Studies on *Cymbidium* rot and its management by biocontrol agents

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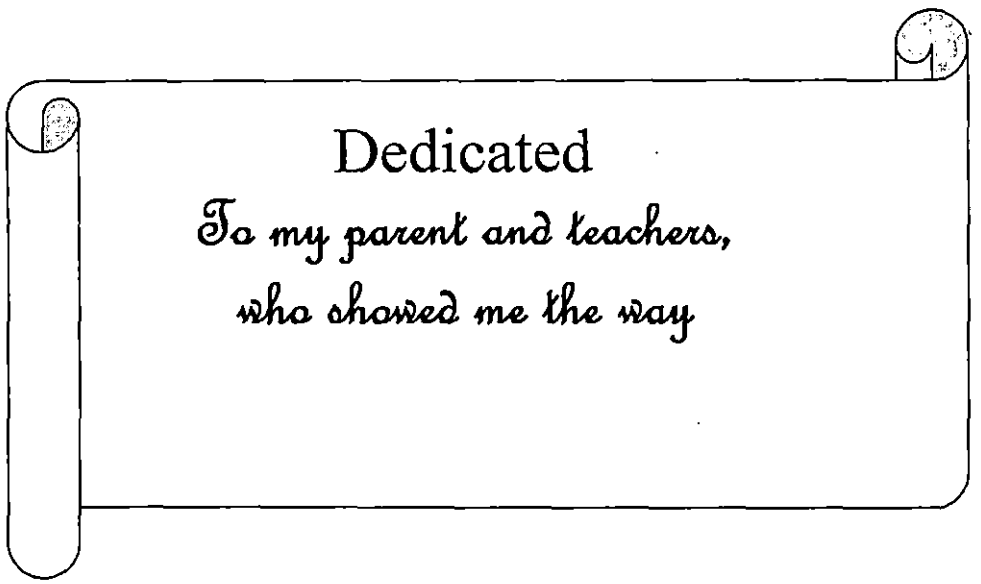
Dedicated
To my parent and teachers,
who showed me the way

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Abbreviations

°C: Degree Centigrade

°F: Degree Fahrenheit

µm: Micrometer

ARI: Agharkar Research Institute

BCAs: Biological Control Agents

C/N: Carbon to Nitrogen ratio

CAS: Chrome Azurol S

cfu: Colony Forming Unit

CITES: Convention of International Trade in Endangered Species

cm: Centimeter

CMC: Carboxy Methyl Cellulose

CymMV: *Cymbidium mosaic virus*

DAPG: 2,4-Diacetylphloroglucinol

DS: Disease Severity

FAA: Formalin-aceto-alcohol solution

gm/lit: Gram per liter

gm: Gram

HCN: Hydrogen Cyanide

IAA: Indole Acetic Acid

ISR: Induced Systemic Resistance

ITS: Internal Transcribed Spacer

kg: Kilogram

LB: Luria Bertani broth

lit: Liter

M: Molar

mg: Miligram

min: Minute

ml: Milliliter

mm: Millimeter

MR: Methyl Red

N: Normal

NA: Nutrient Agar

nm: Nanometer

ORSV: *Odontoglossum ringspot virus*

PCA: Phenazine-1-carboxylic acid

PDA: Potato Dextrose Agar

PGA: Peptone Glucose Agar

PGPB: Plant Growth Promoting Bacteria

PGPR: Plant Growth Promoting Rhizobacteria

Pln: Pyrrolnitrin

Plt: Pyoluterin

PR: Pathogeneis-related protein

PS: Peptone Skim milk media

rpm: Rotation per minute

SAR: Systemic Acquired Resistance

sec: Second

SSF: Solid State Fermentation

TCA: Tri Chloro Acetic Acid

TMV: *Tobacco mosaic virus*

TSA: Tryptone Soya Agar

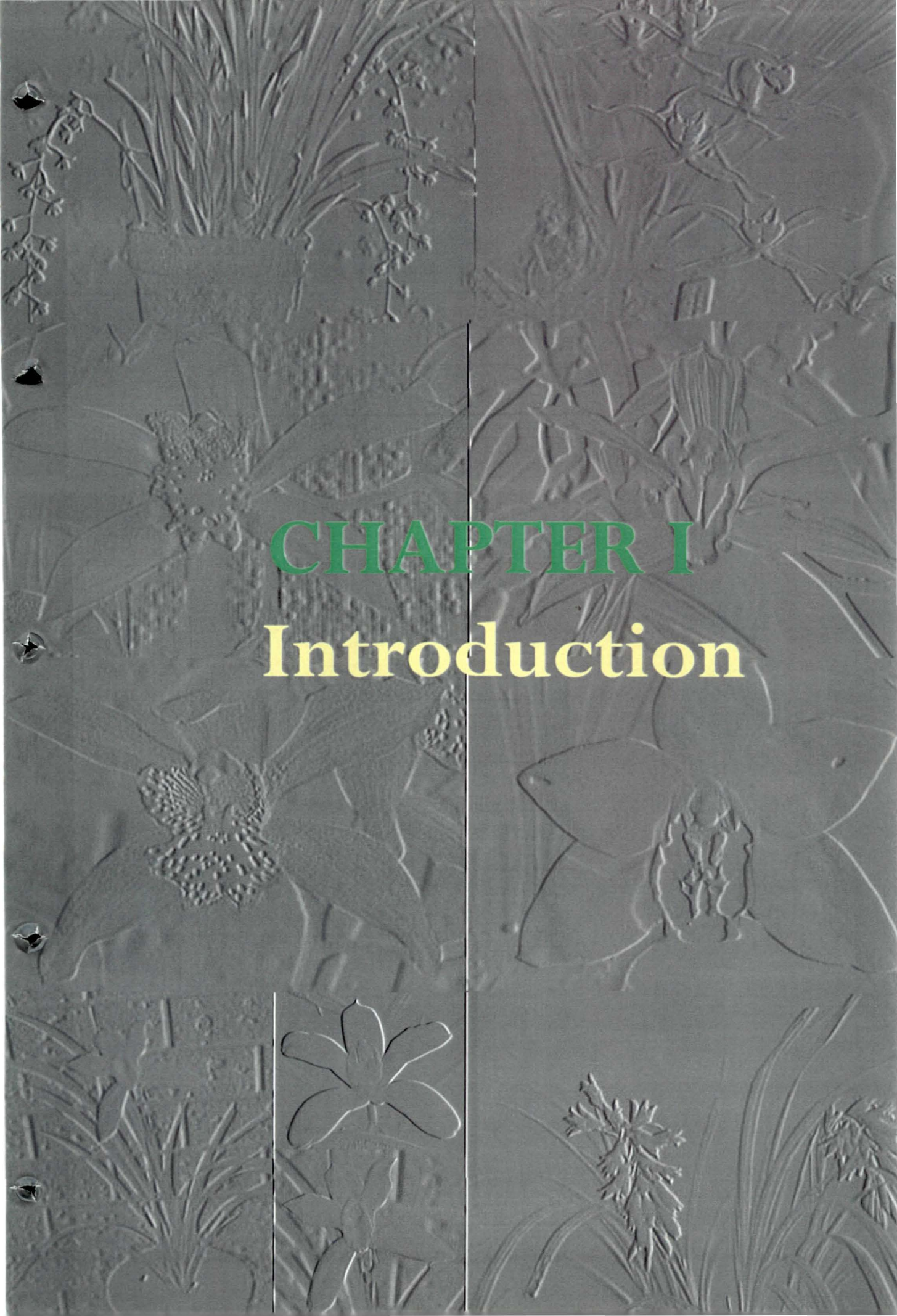
UK: United Kingdom

USA: United State of America

UV: Ultra Violet

VP: Voges-Proskaner

w/v: Weight per volume



CHAPTER I

Introduction

1.1. ORCHID: AN OVERVIEW

Theophrastus, 'the father of botany' (370-285 BC), gave the name "ORCHIDS" to a unique group of plants bearing the most beautiful flowers of nature. Taxonomically, they represent the most highly evolved family among monocotyledons with more than 800 genera and over 25,000 species that are commercially grown all over the world (Arditti, 1992). Orchids exhibit an incredible range of diversity in size, shape and colour of their flowers. They are most pampered of the plants and occupy top position among all the flowering plants valued for cut flower production and as potted plants. In India they are known for their longer lasting and bewitchingly beautiful flowers which fetch a very high price in the international market. The morphological, physiological, and genetic peculiarities inherent in this group of plants have stimulated research to such a degree that Orchidology today is one of the most popular and dynamic branches of Botany (Pathak *et al.*, 2001). Besides their unmatched ornamental values, orchids have some value in traditional medicines also (Srivastava, 1999). The drug obtained from the roots of *Cypripedium pubescens* is useful in the treatment of stomach worms and in allaying pains of the joints (Lewis and Elvin-Lewis, 1977).

In the Indian Vedic scriptures there is a mention of the plants under the name "VANDA", which has been adapted as a generic name in one of the most beautiful group of orchids. Most of the orchids are perennial herbs with simple leaves. Although the specialized flower structure conforms to a standard formula, the vegetative part shows great variation. Large number of them being epiphytes, or terrestrial and a few are saprophytes and leafless in nature. Majority of the cultivated orchids are native of tropical countries and occur in greatest diversity in humid tropical forest of South and Central America, Mexico, India, Ceylon, Burma, South China, Thailand, Malaysia, Philippines, New Guinea and Australia. Brazilian *Cattleya*, Mexican *Laelia* and Indian *Dendrobium*, *Cymbidium* and *Vanda* have played a major role in the development of modern orchid industry in the world (www.orchidsasia.com/orcintro.htm).

The major developments in cultivation of orchids in the world have been due to modern scientific technologies, which have been suitably used in case of orchid seed germination and meristem culture. Today orchids are grown on assembly-line method in extensive glasshouses with controlled environment and the sale of orchid flowers runs in millions of dollars. The modern methods of propagation have brought orchid cultivation on parity with other commercial crops. Horticulturists worldwide today grow orchids not only because they are mysterious, but mainly due to the fact that they are highly priced and occupy 8% share of the Global floricultural trade (www.sikkim.nic.in/nrco/nrco.htm).

