage of presence of such bacteria was evident when isolations were made from woody debries of Shorea, dead-roots of Chorchorus and stubbles of Rice. The total survey clearly revealed the presence of green fluorescent bacteria in different extent under different situations.

5.1.2 Identification and determination of green fluorescent bacteria isolated :

Determinative characteristics of such bacteria and methods of study have been discussed in Grogan 1969, Schroth et al., 1977 and The Manual, 1974. Different cell-morphological, colony morphological and physiological and biochemical characteristics have been studied following standard bacteriological procedure.

Characteristics as revealed from the study on eight bacterial isolates of green fluorescent bacteria clearly established that these bacteria were rodshaped with the size range of $0.3-.8 \times 1.5-2.5 \mu\text{m}$. All the bacteria were found to be motile being flagelated. The isolates were nonspore former and non-capsulated. They were all gram negative in reaction. They formed generally white, slimy colonies with

production of greenish fluorescent water soluble pigments. Some isolates however produced yellowish to yellowish green pigments in KBC-medium at 40-42°C temperature within 24 hrs. Many of the isolates produced a brownish-black pigment in addition after 6-7 days of growth. While the 1st pigment might be fluorescein in the 2nd one was pyocyanin. Studying the characteristics of the isolates and comparing them with those in The Manual the isolates were identified to be strains of Pseudomonas aeruginosa. The identity was later confirmed from CMI, England.

Serological technique was used to determine the natural relationship of the different isolates of the bacterium. Using white-rabbits antisera was produced with three of the ten, isolates. The antisera was prepared and allowed to react with the antigen, prepared from 10 isolates, on glass-slides in agar-gel system. The reaction between three antisera and 10 antigens demonstrated that the isolates have mostly a close relationship excepting the isolate GFB₆. It appeared that serologically a large number of isolates were similar.

5.1.3 Pathogenicity of the green fluorescent bacteria :

Pathogenicity of the 4 selected green fluorescent bacterial isolates were tested against both plant and animal hosts following standard procedure.

(a) With regard to their plant pathogenicity, the isolates were successful causing infection and symptom-production even on uninjured host-plants in case of cabbage. However, it could infect some other

plant hosts like tomato and beet only when there was injury. Its success in infecting cabbage was of great significance because it was possible even at a lower inoculum load. The bacterium as it appeared could produce blotches on this particular host. Although these isolates might be weak opportunistic plant pathogens for number of hosts like tomato and beet it seemed to have acquired pathogenicity toward cabbage. When tried against some other cruciferous hosts like raddish, mustard and cauliflower it was however found that other hosts were not equally susceptible-cauliflower however showed a greater susceptibility.

When the faculty of such bacteria to cause infection in vascular tissue of host plant was examined by conducting root-dipping inoculation, in plants like tomato, beet and cabbage, a successful invasion of the vascular tissue of cabbage alone with all the four isolates of the bacterium could be a possibility. The withered leaves of the invaded plant when examined, revealed presence of the invading bacterium. The failure of the bacteria to parasites tomato or beet indicated a level of specificity also in the isolates with regard to their pathogenicity. The Plant pathogenicity of some of the isolates of green fluorescent bacteria toward cabbage was thus established.

With regard to the investigation on the tissue-retting capability of such bacteria tested on five different plant materials like, potato-tuber, bean-seeds, cucurbit-leaves, onion-bulbs and jute-stems, it was evident that a relatively fast rotting of tissues like potato

and jute bark was possible with the isolates. While no rotting of bean seeds could be caused, a slow rotting of cucurbit leaves and onion bulbs was evident.

(b) Some species of green fluorescent bacteria are known to cause infection in animal under stress. Wheather some of the isolates of the present bacteria had the property of animal pathogenicity was tested on a mammal test animal namely white-mouse. Swiss variety of white mice were inoculated by interperitonal injection at inoculum density of 3×10^3 per inoculum. The animals when injected by 0.5 ml of inoculum died within 72 hours, producing characteristics symptoms. Inoculation with all the isolates resulted in the test animals refusal of food, excessive thirst, and slow or no movement. There were other symptoms like erect hair, fall of hairs, and appearance of red patches on the tail and lower abdomen. The killed animal secreted green juicy fluid from the patches and sores. Establishment of animal pathogenicity particularly against a mammal host in some green fluorescent bacteria is of great significance. While investigating the time required to kill the mice with different isolates it was revealed that isolates GFB₃ and GFB₄ were more virulent killing the animals by 48 hours, while GFB₁ was less virulent requiring at least 72 hrs. The differences in the virulence of the isolates of Ps.aeruginosa was worth of record.