The economic importance of orchid lies mainly in their ornamental value and horticultural uses. They provide cut blooms which remain fresh for long, make pretty corsages and used for long in Europe and the USA as progenitors for the production of some of the famous hybrids and even today are in great demand by orchid dealers abroad (Arora, 1985). The world export/import trade of orchid cut flowers and orchid plants exceeded $150 million in the year 2000. Of this, $128 million was in cut orchid flowers and about $23 million in orchid plants, according to United Nations Comstats. Thailand is the world's largest orchid exporter with 2,240 hectors of orchids in production. Singapore is also an important exporter, with $7.7 million in exports of cut orchids and $8,000 in orchid plants. Malaysia, much smaller, exported $2.8 million in orchid cut flowers and $15,000 in pot plants. New Zealand exported $830,000 in cut orchids and $93,000 in orchid plants. Only one European country Italy, is a significant exporter of orchids, which exported $652,000 in cut orchids and $399,000 in potted plants. The world commerce in orchid cut flowers is eight times as important as the world trade in orchid plants (Laws, 2003).

From a commercial standpoint, the most important orchids are those grown for cut flowers, which include species and hybrids of *Arachnis*, *Aranda*, *Asocentrum*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Laelia*, *Oncidium*, *Paphiopedilum*, *Phalenopsis*, *Renanthera*, and

Vanda. Annually, US$ 35 million worth of orchid cut flowers are produced and exported by Thailand (Zettler *et al.*, 1990). Singapore, being the world's second largest exporter of orchid cut flowers, exports US$ 24 million worth of cut flowers annually (Zettler *et al.*, 1990). *Dendrobium* orchid hybrids, one of the most economically important cut and potted floricultural crops grown in Hawaii, commanded a wholesale value of US$ 6.3 million in 1990 (Hu *et al.*, 1993). With the help of various mass-production methods, orchids account for 23 percent of the total production value of Taiwan's domestic flower industry. The total area of land devoted to orchid growing in Taiwan today is about 460 hectares, with an annual export value estimated at US $45 million (Chu, 2004).

Considering the number of species, India is certainly rich in orchid flora (Jain, 1985). India is not only rich in number of orchid taxa which grow profusely in nature, but most species are at top of the list of ornamental orchids (Sharma, 1996). Orchids form 9% of our flora and are the largest botanical family of higher plants in India. India is home to about 1,700 species of orchids (Varmah and Sahni, 1976), of which about 800 are found in the North Eastern region of the country where North Eastern Himalayas ranks the top in the list. Within these areas, Sikkim Himalaya, comprising the hills of Sikkim and Darjeeling, harbours about 450 species and the centre of origin for important species like *Cymbidium*. Mehra and Vij (1974) studied ecological adaptations and distribution pattern of East Himalayan orchids. Similar data was presented from the Simla (Vij *et al.*, 1982) and Nainital (Vij *et al.*, 1983) hills in the North-Western Himalayas.

India has been mainly exporting native species of orchids. In accordance with the recent Convention of International Trade in Endangered Species (CITES), the trade in native species from wild sources has been banned. India's export of the orchid flowers is yet to reach a sizeable figure. Some reputed sources indicate that the country exports orchid flowers to Europe, Hong Kong, Japan and Australia. However, India's potential

for the export of orchid flowers is rated as high. Though India's present export of orchids is meager, its future potential is high in view of the following reasons:

A large number of orchid species grow naturally in the congenial climate of India. The northeast and southwest regions are considered as two "hot spots" of the orchids. Of the numerous species of orchids only a few can be successfully grown in the plains.

- The climatic conditions may permit the cultivation of the orchids, either in the open or in simple green house, so as to export round the year.
- The cheap labour, land and input cost enable Indian orchids to be cost effective.
- India is strategically located between Middle East, Far East and EEC countries viz. Italy, the Netherlands, Germany, France, U.K. markets.
- Decreasing availability of land area, due to rapid urbanization in the South East Asian countries, which are the major exporters of orchids, is opening up the possibility of shifting the orchid production centers to South Asian countries.
- The recent economic liberalization initiated by the Union Government, and emphasis on the promotion of agri-export and the development of infrastructure have created congenial conditions for setting up of export oriented units of orchids.
- A domestic market for the orchids is fast developing, which can absorb, non-exportable surplus. (www.nabard.org/whats/orchid.htm)

The *Cymbidium*

Many people think first of a *Cymbidium* flower when they hear the word 'orchid'. Cymbidiums are widely grown by orchid enthusiasts throughout the cooler parts of the world and they form the basis of a significant cut-flower production. The flowers are long-lasting, both on the plant and when cut, large, attractive and available in a wide range of colours (Figure 1.1). This popular ornamental genus of the orchids is reported

to have 300 species. *Cymbidium* is truly an Asiatic orchid since it is most widely distributed in Asian countries. The North-East Indian hills are the richest phyto-geographical habitat for this genus in India because of the prevailing supporting climate (Munsi *et al.*, 2004). It thrives well in the temperature ranging from 10^0C (night) to 25^0C (day). Depending on the temperature requirements, there are two broad groups of *Cymbidium*: (a) tropical, which requires higher and (b) temperate, which requires lower temperature. However, most species flowers, when the night temperature is about 10^0C, but it should not be less than 4-5^0C. The *Cymbidium* species in nature are usually tree dwellers (epiphytic) or live on rocks (lithophytic), but some are even ground dwellers (terrestrial). However, this group of orchids is treated as semi-terrestrial in commercial cultivation and grows on pots containing a medium with the characteristics of rapidly drainable yet moist. The Cymbidiums belong to sympodial group of orchids, and can be propagated by the division of rhizomatus clump when the plants have more than 8 pseudobulbs (swollen stems) (DuPuy and Cribb, 1988). Plants which normally grow on trees are sometimes encountered on rocks, and one species, *C. macrorhizon*, is a saprophyte (Hooker, 1890) which grows entirely beneath the soil surface except when the flower spike emerges. Most species have thick roots which are covered in a spongy white velamen and have only a thin core of vascular tissue. The erect stems are usually short and swollen to form a prominent pseudobulb which is often slightly flattened. Many species produce a new growth annually. In one section of the genus the pseudobulbs grow and flower for several years before a new shoot is produced, and in *C. mastersii*, *C. elongatuni* and *C. suave*, each shoot grows continuously for many years producing an elongated stem rather than a typical pseudobulb.

Fifure 1.1: Different species of Cymbidium

1.2. GLOBAL OCCURRENCE OF ORCHID DISEASES: A REVIEW

The orchids are adapted to withstand a variety of environmental stresses but under highly adverse growing condition their natural defense mechanism generally gets weakened and they succumb to a variety of diseases. The susceptibility however, varies with the geographical regions, genera, species, hybrids and even individual clones. Besides the physiological factors, the orchid diseases are usually caused by fungi, bacteria, viruses, algae, weeds, insects etc. the main cause of stress in orchids are related to water. Too much water, due to over watering of poorly drained medium, leads to their rot and subsequent attack by fungi and bacteria. The impaired health status of the diseased plant detrimentally affects their commercial significance and they are outrightedly rejected in the floriculture trade (Vij and Kaur, 1985).

Information on orchid diseases and their control has been accumulating over the years. It can be effectively used to ameliorate the health and commercial status of the diseased plants and is of great interest to professional and amateur orchid growers. It is better to prevent diseases from its initial development and it is possible if a grower uses good cultural practices unfavourable for disease infection or pest attack (Burnett, 1965). Proper sanitation in the greenhouse and application of preventive steps are very important to keep the plants free from various pests and diseases. Various orchid diseases are discussed here:

1.2.1. FUNGAL DISEASES: -

1.2.1.1. Leaf Spot: - This disease is very common and can be found in almost all cultivated species of orchids. It is caused by species of *Gloeosporium* (Alexopoulos, 1935), *Colletotrichum, Phyllostictina*. This disease have been reported from Germany, England and other countries by Pirone *et al.*, (1960). Within a few days of infection sunken spots appear at any place on the leaves which later turn brown. Warm humid weather and lack of light encourage the spread of disease. Spraying of Bordeaux mixture effectively controls the

disease. Yellow oxide of copper, 1lb in 100 gallons of water, also controls the disease if applied regularly to the plants (Ark, 1959).

Another leaf spot disease of orchids was reported from Wutong Shan Orchid Nurseries, Shenzhen, Guangdong, China. The fungus produced spots on orchid leaves in mid-February. The disease epidemic peak occurred from April to June when cloudy and damp weather prevailed. The disease was more serious when plants were cultivated under hot, windless conditions. After applying Koch's postulates and a comparative inoculation test, the pathogen was identified as *Stagonospora curtisii* (Wu *et al.*, 1997).

Cymbidium leaf spot is caused by *Fusarium* species which was consistently isolated from yellow, swollen spots with reddish brown centers and small black spots on leaves of *Cymbidium* plants in the greenhouse. *Fusarium subglutinans* caused the yellow spots and *Fusarium proliferatum* caused either the yellow or the black spots. Kazunori and Takayuki (2000) propose the name "yellow spot" for the new disease.

1.2.1.2. *Pythium* Black rot: -This disease affects mostly seedlings in community pots and is caused by *Pythium ultimum* (Ark and Middleton, 1949). Affected plants turn black and leaves start falling. The pseudobulbs also start to rot within. Since in community pots the plants are closed to each other, the disease spreads from plant to plant at a very rapid rate. Orchid such as *Epidendrum* sp were found to be susceptible to *Pythium ultimum*. Withholding of watering for a few days and shifting the plants to less humid part of the green house help to check the disease. Infected plants should be removed and the remaining healthy ones spread with fungicide like mancozeb @ 2.5 gm/lit. Irrigation with a mild solution of a copper fungicide once a month till the plants are tender, is an effective preventive measure.

1.2.1.3. Heart Rot: - *Cattleya, Phalaenopsis* and *Vanda* are the important orchid genera susceptible to the disease, which is caused by *Phytophthora palmivora*. The leaves of the infected plants turn yellow and drop off. Pseudobulbs have dark rotted areas inside. The infected pseudobulbs should be removed and the plants repotted in fresh media after dipping in fungicide. Gilbert et. al. (1950) tried two different methods of healing orchid plants to control a species of *Phytophthora*. In the first step inoculated *Cattleya* plants were placed in a forced draft type poultry incubated at 107^0F, 90% relative humidity for 24 to 66 hours. All plants showed injury but new healthy growths were produced later. In the second method, inoculated plants were exposed to vapour heat at 116^0 or 120^0 F for 39 min to 1 hour and 25 min. With this treatment orchid plants showed no apparent heat injury but the fungal growth was checked.

1.2.1.4. Brown Speck and Blight of Flowers: - The disease affects a number of orchids like *Cattleya, Phalaenopsis, Dendrobium Onchidium* and *Vanda*. It is caused by *Botrytis cinerea*. When infection is mild, spots appear on the flowers in severe cases, the entire flower may be covered with spore masses and a blighting effect is produced. All infected flowers should be cut off and destroyed and the plants be kept in a place where humidity is low, temperature is high and there is good ventilation. For prevention of *Botrytis* it is important to keep the plants dry during cool whether. The best control may be achieved by careful spraying with fungicide and by eliminating of host plants near the orchid house. A black spot disease on blooms of *Vanda tricolor* caused by *Glomerella* sp is controlled by spraying with a solution of bioquin 700 (o-quinolinol benzoate) before the appearance of the symptoms (Ark and Snyder, 1951).

1.2.1.5. Root Rots: - The fungus *Fusarium* is responsible for this disease which causes the destruction of the root tissue. *Pellicularia filamentosa* is also said to cause root

rot. The disease kills the seedling and retards the growth of mature plants. Affected plants should be treated with zineb or tersan (Bose and Yadav, 1989).

1.2.1.6. Orchid wilt: - This disease is caused by *Sclerotium rolfsii* which affects mostly *Cymbidium* and *Paphiopedilum* plants. The leaf base initially becomes yellow later turning brown. Warm humid weather encourages spread of the disease. Affected leaves should be removed and plants repotted in fresh medium after treatment with protective fungicide like pentachloro-nitro-benzene. Treatment with zineb or natriphene is effective (Bose and Yadav, 1989).

1.2.1.7. Anthracnose Orchid Spot: - It is a major disease, often superficial, caused by a variety of fungi. Plants damaged or weakened by poor growing conditions are often attacked. Excessive fertilization with nitrogen also makes plants susceptible to infection. The disease is caused by *Colletotrichum gleosporiodes*, C. *cinctum* several species and hybrids in the genera *Aerides, Cymbidium, Dendrobium, Vanda* etc. are the common host of this disease. The basic symptoms of this disease are raised pustules on flower buds, petals and sepals. Target spot type lesion often developed at tip or on the middle area of the leaves. The spots may be circular, oval, sunken and reddish in colour. Eradication of disease parts and / or organs by burning has been suggested. Spraying with captan or benomyl proves useful in controlling the disease (Ciferri, 1926).

1.2.1.8. *Cymbidium* tip burn: - The disease is caused by *Botrytis* species; the pathogen penetrates the leaf tips damaged by exudation of salts and spreads rapidly into the healthy tissues of the plant. The infected leaf tips becomes spotted the spots coalesce to ultimately result in the death of entire tip. Dead tips usually get coated by mass of powdery spores. Removal of dead tips and spray with fungicides such as bavistin, captan or dithane was suggested by Vij and Kaur, 1985.

1.2.1.9. Rust: - In the United States and other parts of the world, *Cattleya* and other orchids are subjected to the attack of a rust fungus *Hemileia americana.* The disease is characterized by yellow pustules covered with fine powdery masses of spores (Ark 1959). Treatment with copper fungicide or terson has been suggested.

1.2.1.10. Collar rot: - It is caused by *Sclerotium rolfsii.* The stem and leaf bases of *Cymbidium, Paphiopedilum* loose chlorophyll. The infected parts usually harbour white mycelium and sclerotia. The sclerotia may survive in soil, potting mixes, and on benches and pots for many years. The disease is checked by destroying the infected plants, practice of strict sanitation, proper sterilization of potting medium and use of carboxin like fungicide is most effective (Ark, 1959).

1.2.1.11. Damping off: - It is caused by number of pathogens like *Phytophthora palmivora, P. omnivora, Pythium ultimum* etc. The pathogen attacks seedlings of many species and hybrids including *Cattleya, Vanda.* Watery spots appear in leaves, which turn yellow/ black and die, the roots often collapse and eventually the entire plant dies. Sanitation, controlled irrigation, sterile potting media and containers and clean benches are usually recommended to prevent damping off. Seedling should be drenched at seven-day intervals with 50 mg/l Dexon or 1 part copper sulphate in 100,000 part of water (Vij and Kaur, 1985).

1.2.1.12. Botrytis flower spotting: - This disease affects *Cymbidium*, *Cattleya*, *Dendrobium* and *Phalaenopsis* particularly, and generally the older flowers of other genera. Very small dark spots appear on the flowers. If conditions are very moist, then a grey mould growth will appear. The best control is prevention: Removal and eradication of infected materials reduce the source of inoculum, increase the air movement, reduce humidity and increase night time temperatures (Wright, 1994).

1.2.1.13. Leaf and Stem rot of *Cymbidium*: - A *Phytophthora* species was isolated from blackened leaves and stems of infected *Cymbidium* plants. Cultural characters did not fit descriptions of any known *Phytophthora* species. It was concluded that a new *Phytophthora* species, described here as *Phytophthora multivesiculata* is the causal agent (Ilieva *et al.*, 1998).

1.2.1.14. Leaf Bloch of *Cymbidium*: The pathogen that causes a leaf blotch and a rot of cymbidium orchids in New Zealand has been identified as *Phytophthora multivesiculata* using morphological characteristics and Internal Transcribed Spacer (ITS) fingerprinting. It is widespread in the North Island of the country (Hill, 2004).

1.2.2. BACTERIAL DISEASES: -

1.2.2.1. Bacterial Soft Rot: - It is a serious disease of *Cattleya* orchids and is caused by *Erwinia carotovora*. The disease starts at the upper end of the leaf as small, water soaked and somewhat darker than normal green spot. Pseudobulb of the infected plants turns soft and pulpy and become yellow in colour. A foul smelling liquid sometimes starts to ooze from the bulb. It is difficult to control the disease, but spraying with agrimycin has certain beneficial effect (Limber, and Friedman, 1943).

1.2.2.2. Bacterial Brown Rot: - This disease is serious on *Paphiopedilum*. It is caused by a rod shaped bacteria *Erwinia cypripedii* as described by Hori (1911). The organism attacks the plants through wounds and cracks. Yellowing and browning of leaves indicate the presence of the disease. The infection spreads into the crown and causes death of the plant. The disease could be suppressed by merging infected plants in 1:2000 solution of quinolinol benzoate or natriphene for 1 to 2.5 hours.

1.2.2.3. Bacterial Brown Spot: - This is a disease of both seedling and full-grown plants and caused by *Xanthomonas cattleyae*. In the beginning, there appear a small water soaked spot on any part of the leaves. The lesion enlarges and soon the leaf become soft and dies. The disease may progress into the growing point often killing the plant (Bose and Yadav, 1989).

1.2.2.4. Bacterial leaf rot of *Odontioda*: An unknown disease causing leaf rot on leaves of *Odontioda* sp., which is an intergeneric hybrid of *Odontoglossum*, occurred in Tochigi Prefecture, Japan, during the summer of 1995. One bacterial species was almost purely isolated from the infected leaves. The isolated strains were pathogenic to *Odontioda* cv. Baiser orchids in the dark with high temperature stress and high humidity. Based on the results of API 20E strips and other physiological and biochemical tests, the strain was identified as *Enterobacter cloacae*. This is the first report of an orchid disease caused by *E. cloacae* (Takahashi, *et al.*, 1997).

1.2.2.5. Bacteria Leaf spot: The disease caused by *Acidovorax avanae* subsp. *cattleyae* has been reported on three genera of orchids viz. *Dendrobium, Oncidium, Rhynchostylis*, usually start as small brown soft water soaked areas which quickly expand over the entire leaf. The covering infected area is easily damaged releasing a bacterial 'soup' which is spread to healthy leaves by splashing water (Duff and Daly, 2002).

1.2.3. VIRAL DISEASES: -

Orchids are affected by more virus disease problems than most crops (Zettler *et al.*, 1990), reducing their commercial values considerably. Viruses cause a number of orchid diseases which can be prevented but are difficult to control, impossible to eliminate or cure, and generally lead to loss of plant. Viral infections are rare in wild orchid. Jensen (1952a; 1952b) listed 32 virus diseases to occur in orchids but it does not mean that there were 32 different kinds of

viruses. Viruses can be transmitted by insects, mites, nematodes, fungi, and parasitic plants. Human handling may also transmit viral diseases. Viruses can also be transmitted through vegetative propagation, sap and contact between plants, pots and greenhouse benches. *Cymbidium mosaic virus* (CymMV), tobacco mosaic virus-o (TMV-o) and *Odontoglossum ringspot virus* (ORSV) are the most common among orchid viruses. Viral diseases are not always easy to recognize. Sherpa *et al.* (2003) reported *Cymbidium mosaic virus* on *Cymbidium* for the first time from India. In Thailand CymMV was detected in 17 genera in 93% nurseries, while ORSV was detected in 4 genera (*Arachnis, Cattleya, Oncidium* and *Vanda*) in 40% of nurseries tested (Tanaka *et al.*, 1997). Symptoms of viral diseases often take the form of chlorotic spots of mottled areas, or sometimes more prominent rings, streaks and necrotic areas. In situation where certain viruses commonly cause symptomless infections, the viruses may spread systematically through the entire plant. These diseases are incurable. Orchid growers are likely to spread viral diseases unwittingly in the normal cause of horticulture operations. The best means of controlling viral diseases are cleanliness, eradication of insect vector and infected plants, use to clean tools, removal of weeds and other sanitation procedures. A solution of 2% formalin and 2% NaOH in water is an excellent disinfectant for tools. Infected plants must be destroyed or isolated carefully to prevent the spread of the disease. Shoot tip culture is the only way to save infected plants. Unfortunately, however, even this procedure is not complete cure because shoot tips of some plants are difficult or impossible to culture or cannot be freed of viruses through tissue culture.