5.4 Growth of green fluorescent bacteria in different physical environments :

In order to understand the liking and requirement of such bacteria with regard to some parameters of physical environment like

atmospheric temperature, pH of the growing environment and dessication number of experiments were conducted.

(a) Under laboratory conditions growth of the bacteria was based around 40°C while marginal increase from 40 were recorded a lower decline of growth rate compaired to the decline in atemperature on the lower side. With regard to the production of green fluorescin pigment again the temperature around 40 was the most favourable. These clearly indicated that both in terms of increasing their population and intensifying their physiological activities the isolates of the bacteria were in favourable position at temperatures around 40°C.

(b) The isolates of the bacteria recorded significantly better growth at pH 7.0 - 7.5. Both increase and decrease of the pH of the growing environment resulted in decline of growth of the bacteria. Evidently the bacteria were favoured generally at near neutral pH.

(c) As regards the tolerance to desiccation of these isolates of the bacteria they were able to withstand desiccation upto a period of 40-60 days under different temperature conditions. Generally in winter the bacteria were more tolerant to higher desiccation and in summer they were less tolerant. Although the behaviour remained to be further explained through additional experimentation, the ability of the bacteria as determined, to withstand desiccation could partly explain their wide-spread occurrence in these part of the sub-tropics.

5.5 Growth of green fluorescent bacteria in different natural nutritional environment:

Growth of the isolates of the bacteria on different substrates like FYM (Farm Yard Manure), oil cake, leaf mould and drainage silt on the one hand and crop field-water and pond water on the other was examined. Although no visible growth was evident on these organic substrates, 100 % survival of the bacteria for at least 20 days was recorded on many of these substrates. While tried for their growth on dusts and meals of different grains like maize, gram and wheat a positive but slow growth was recorded for all the isolates. Isolates of the bacteria differed in their capability to grow on such substrates.

When grown on crushed-leaf-tissue of plants like Ludwigia, Hygrophiza, and Eichhornia- all aquatic and Imperata, a terrestrial, the bacteria were occasionally able to grow on such surfaces although they remained viable on them for more than a months.

The isolates of the bacteria recorded very little growth on animal products like hairs, feathers, and shells of egg. They produced white to creamy, slimy colonies along with only black pigment production. Their growth on animal excreta like cow-dung and goat-droppings were however unimpressive. All these results on growth of such bacteria on different types of organic substrates found around in nature however, established the ability of such bacteria to continue on such surfaces even though they grew poorly.

5.6 Survival of green fluorescent bacteria in the environment :

The ability of the green fluorescent bacteria to survive on 8 different substrate in nature has been examined. They survived for 220 or more days on sterilized soils from river Ganga, agricultural fields, and ponds but gradually declined from 30 days onwards on unsterilized soils from the same sources.

But in both unsterilized and sterilized waters from different sources like ponds and rivers the bacteria could survive for a year or more. Under such conditions they could remain physiologically active to a satisfactory level which was revealed from their heavy rate of pigment production. Survival of the isolates of Ps. aeruginosa in different waters must be of great significant.

Experiments conducted to understand the level of their accommodation in the rhizospheres of some crop plants like cabbage, tomato, and beet clearly indicated that the bacteria were able to make a stay in the rhizosphere competing with other rhizosphere microflora. Survival of the bacteria in such a highly competitive sphere in soil also indicated one reason of their wide occurrence.

The bacteria were also able to survive as epiphytes on foliages of crop plants like cabbage, beet and tomato when applied externally. On surfaces of cabbage which has already been established as their host, they could even increase their population. The bacteria

were also able to survive competitively on surfaces of four weed plants namely, Ludwigia, Hygrophiza, Eichhornia (aquatic) and Imperata (terrestrial). On being applied from outside they could establish on the leaf surfaces successfully within 45 days on a 100 % dominance basis.