1.3. BIOLOGICAL CONTROL: A CONCEPT

Biological control was established by trial and error and then practiced in agriculture long before the term itself came into use (Baker and Cook, 1974). It is conceivable to believe that the control of plant diseases can be done with chemicals but alternate method of controlling plant diseases are needed as the use of chemicals and pesticides is disturbing the natural ecosystem with sometime disastrous consequences. Baker and Cook (1974) have discussed the 'how and why' of biological control. Biological control offers a powerful means to increase yield by destruction or suppression of pathogen inoculums, protect plant against infection or increase the ability of plant to resist pathogen. The era of modern biological control, involving the deliberate transfer and introduction of natural enemies of insect pests, was launched 100 years ago with the highly successful introduction of the vadalia beetle from Australia to California in 1888 to control the cottony cushion scale of citrus. In 1914, the German plant pathologist C. F. von Tubuef wrote a somewhat speculative article entitled "Biologische Bekampfung von Pilzkrankheiten der Pflanzen." This is apparently the first reference in the scientific literature to the term "Biologische Bekampfung" or "biological control" (Baker, 1987). With the increasing public awareness of the environmental implications of the use of large quantities of pesticides in agricultural practices, alternative strategies for the control of plant diseases are being sought (Weller 1988). Many of these strategies involve the use of antibiotic-producing fluorescent pseudomonads, and other bacteria, as effective biological control agents (BCAs) (O'Sullivan and O'Gara 1992). Several commercial biocontrol products are available which protect seedlings from fungal diseases, e.g. 'No Gall', an *Agrobacterium* strain antagonistic to crown gall disease of fruit, 'Dagger G', a *Pseudomonas fluorescens* strain active against damping-off of cotton, and 'GlioGard', based on a strain of *Gliocadium virens* (Cook 1993; Ryder 1994). Despite these apparent successes, the uses of BCAs are generally not as

reliable as their chemical counterparts (Weller 1988). This may be due to the inherent variability of applying a living organism to the environment. As environmental conditions fluctuate, so the colonization and *in planta* activity of inocula may vary (Weller and Thomashow 1994). Biological control of foliar pathogens with antagonistic fungi offers an ideal remedy to problems arising out of constant fungicide application (Mathivanan *et al.*, 2000). Govendasamy and Balasubramaniam (1989) have reported the biocontrol potential of *Trichoderma harzianum* in controlling rust disease in groundnut under green house condition. The potential of *Trichoderma* spp. in foot rot management in black pepper has been clearly established (Rajan *et al.*, 2002). The key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in phylosphere and rhizosphere is required to predict the conditions under which biocontrol can be achieved (Deacon, 1994; Whipps, 1997) and, indeed, may be part of the reason why more biocontrol agents are reaching the market-place (Fravel, 1999; Whipps and Lumsden, 2001; Whipps and Davies, 2000). This type of work requires a study not only of any potential biocontrol agent *per se* but also its interactions with the crop, the natural resident microbiota and the environment as well. Biocontrol involves harnessing disease-suppressive microorganisms to improve plant health. Disease suppression by biocontrol agents is the sustained manifestation of interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant, and the physical environment. Even in model laboratory systems, the study of biocontrol involves interactions among a minimum of three organisms. Therefore, despite its potential in agricultural applications, biocontrol is one of the most poorly understood areas of plant-microbe interactions. The complexity of these systems has influenced the acceptance of biocontrol as a means of controlling plant diseases in two ways. First, practical results with biocontrol have been variable. Thus, despite some stunning successes

with biocontrol agents in agriculture, there remains a general skepticism born of past failures (Weller, 1988). Second, progress in understanding an entire system has been slow. Recently, however, substantial progress has been made in a number of biocontrol systems through the application of genetic and mathematical approaches that accommodate the complexity. Biocontrol of soilborne diseases is particularly complex because these diseases occur in the dynamic environment at the interface of root and soil known as the rhizosphere, which is defined as the region surrounding a root that is affected by it. The rhizosphere is typified by rapid change, intense microbial activity, and high populations of bacteria compared with non-rhizosphere soil. Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere microorganisms. The rhizosphere is subject to dramatic changes on a short temporal scale rain events and daytime drought can result in fluctuations in salt concentration, pH, osmotic potential, water potential, and soil particle structure. The complexity of the root-soil interface must be accommodated in the study of biocontrol, which must involve whole organisms and ultimately entire communities, if we are to understand the essential interactions in soil in the field. The challenge in elucidating mechanisms of biocontrol is in reducing the complexity to address tractable scientific questions. One of the most effective approaches toward the identification of critical variables in a complex system has been genetics. The study of mutants can be conducted in simplified laboratory systems or in the field, thus making accessible the examination of particular genetic changes and the associated biochemical characteristics in the real world.

1.4. MECHANISMS OF BIOCONTROL

The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been a subject of many studies in the past two decades (Janisiewicz, *et al.,* 2000). Several mechanisms, operating alone or in concert, are known to be involved in antagonistic interactions in the phyllosphere and rhizosphere. Nutrient competition, antibiosis and mycoparasitism are the major mechanisms. Additional mechanisms, such as induced resistance, production of biosurfactants, interference with pathogen-related enzymes and undoubtedly a number of still unknown mechanisms may complete the microbial arsenal (Elad, 1996). In particular, mechanistic studies have benefited from molecular biology by identifying, deleting and supplementing genes responsible for mechanisms such as antibiotic production. Knowledge of mechanisms involved in biocontrol is important for estimating and predicting its reliability and selection of better strains. Besides other criteria, the choice of an antagonist with its characteristic mechanisms depends on the stage of the life cycle of the pathogen the antagonist is aimed at. Allowable interaction times and niche characteristics determine the suitability of certain mode of action during different developmental stages of the pathogen.

1.4.1. Mechanism of action of bacterial antagonist against bacterial pathogen

In the last few years there have been relatively few studies of bacteria applied to seeds and roots for the purpose of controlling bacterial diseases. One example, is the application of non-pathogenic strains of *Streptomyces* to control scab of potato (*Solanum tuberosum*) caused by *Streptomyces scabies* (Ryan and Kinkel, 1997; Neeno-Eckwall and Schottel, 1999). Here biocontrol may operate through antibiosis or competition for space or nutrients in the rhizosphere. Antagonism of *Pseudomonas* strains toward *Erwinia carotovora* can be attributed to production of the siderophore pseudobactin (Kloepper *et al.,* 1980). In contrast, *Pseudomonas*

fluorescens F113 was shown to control the soft rot potato pathogen *Erwinia carotovora* subsp *atroseptica* by production of the antibiotic 2, 4-diacetylphloroglucinol (DAPG). Some evidence was also obtained that siderophore production by *P. fluorescens* F113 may play a role in biocontrol of potato soft rot under iron-limiting conditions, but DAPG appears to be the major biocontrol determinant. *Pseudomonas* species may also control crown gall disease in many dicotyledonous plants caused by *Agrobacterium tumefaciens* (Khmel *et al.*, 1998).

1.4.2. Mechanism of action of bacterial antagonist against fungal pathogen

The study in this area continues to increase at a rapid rate, stimulated by the increasing ease with which molecular techniques can be applied to answer questions concerning distribution, and occurrence and relative importance of specific modes of action. Although a range of different bacterial genera and species has been studied, the overwhelming numbers of papers have involved the use of *Pseudomonas* species (Whipps, 2001). Clearly, *Pseudomonas* species must have activity but it begs the question as to the features that make this genus so effective and the choice of so many workers. Pseudomonads are characteristically fast growing, easy to culture and manipulate genetically in the laboratory, and are able to utilize a range of easily metabolizable organic compounds, making them amenable to experimentation. But, in addition, they are common rhizosphere organisms and must be adapted to life in the rhizosphere to a large extent (deWeger *et al.*, 1995; Marilley and Aragno, 1999). A few specific examples of the modes of action involved with bacterial biocontrol of fungal pathogens in the rhizosphere and spermosphere are given below.

1.4.2.1. *Antibiosis*

Biocontrol is often attributed to antibiosis. In many biocontrol systems that have been studied, one or more antibiotics have been shown to play a role in

disease suppression. The fact that antibiosis is a common mechanism of biocontrol may be due to a bias in choice of organisms for study. Alternatively, it may be due to the attractiveness of the antibiosis hypothesis, or antibiosis may be simply a highly effective mechanism for suppressing pathogens in the rhizosphere (Handelsman and Stabb 1996). There are numerous reports of the production of antifungal metabolites (excluding metal chelators and enzymes) produced by bacteria *in vitro* that may also have activity *in vivo*. These include ammonia, butyrolactones, 2,4-diacetylphloroglucinol (Phl), HCN, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid (PCA), pyoluterin (Plt), pyrrolnitrin (Pln), viscosinamide, xanthobaccin, and zwittermycin A as well as several other uncharacterized moieties (Milner *et al.*, 1996; Kang *et al.*, 1998; Kim *et al.*, 1999; Nakayama *et al.*, 1999). To demonstrate a role for antibiotics in biocontrol, mutants lacking production of antibiotics or over-producing mutants have been used (Bonsall *et al.*, 1997; Chin-A-Woeng *et al.*, 1998; Nowak-Thompson *et al.*, 1999). Alternatively, the use of reporter genes or probes to demonstrate production of antibiotics in the rhizosphere is becoming more commonplace (Kraus and Loper, 1995; Raaijmakers *et al.*, 1997; Chin-A-Woeng *et al.*, 1998). Significantly, both Phl and PCA have been isolated from the rhizosphere of wheat following introduction of biocontrol strains of *Pseudomonas* (Thomashow *et al.*, 1990; Bonsall *et al.*, 1997; Raaijmakers *et al.*, 1999), finally confirming that such antibiotics are produced *in vivo*. Further, Phl production in the rhizosphere of wheat was strongly related to the density of the bacterial population present and the ability to colonize roots (Raaijmakers *et al.*, 1999). PCA from *Pseudomonas aureofaciens* Tx-1 has even been used as a direct field treatment for the control of dollar spot (*Sclerotinia homeocarpa*) on creeping bentgrass (*Agrostis palustris*) (Powell *et al.*, 2000).

1.4.2.2. *Competition for iron*

Although competition between bacteria and fungal plant pathogens for space or nutrients has been known to exist as a biocontrol mechanism for many years (Whipps, 1997) the greatest interest recently has involved competition for iron. Under iron-limiting conditions, bacteria produce a range of iron chelating compounds or siderophores which have a very high affinity for ferric iron. These bacterial iron chelators are thought to sequester the limited supply of iron available in the rhizosphere making it unavailable to pathogenic fungi, thereby restricting their growth (O'Sullivan and O'Gara, 1992; Loper and Henkels, 1999). Studies have clearly shown that the iron nutrition of the plant influences the rhizosphere microbial community structure (Yang and Crowley, 2000). In general siderophores are grouped as hydroxamate, phenol /catecholates and carboxylate (Neilands, 1981). Ferric hydroxamate complex is more stable and predominant in rhizosphere (O'Sullivan and O'Gara, 1992) Iron competition in pseudomonads has been intensively studied and the role of the siderophore known as pyoverdine or pseudobactins that fluoresce under UV light (Buysens, *et al.*, 1996) produced by many *Pseudomonas* species has been clearly demonstrated in the control of *Pythium* and *Fusarium* species, either by comparing the effects of purified pyoverdine with synthetic iron chelators or through the use of pyoverdine minus mutants (Loper and Buyer, 1991; Duijff *et al.*, 1993). Pseudomonads also produce two other siderophores, pyochelin and its precursor salicylic acid, and pyochelin is thought to contribute to the protection of tomato plants from *Pythium* by *Pseudomonas aeruginosa* 7NSK2 (Buysens *et al.*, 1996). The dynamics of iron competition in the rhizosphere are often complex. For example, some siderophores can only be used by the bacteria that produce them (Ongena *et al.*, 1999), whereas others can be used by many different bacteria (Loper and Henkels, 1999). Different environmental factors can also influence the quantity of siderophores produced (Duffy and

Defago, 1999). There is also the further complication that pyoverdine and salicylate may act as elicitors for inducing systemic resistance against pathogens in some plants (Metraux *et al.*, 1990; Leeman *et al.*, 1996b).

1.4.2.3. *Parasitism and production of extracellular enzymes*

The ability of bacteria, especially actinomycetes, to parasitize and degrade spores of fungal plant pathogens is well established (El-Tarabily *et al.*, 1997). Assuming that nutrients pass from the plant pathogen to bacteria, and that fungal growth is inhibited, the spectrum of parasitism could range from simple attachment of cells to hyphae, as with the *Enterobacter cloacae–Pythium ultimum* interaction (Nelson *et al.*, 1986) to complete lysis and degradation of hyphae as found with the *Arthrobacter–Pythium debaryanum* interaction (Mitchell and Hurwitz, 1965). If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort has gone into identifying cell wall degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. For example, biocontrol of *Phytophthora cinnamomi* root rot of *Banksia grandis* was obtained using a cellulase-producing isolate of *Micromonospora carbonacea* (El-Tarabily *et al.*, 1996) and control of *Phytophthora fragariae* var. *rubi* causing raspberry root rot was suppressed by the application of actinomycete isolates that were selected for the production of ß-1,3-, ß-1,4- and ß-1,6-glucanases (Valois *et al.*, 1996). The gram negative rod *Stenotrophomonas maltophila* W81 is a sugar beet rhizosphere isolate capable of conferring protection against *Pytium ultimum*-mediated damping off (Dunne *et al.*, 1997, 1998). Mutagenesis of *S. maltophila* W81 with Tn*5*-746cd demonstrated that this ability is mediated by proteolytic enzyme production (Dunne *et al.*, 1997). Protease and chitinase

activity has also been reported among fluorescent pseudomonds (Nielsen *et al.*, 1998; Nielsen, *et al.*, 2002). Chitinolytic enzymes produced by both *Bacillus cereus* and *Pantoea* (*Enterobacter*) *agglomerans* also appear to be involved in biocontrol of *Rhizoctonia solani* (Chernin *et al.*, 1995; Pleban *et al.*, 1997). Tn5 mutants of *E. agglomerans* deficient in chitinolytic activity were unable to protect cotton (*Gossypium barbardense*) and expression of the *chiA* gene for endochitinase in *Escherichia coli* allowed the transformed strain to inhibit *R. solani* on cotton seedlings.

1.4.2.4. *Induced resistance*

The definition of induced resistance was suggested by Kloepper *et al.* (1992) covered both biotic and abiotic inducers. Perhaps the greatest growth area in biocontrol in the last few years has been concerned with induced resistance defined as 'the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (inducing agents)' (Kloepper *et al.*, 1992). This has come about through the synergistic interaction of microbiologists, plant pathologists and plant scientists armed with an appropriate battery of molecular tools. The effect had previously often been overlooked through inadequate techniques or controls as well as the biocontrol agent exhibiting other modes of action at the same time. Most work has focused on the systemic resistance induced by non-pathogenic rhizosphere-colonizing *Bacillus* and *Pseudomonas* species in systems where the inducing bacteria and the challenging pathogen remained spatially separated and no direct interaction between the bacteria and pathogen was possible (Sticher *et al.*, 1997; van Loon, 1997). Such split root or spatial root inoculation experiments were used to demonstrate the phenomenon in radish (*Raphanus sativus*) and *Arabidopsis* against *Fusarium oxysporum* (Leeman *et al.*, 1996*a*; van Wees *et al.*, 1997) and in cucumber (*Cucumis sativus*) against *Pythium aphanidermatum* (Chen *et al.*, 1998). Various combinations of timing and position have indicated that induced

resistance also occurs in carnation (*Dianthus caryophyllus*) (van Peer *et al.*, 1991), tobacco (*Nicotiana tabacum*) (Maurhaufer *et al.*, 1994) and tomato (*Lycopersicon esculentum*) (Duijff *et al.*, 1997). Bacteria differ in ability to induce resistance, with some being active on some plant species and not others; variation in inducibility also exists within plant species (van Loon, 1997). The full range of inducing moieties produced by bacteria is probably not yet known, but lipopolysaccharides (Leeman *et al.*, 1995) and siderophores (Metraux *et al.*, 1990; Leeman *et al.*, 1996b) are clearly indicated. Although the phenotypic effects of root inoculation with bacteria may be similar to treatment with abiotic agents or microorganisms that cause localized damage, the biochemical and mechanistic changes appear to be subtly different. This has resulted in the term induced systemic resistance (ISR) for bacterially-induced resistance and systemic acquired resistance for the other forms (Pieterse *et al.*, 1996). The major differences are that pathogenesis-related (PR) proteins such as chitinases, ß-1,3-glucanases, proteinase inhibitors and one or two other rarer types, are not universally associated with bacterially induced resistance (Hoffland *et al.*, 1995) and salicylic acid (a known inducer of SAR) is not always involved in expression of ISR, but this is dependent on bacterial strain and host plant involved (Pieterse *et al.*, 1996; de Meyer *et al.*, 1999;). Ethylene responsiveness may also be required at the site of inoculation of the inducing bacteria for ISR to occur (Knoester *et al.*, 1999).

1.4.2.5. *Plant growth-promoting rhizobacteria (PGPR)*

In recent years, considerable attention has been paid to Plant Growth Promoting Rhizobacteria (PGPR) as the best choice in place of agrochemicals, to facilitate the biocontrol of soil and seed borne plant pathogens (Weller, 1988; Haas and Defago, 2005). Most of the bacteria exhibiting Plant Growth Promotory (PGP) activity belong to Gram-negative group and among these

fluorescent pseudomonads are the most widely studied (Duffy and Defago, 1999; Siddiqui and Shaukat, 2003). PGPR increase plant growth indirectly either by the suppression of well-known diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens (microorganisms which reduce plant growth but without obvious symptoms). Alternatively, PGPR may increase plant growth in other ways, for example, by associative N_2 fixation (Hong *et al.*, 1991), solubilizing nutrients such as phosphorus (Whitelaw, 2000), promoting mycorrhizal function (Garbaye, 1994), regulating ethylene production in roots (Glick, 1995), releasing phytohormones (Arshad and Frankenberger, 1991; Beyeler *et al.*, 1999), and decreasing heavy metal toxicity (Burd *et al.*, 1998). It has been suggested that the two groups should be reclassified into biocontrol plant growth-promoting bacteria (biocontrol PGPB) and PGPB (Bashan and Holguin, 1998). To date this proposal does not seem to have been widely accepted, but it does highlight the need to consider the full ecological interactions taking place following application of bacteria to seeds and roots that lead to plant growth promotion. It is also important to remember that deleterious rhizobacteria that inhibit plant growth are also known (Nehl *et al.*, 1996) which can influence such interactions.