5.7 Search for suppressive agents for green fluorescent bacteria :

From the data produced so far it has become clear that a bacterium like Ps. aeruginosa is occurring in the natural surroundings of a river under sub-tropical conditions. It seems that its population on different substrates on nature depend on different factors. Logically it was thought that there might be other factors useful for restraining their population in the environment.

Preliminary attempts have been made to identify such factors or agents, chemical or biological which may be fruitfully utilized for the purpose. Number of different chemical compounds namely HCl, NaOH, NaCl₂, H₃PO₄, K₂SO₄ and CaO among the inorganics, formaldehyde and phenol among the organics and Dettol, Phenyl and bleaching powder among the marketed disinfectants and antiseptics were included in the study of sensitivity of the bacterium towards the chemicals.

Data from fish-spine method of assessment revealed that Sodium hydroxyde and HCl were inhibitory to all the isolates of the bacteria at 2-5 % concentration. Among the organic compounds both

formaldehyde and phenols were good inhibitors at 5% concentration. Between the two phenol was a much better chemical. The identified disinfectants thus hold a good prospect of trial against the bacteria.

Antiseptics at 5% concentration inhibited two of the isolates only; hence to cover suppression of all or most of the isolates the antiseptics did not stand a prospects.

Among the antibiotics tested in a different experiments following the fish-spine and paper-disc method gentamycin was more effective. But antibiotics like chloramphenical, streptomycin and penicillin were of no use. Ciprafloxacin was a more effective inhibitor than others followed by bacterionol and norfloxin in order. Inhibitor antibiotics are also thus identified.

Experiment for engaging natural resources like plant extracts, straw and pure fungal cultures have not been able to produce any positive result. Five percent methanol extract of rice bean, Tephrosia and Coriaria , 5 % pet-ether extract of Rungia, and Tephrosia, 5 % chloroform extract of rice bean and 5 % water extract of Azadirachta were found to be without any inhibitory effect. Dried straw which are likely carrier of Bacillus species was also not effective. None of the fungi like Penicillium italicum, Trichoderma viride, Alternaria solani. Rhizopus stolonifer and Helminthosporium oryzae were inhibitory towards the bacteria.

Evidently barring identification of a few inorganic compounds like HCl, NaOH, all other agents tested failed to suppress the bacterium in vitro. As such the attempts to suppress the bacterium under its natural habitats has to centre so far round the identified chemicals alone.

B. Conclusion :

The work has been an intensive investigation for a bacterium which has not been explored so far in the country. Even though such investigations has started in other countries, nothing has so far been done in our country.

The extent of occurrence of Pseudomonas aeruginosa in the locality has been worked out from this study. The bacterium has been identified and characterised. The importance of the bacterium has also been realised after their pathogenicity toward plants and animals has been established.

The epidemiological aspects for such bacterium infecting host plants has also been explored to some extent. Their resistance toward drugs or chemicals have made the situation more complex.

The bacterium, due to its diverse biological capabilities could adjust or overcome different adverseries in the environment. As a result Ps.aeruginosa identified in the present situation, required further and deep attention.

Chapter VI

FUTURE SCOPE OF RESEARCH

FUTURE SCOPE OF RESEARCH

The studies made an ecology and epidemiology of green fluorescent bacteria particularly Pseudomonas aeruginosa has brought out many valuable informations. It has also identified some areas where further research programmes need to be taken up. The future research programme may be outlined as follows :

- 1) Further detailed survey for the existence or occurrence of Ps.aeruginosa on other crop plants and produces in other areas of the State.
- 2) Elaborate studies on different aspects of plant pathogenicity and plant tissue rotting ability requires to be taken up.
- 3) The nature and distribution of the different strains of this bacterium following modern techniques of serotyping and phage-typing.
- 4) Intensive search for natural substrates that may act as source of microorganisms suppressive for Ps.aeruginosa may be taken up.
- 5) Technologies for engaging suppressive natural substrates may be worked out and tested.
- 6) Regular and continuous monitoring system of estimation of population of Ps.aeruginosa in different water bodies needs to be taken up.

Chapter VII

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