Irrespective of mode of action, a key feature of all PGPR is that they all colonize roots to some extent. In some cases this may involve specific attachment through, pili, as with the attachment of *Pseudomonas fluorescens* 2-79 to the surface of wheat roots (Vesper, 1987). Differences in colonization between a fluorescent pseudomonad and isogenic flagella mutants prompted the conclusion that flagella are required for colonization of potato roots (de Weger *et al.*, 1995). When colonizing a root environment, an organism is confronted with a complex array of parameters such as water content, temperature, pH, soil types, composition of root exudates, mineral content and other microorganisms. Numerous studies have been conducted to assess

the contribution of each of these parameters. For example, the colonization of a fluorescent *Pseudomonas* strain in the potato rhizosphere was reported to be 10-fold greater in a sandy loam soil than in a clay loam soil (Bahme and Schroth, 1987) and another strain performed better in a sandy soil than in a peat soil (Kloepper *et al.*, 1980). Although these experiments suggest that soil texture may have a direct influence on the colonization of these strains, other indirect factors associated with these soil types could also provide underlying reasons for the differences in colonization. Colonization may involve simply root surface development but, endophytic colonization of the root is also known, and the degree of endophytic colonization depends on bacterial strain and plant type. Endophytic growth in roots has been recorded with the PGPR *Bacillus polymyxa* Pw-ZR and *Pseudomonas fluorescens* Sm3-RN on spruce (*Picea glauca x P. engelmannii*) (Shishido *et al.*, 1999), with the biocontrol strains of *Bacillus* sp. L324-92R_{12} and *P. fluorescens* 2-79RN_{10} on wheat (Kim *et al.*, 1997) and with several that induce resistance such as *Bacillus pumilus* SE34 and *P. fluorescens* 63-28 on pea (*Pisum sativum*) (Benhamou *et al.*, 1996*a*, *b*; M'Piga *et al.*, 1997), *P. fluorescens* CHA0 on tobacco (Troxler *et al.*, 1997) and *P. fluorescens* WCS417r on tomato (Duijff *et al.*, 1997). These endophytic bacteria may be in a particularly advantageous ecological position in that they may be able to grow and compete on the root surface, but also may be capable of developing within the root, relatively protected from the competitive and high-stress environment of the soil. Indeed, many seeds, roots and tubers are normally colonized by endophytic bacteria (McInroy and Kloepper, 1995; Sturz *et al.*, 1999). Any plant resistance encountered must be minimal, although, in many cases, sufficient to allow ISR to develop. The localized signaling between plant and bacteria within the root environment deserves further study. Certainly, use of mutants and promoter probe techniques are beginning to identify genes in bacteria that are important in colonization and these are often related to

nutrient uptake (Bayliss *et al.*, 1997; Roberts *et al.*, 2000). Such nutrient uptake genes may also play a role in biocontrol by aiding the uptake and metabolism of nutrients that stimulate germination of pathogen propagules (Maloney *et al.*, 1994).

The ability to colonize seeds is also an important feature for many bacterial biocontrol agents. *Pseudomonas chlororaphis* MA342 is applied to cereal seeds to control many seed and soil-borne pathogens and has been found to colonize specific areas of the seed coat (Tombolini *et al.*, 1999). After inoculation, the bacteria were found under the seed glume (or husk), but after planting they were found to colonize the glume cells epiphytically. At present, a good colonizing *Pseudomonas* strain is determined by testing its performance *in vivo*. As more knowledge on the traits needed for efficient colonization becomes available, it may be possible to select strains with defined characteristics.

1.4.3. Mechanism of action of fungal antagonist against bacterial pathogen

In the last few years there have been no clear examples of fungi used to control bacterial plant pathogens in the rhizosphere or spermosphere. The reasons for this are unclear but could perhaps indicate an area that deserves further research in the future (Whipps, 2001).

1.4.4. Mechanism of action of fungal antagonist against fungal pathogen

There are a variety of fungal species and isolates that have been examined as biocontrol agents but *Trichoderma* species clearly dominate, perhaps reflecting their ease of growth and wide host range (Whipps and Lumsden, 2001). There has been an upsurge in interest in non-pathogenic *Pythium* species, particularly *P. oligandrum* where additional modes of action have been determined recently, and a continued interest in well-established saprotrophic antagonists such as non-pathogenic *Fusarium* species, non-pathogenic binucleate *Rhizoctonia* isolates and *Phialophora* species, as well as mutualistic symbionts including mycorrhizal fungi

such as *Glomus intraradices*. The most common pathogen targets are *Pythium* species, *Fusarium* species and *Rhizoctonia solani* reflecting their world-wide importance and perhaps their relative ease of control under protected cropping systems, although numerous other pathogens have been examined. Some specific examples of the modes of action found to occur in the rhizosphere and spermosphere during interactions between fungi and fungal plant pathogens are given below.

1.4.4.1. *Competition*

There have been relatively few studies on competition for nutrients, space or infection sites between fungi in the rhizosphere and spermosphere recently. Competition for carbon, nitrogen and iron has been shown to be a mechanism associated with biocontrol or suppression of *Fusarium* wilt in several systems by non-pathogenic *Fusarium* and *Trichoderma* species (Mandeel and Baker, 1991; Couteadier, 1992; Sivan and Chet, 1989) and competition for thiamine as a significant process in the control of *Gaeumannomyces graminis* var. *tritici* by a sterile red fungus in the rhizosphere of wheat (Shankar *et al.*, 1994). Many studies have shown a relationship between increased colonization of the rhizosphere by a non-pathogen, associated subsequently, with disease suppression. This is well established for non-pathogenic strains of *Fusarium oxysporum* controlling pathogenic *F. oxysporum* on a variety of crop plants (Eparvier and Alabouvette, 1994; Postma and Rattink, 1991), hypovirulent or non-pathogenic binucleate strains of *Rhizoctonia* species to control pathogenic isolates of *R. solani* (Herr, 1995) and several fungi including *Phialophora* species, *Gaeumannomyces graminis* var. *graminis* and *Idriella bolleyi* as well as several non-sporulating fungi, to control *G. graminis* var. *tritici* (Deacon, 1974; Wong and Southwell, 1980; Kirk and Deacon, 1987; Shivanna *et al.*, 1996). As just one example, *I. bolleyi* exploits the naturally senescing cortical cells of cereal roots

during the early stages of the crop and out competes *G. graminis* var. *tritici* for infection sites and nutrients. Rapid production of spores, which are then carried down the root by water, continue the root colonization process and this is suggested to be a key feature in the establishment of the biocontrol agent on the root (Lascaris and Deacon, 1994; Allan *et al.*, 1992; Douglas and Deacon, 1994). Mycorrhizal fungi are also strong candidates for providing biocontrol through competition for space by virtue of their ecologically obligate association with roots. Ectomycorrhizal fungi because of their physical sheathing morphology may well occupy normal pathogen infection sites.

1.4.4.2. *Antibiosis*

Although production of antibiotics by fungi involved in biocontrol is a well-documented phenomenon (Howell, 1998; Sivasithamparam and Ghisalberti, 1998), there is little recent work clearly demonstrating production of antibiotics by fungi in the rhizosphere and spermosphere. Unlike the situation with biocontrol bacteria, there appear to be no detailed studies in biocontrol fungi of genes coding for antibiotic synthesis. Mutants with raised or decreased production of antibiotics are either natural spontaneous ones or generated by UV or chemical mutagenesis, with inherent problems of pleiotropic gene effects, rather than targeted gene disruption (Howell and Stipanovic, 1995; Graeme-Cook and Faull, 1991; Wilhite *et al.*, 1994; Fravel and Roberts, 1991). Consequently, clear identification and understanding of the role of antibiotics in disease control lags far behind that in bacteria and needs to be addressed. Antibiotic production by fungi exhibiting biocontrol activity has most commonly been reported for isolates of *Trichoderma / Gliocladium* (Howell, 1998) and *Talaromyces flavus* (Kim *et al.*, 1990; Fravel and Roberts, 1991) although in the last few years antibiotics have been at least partially characterized in *Chaetomium globosum* (Di Pietro *et al.*, 1992). *Minimedusa*

polyspora (Beale and Pitt, 1995) and *Verticillium biguttatum* (Morris *et al.*, 1995). Of particular interest are those studies where antibiotic production has a definite link to biocontrol. For example, *Trichoderma* (*Gliocladium*) *virens* comprises P and Q group strains, based on their antibiotic profiles (Howell, 1999). Strains of P group produce the antibiotic gliovirin which is active against *Pythium ultimum* but not against *Rhizoctonia solani* AG-4. Strains of the Q group produce the antibiotic gliotoxin which is very active against *R. solani* but less so against *P. ultimum*. Gliotoxin production by *Trichoderma* is also thought to be responsible for cytoplasmic leakage from *R. solani* observed directly on membranes in potting mix (Harris and Lumsden, 1997).

1.4.4.3. *Induced resistance*

As with bacteria, the ability of fungi to induce resistance in plants and provide biocontrol has gradually been receiving more attention in the last few years. A considerable number of fungi previously described to provide biocontrol by mechanisms such as competition, antibiosis, mycoparasitism or direct growth promotion are now thought to provide control, at least in part, by this mechanism. These include saprotrophs such as non-pathogenic *Fusarium* isolates (Hervas *et al.*, 1995; Larkin *et al.*, 1996; Postma and Luttikholt, 1996; Fuchs *et al.*, 1997, 1999; Duijff *et al.*, 1998; Larkin and Fravel, 1999), *Trichoderma* species (Yedidia *et al.*, 1999), *Pythium oligandrum* (Benhamou *et al.*, 1997; Rey *et al.*, 1998), non-pathogenic binucleate *Rhizoctonia* isolates (Poromarto *et al.*, 1998; Xue *et al.*, 1998; Jabaji-Hare *et al.*, 1999), and *Penicillium oxalicum* (de Cal *et al.*, 1997) as well as mutualistic biotrophs such as mycorrhizal fungi (Volpin *et al.*, 1995; Dugassa *et al.*, 1996; Morandi, 1996; St Arnaud *et al.*, 1997). However, not all these studies used the strict criterion of spatial separation between application of the biocontrol fungus and the challenging pathogen to define induced resistance. Indeed, some simply measured changes

in enzymes, PR-proteins or cell wall characteristics found to be induced in plants through SAR without involvement of a pathogen at all (Volpin *et al.*, 1995; Morandi, 1996; Yedidia *et al.*, 1999; Rey *et al.*, 1998). However, spatial or temporal separation experiments have indicated that increased levels of chitinases, ß-1,3 glucanases, ß-1,4 glucosidase, PR-1 protein, and peroxidase as well as cell wall appositions and phenolics may be associated with induced resistance due to fungi (Benhamou *et al.*, 1997; Fuchs *et al.*, 1997; Duijff *et al.*, 1998; Xue *et al.*, 1998; Jabaji-Hare *et al.*, 1999). The elicitors responsible for inducing resistance are not known in detail. *Trichoderma* species produce a 22 kDa xylanase that, when injected in plant tissues, will induce plant defense responses including K^+, H^+ and Ca^{2+} channeling, PR protein synthesis, ethylene biosynthesis, and glycosylation and fatty acylation of phytosterols (Bailey and Lumsden, 1998). However, whether such a system is active in roots exposed to *Trichoderma* is not known. Pectic oligogalacturonides released after hydrolysis by a non-pathogenic binucleate *Rhizoctonia* isolate may act as elicitors of defense responses in bean (*Phaseolus vulgaris*) (Jabaji-Hare *et al.*, 1999).

1.4.4.4. *Mycoparasitism*

There is a huge literature on the ability of fungi to parasitize spores, sclerotia, hyphae, and other fungal structures and many of these observations are linked with biocontrol (Jeffries and Young, 1994; van den Boogert and Deacon, 1994; Madsen and de Neergaard, 1999; Mischke, 1998; Al-Rawahi and Hancock, 1998; Davanlou *et al.*, 1999). However, most of the microscopical observations concerning mycoparasitism have come from *in vitro* studies or sterile systems (Benhamou and Chet, 1997; Inbar *et al.*, 1996; Cartwright *et al.*, 1997; Benhamou *et al.*, 1999; Davanlou *et al.*, 1999) and examples clearly demonstrating mycoparasitism in the rhizosphere or spermosphere are rare (Lo *et al.*, 1998). However, indirect population dynamic studies showed that

mycelium of *Rhizoctonia solani* in the rhizosphere of potato was a prerequisite for development of the mycoparasite *Verticillium biguttatum* (van den Boogert and Velvis, 1992) and rhizosphere competence was strongly related to biocontrol in mycoparasite isolates of *Trichoderma* species (Sivan and Harman, 1991; Peterbauer *et al.*, 1996; Thrane *et al.*, 1997; Harman and Björkman, 1998).

The process involved in mycoparasitism may consist of sensing the host, followed by directed growth, contact, recognition, attachment, penetration, and exit. Although not all these features occur in every fungal–fungal interaction, the key factor is nutrient transfer from host to mycoparasite. Directed growth of hyphae of *Trichoderma* to hyphae of *Rhizoctonia solani* prior to penetration has often been observed (Chet *et al.*, 1981). However, the factors involved in controlling directed growth in these systems have not been fully characterized. Similarly, the factors controlling recognition and binding between fungal host and parasite are not yet clear. This process may involve hydrophobic interactions or interactions between complementary molecules present on the surface of both the host and the mycoparasite such as between lectins and carbohydrates. With *Trichoderma*, there is good evidence of lectin production by both parasite and host *Corticum* (*Sclerotium*) *rolfsii* and involvement of lectins in the differentiation of mycoparasitism-related structures (Inbar and Chet, 1994; Neethling and Nevalainen, 1996). As penetration or cell wall degradation are frequently observed during mycoparasitism, great emphasis has been placed on characterizing and cloning the extracellular enzymes such as ß-1,3 glucanases, chitinases, cellulases, and proteases produced by fungal biocontrol strains (Haran *et al.*, 1996; Peterbauer *et al.*, 1996; Archambault *et al.*, 1998; Deane *et al.*, 1998). By manipulating their activity through construction of 'overproducing' mutants, enzyme-negative mutants or even transgenic plants expressing the enzyme, a role for their production in biocontrol has been implied. Several fungi have been

examined in this way including *Talaromyces flavus* (Madi *et al.*, 1997), but this type of work has essentially focused on *Trichoderma* species. Transformants of *T. harzianum*, overproducing proteinase encoded by *prb1*, provided up to a 5-fold increase in control of damping-off in cotton caused by *Rhizoctonia solani* (Flores *et al.*, 1996). Interestingly, the best protection was provided by a strain which produced only an intermediate level of proteinase activity and it was suggested that very high levels of proteinase production might cause degradation of other enzymes which are important in the mycoparasitic process (Flores *et al.*, 1996). In this regard, chitinases have received the greatest attention in mycoparasitism. The combination of chitinases as well as other cell wall-degrading enzymes differs between species and strains (Lorito, 1998) and chitinases are differently expressed during mycoparasitism (Mach *et al.*, 1999; Zeilinger *et al.*, 1999). For example, an *N*-acetyl hexosaminidase (CHIT 102) was the first to be induced in *T. harzianum* T-Y, but as early as 12 h after contact with its host *Sclerotium rolfsii*, its activity diminished, while that of another *N*-acetylhexosaminidase increased (Haran *et al.* 1996).

The final evidence for a role for cell wall-degrading enzymes in biocontrol involves the expression of fungal genes in transgenic plants. For example, an endochitinase from *Trichoderma harzianum* has been transformed into tobacco and potato and the transgenic plants showed a high level of resistance to a broad spectrum of diseases (Lorito, 1998). Similarly, transgenic apple trees expressing an endochitinase from *T. harzianum* also exhibited increased resistance to apple scab caused by *Venturia inaequalis* although plant growth was reduced (Bolar *et al.*, 2000). The potential consequently exists to combine different enzymes in transgenic plants to obtain synergistic biocontrol and these experiments are underway (Lorito, 1998; Bolar *et al.*, 2000).

1.4.4.5. *Plant growth promotion and rhizosphere competence*

The terminology associated with biocontrol in the rhizosphere and with soil–plant–microbe interactions has gradually become more complex through the use of a range of descriptive rather than mechanistic terms such as plant growth promotion and rhizosphere competence. Much like the situation with PGPR, many saprotrophic fungi, particularly certain isolates of *Trichoderma* species, can provide plant growth promotion in the absence of any major pathogens (Whipps, 1997; Inbar *et al.*, 1994). In many cases these studies are restricted to simple observations of improved plant growth with no indication of the possible mechanisms involved, although there are exceptions. For example, *Trichoderma harzianum* 1295–27 was shown to solubilize phosphate and micronutrients that could be made available to provide plant growth (Altomare *et al.*, 1999). This situation is compounded by the fact that many proven fungal biocontrol agents including some *Trichoderma* species, binucleate *Rhizoctonia* isolates and *Pythium oligandrum* can provide improved plant growth in the absence of pathogens (Windham *et al.*, 1986; Shivanna *et al.*, 1996; Wulff *et al.*, 1998; Harris, 1999). Further, colonization of the surface of the seeds or roots or behaviour as endophytes has frequently been seen to be a desirable trait for biocontrol activity (Kleifeld and Chet, 1992; Harman and Bjorkman, 1998) and although there is a clear relationship between rhizosphere colonization and biocontrol activity with some isolates of biocontrol fungi such as *Trichoderma* species, non-pathogenic *Fusarium* species, *P. oligandrum*, *Verticillium biguttatum*, and *Talaromyces flavus* (Ahmad and Baker, 1988; Couteadier *et al.*, 1993; van den Boogert and Velvis, 1992; Al-Rawahi and Hancock, 1997; Lo *et al.*, 1996; Tjamos and Fravel, 1997; Nagtzaam and Bollen, 1997; Bjorkman *et al.*, 1998), this is not always the case. Indeed transient plant growth inhibition following application of some biocontrol agents to seeds or roots is known (Wulff *et al.*, 1998; Bailey and Lumsden, 1998). Consequently, it is important to appreciate

that just because a microorganism can grow in the rhizosphere or spermosphere; it may not automatically provide biocontrol or plant growth promotion. Similarly, the converse is true. A proven biocontrol agent of a soilborne plant pathogen may not always be capable of colonizing the rhizosphere or providing plant growth promotion.

1.5. THESIS OBJECTIVE

Literature survey of the introductory chapter leads to formulate the objective stated below:

- Field survey, symtomological and histopathological study, isolation of pathogen(s) from infected region of the plant parts and pathogenicity test to confirm pathogens.
- Isolation and characterization of biocontrol agents from rhizospheric soil of healthy plant as well as from soils of different places of Darjeeling and its adjoining area.
- Study on biocontrol potentiality of the isolated organisms against the causal organism(s) of *Cymbidium* rot and finally screening out the potential biocontrol organism which will be used for further study.
- Study of the mechanism of action of the biocontrol agents.
- Optimization of different physiological factors on growth of the biocontrol agent for large-scale biomass production; powder formulation of the biocontrol agent and test for survivality.
- *In vivo* evaluation on the potentiality of the biocontrol agent.

These objectives are pursued via accumulation of literature on the subject and the aim of the following chapters of this thesis is to satisfy the said objectives.

1.6. REFERENCES

- Ahmad, J.S. and Baker, R. 1988. Rhizosphere competence of *Trichoderma harzianum*. *Phytopathology*. **77**: 182–189.

- Alexopoulos, C.J. 1935. *Gleosporium* leaf spot, a serious disease of orchids. *Phytopathology*. **25**: 435-437.

- Allan, R.H., Thorpe, C.J. and Deacon, J.W. 1992. Differential tropism to living and dead cereal root hairs by the biocontrol fungus *Idriella bolleyi*. *Physiol. Mol. Plant Pathol.* 41: 217–226.

- Al-Rawahi, A.K. and Hancock, F.G. 1997. Rhizosphere competence of *Pythium oligandrum*. *Phytopathology*. **87**: 951–959.

- Al-Rawahi, A.K. and Hancock, F.G. 1998. Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. *Plant Dis.* **82**: 1100–1106.

- Altomare, C., Norvell, W.A., Björkman, T. and Harman, G.E. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295–22. *Appl. Environ. Microbiol.* **65**: 2926–2933.

- Archambault, C., Coloccia, G., Kermasha, S. and Jabaji-Hare, S. 1998. Characterization of an endo-1,3-ß-D-glucanase produced during the interaction between the mycoparasite *Stachybotrys elegans* and its host *Rhizoctonia solani*. *Can. J. Microbiol.* 44: 989–997.

- Arditti, J. 1992. In: Fundamentals of orchid biology. pp 691. John Wiley & Sons, New York.

- Ark, P.A. 1959. *Fungal and Bacterial Diseases of Orchids*. In: The Orchids- A Scientific Survey. pp. 419-430. ed. Withner, C. L. Ronald Press Co., New York.

- Ark, P.A. and Middleton, J.T. 1949. Pythium black rot of *Cattleya*. *Phytopathol.* **39**: 1060-1064.

- Ark, P.A. and Snyder, W.C. 1951. *Plant Dis. Reptr.* **35**: 43-44.

- Arora, Y.K. 1985. *Conservation and trade of orchids*. In: Biology, Conservation and Culture of Orchid. pp. 381-386. ed. Vij, S. P. East West Press Pvt. Ltd., New Delhi.

- Arshad, M. and Frankenberger, Jr W.T. 1991. *Microbial production of plant hormones.* In: The rhizosphere and plant growth. pp. 327-334. eds. Keister, D.L. and Cregan, P.B. Kluwer Academic Publishers, Dordrecht, The Netherlands.

- Bahme, J.B. and Schroth, M.N. 1987. Spatial-temporal colonization patterns of a rhizobacterium on underground organs of potato. *Phytopathology.* 77:1093-1100.

- Bailey, D.J. and Lumsden, R.D. 1998. *Direct effects of Trichoderma and Gliocladium on plant growth and resistance to pathogens.* In: Trichoderma and Gliocladium, Vol. 2. *Enzymes, biological control and commercial applications.* pp. 185–204. eds. Harman, G.E. and Kubicek, C.P. Taylor & Francis, London.

- Baker, K.F. 1987. Evolving concepts of biological control of plant pathogens. *Annu. Rev. Phytopathol.* 25: 67-85.

- Baker, K.F. and Cook, R.J. 1974. Biological Control of Plant Pathogens, W. H. Freeman and Co, San Francisco, California. pp. 433 (Book, reprinted in 1982, Am. Phytopathol. Soc. St. Paul, Minnesota).

- Bashan, Y. and Holguin, G. 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol. Biochem.* **30:** 1225–1228.

- Bayliss, C, Bent, E., Culham, D.E., MacLellan, S., Clarke, A.J., Brown, G.L. and Wood, J.M. 1997. Bacterial genetic loci implicated in the *Pseudomonas putida* GR12-2R3 – canola mutualism: identification of an exudate-inducible sugar transporter. *Can. J. Microbiol.* **43:** 809–818.

- Beale, R.E. and Pitt, D. 1995. The antifungal properties of *Minimedusa polyspora. Mycol. Res.* **99:** 337–342.

- Benhamou, N. and Chet, I. 1997. Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and *Pythium ultimum. Appl. Environ. Microbiol.* **63:** 2095–2099.

- Benhamou, N., Belanger, R.R. and Paulitz, T.C. 1996*b*. Pre-inoculation of Ri T-DNA-transformed pea roots with *Pseudomonas fluorescens* inhibits colonization by *Pythium ultimum* Trow: an ultrastructural and cytochemical study. *Planta.* **199:** 105–117.

- Benhamou, N., Kloepper, J.W., Quadt-Hallman, A. and Tuzun, S. 1996*a*. Induction of defense-related ultrastructural modifications in pea root tissues inoculated with endophytic bacteria. *Plant Physiol.* **12:** 919–929.

- Benhamou, N., Rey, P., Cherif, M., Hockenhull, J. and Tirilly, Y. 1997. Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Phytopathology*. **87**: 108–122.

- Benhamou, N., Rey, P., Picard, K. and Tirilly, Y. 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soil-borne plant pathogens. *Phytopathology*. **89:** 506–517.

- Beyeler, M., Keel, C., Michaux, P. and Haas, D. 1999. Enhanced production of indole-3-acetic acid by a genetically modified strain of *Pseudomonas fluorescens* CHA0 affects root growth of cucumber, but does not improve protection of the plant against *Pythium* root rot. *FEMS Microbiol. Ecol.* **28:** 225–233.

- Bjorkman, T., Blanchard, L.M. and Harman, G.E. 1998. Growth enhancement of shrunken-2 sweet corn by *Trichoderma harzianum* 1295–22: effect of environmental stress. Journal of the *Am. Hort. Soc.* **123**: 35–40.

- Bolar, J.P., Norelli, J.L., Wong, K.W., Hayes, C.K., Harman, G.E. and Aldwinckle, H.S. 2000. Expression of endochitinase from *Trichoderma harzianum* in transgenic apple increases resistance to apple scab and reduces vigor. *Phytopathology*. **90:** 72–77.

- Bonsall, R.F., Weller, D.M. and Thomashow, L.S. 1997. Quantification of 2, 4-diacetylphloroglucinol produced by fluorescent *Pseudomonas* spp. *in vitro* and in the rhizosphere of wheat. *Appl. Environ. Microbiol.* **63:** 951–955.

- Bose, T.K. and Yadav, L.P. 1989. *Orchids*. In: Commercial Flowers. pp. 151-265. eds. Bose, T.K. and Yadav, L.P. Naya Prokash, Calcutta.

- Burd, G.I., Dixon, D.G. and Glick, B.R. 1998. A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. *Appl. Environ. Microbiol.* **64:** 3663–3668.

- Burnett, H. C. 1965. Orchid Diseases. Vol. 1, No. 3. State of Fla. Dept. of Agriculture.

- Buysens, S. Heungens, K. Poppe, J. and Hofte, M. 1996. Involvement of pyochelin and pyoverdin in suppression of pythim-induced damping off of tomato by *Pseudomonas aeruginosa* 7 NSK2. *Appl. Environ. Microbiol.* **62:** 865-871.

- Cartwright, R.D., Webster, R.K., Wick, C.M. 1997. *Ascochyta mycoparasitica*sp. nov., a novel mycoparasite of *Sclerotium oryzae* in California rice fields. *Mycologia*. **89:** 163–172.

- Chen C, Belanger R.R., Benhamou, N. and Paulitz, T.C. 1998. Induced systemic resistance (ISR) by *Pseudomonas* spp. impairs pre- and post-infection development of *Pythium aphanidermatum* on cucumber roots. *Eur. J. Plant Pathol.* **104:** 877–886.

- Chernin, L., Ismailov, Z., Haran, S. and Chet, I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl. Environ. Microbiol.* **61:** 1720–1726.

- Chet, I., Harman, G.E. and Baker, R. 1981. *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecol.* **7:** 29–38.

- Chin-A-Woeng, T.F.C., Bloemberg, G.V. and van der Bij, A.J. 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL 1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol. Plant-Microbe Interact.* **11:** 1069–1077.

- Chu, O. 2004. Taiwan floriculturists vie for top spot in orchid market. *TaiwanJournal.*http://www.taiwan.com.au/Polieco/Industry/Agricultur/20041029.html

- Ciferri, R. 1926. The pathogenic capacities of fungi causing anthracnose of orchids. *Rivista Patol. Veg.* **16:** 1-16.

- Cook, R.J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Ann. Rev. Phytopathol.* **31:** 53–80.

- Couteadier, Y. 1992. *Competition for carbon in soil and rhizosphere, a mechanism involved in biological control of Fusarium wilts.* In: Biological control of plant diseases. pp. 99–104. eds. Tjamos, E.C., Papavizas, A.C. and Cook, R.J. Plenum Press, New York.

- Couteadier, Y., Daboussi, M.J., Eparvier, A., Langin, R. and Orcival, J. 1993. The GUS gene fusion system (*Escherichia coli* ß-D-glucuronidase gene), a useful tool in studies of root colonization by *Fusarium oxysporum*. *Appl. Environ. Microbiol.* **59:** 1767–1773.

- Davanlou, M., Madsen, A.M., Madsen, C.H. and Hockenhull, J. 1999. Parasitism of macroconidia, chlamydospores and hyphae of *Fusarium culmorum* by mycoparasitic *Pythium* species. *Plant Pathol.* **48:** 352–359.

- de Cal, A., Pascual, S. and Melgarejo, P. 1997. Involvement of resistance induction by *Penicillum oxalicum* in the biocontrol of tomato wilt. *Plant Pathol.* **46:** 72–79.

- de Meyer, G., Capieau, K., Audenaert, K., Buchala, A., Metraux, J.P. and Hofte, M. 1999. Nanogram amounts of salicylic acid produced by the rhizobacterium

Pseudomonas aeruginosa 7NSK2 activate the systemic acquired resistance pathway in bean. *Mol. Plant-Microbe Interact.* **12:** 450–458.

- de Weger, L.A., van der Bij, A.J., Dekkers, L.C., Simons, M., Wijffelman, C.A. and Lugtenberg, B.J.J. 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiol. Eco.* **17:** 221–228.

- Deacon, J.W. 1974. Further studies on *Phialophora radicicola* and *Gaeumannomyces graminis* on roots and stem bases of grasses and cereals. *Trans. Br. Mycol. Soc.* **63:** 307–327.

- Deacon, J.W. 1994. *Rhizosphere constraints affecting biocontrol organisms applied to seeds.* In: Seed treatment: prospects and progress. BCPC Monograph 57. Thornton Heath: pp. 315-327. British Crop Protection Council.

- Deane, E.E., Whipps, J.M., Lynch, J.M. and Peberdy, J.F. 1998. The purification and characterization of a *Trichoderma harzianum* exochitinase. *Bioenergetics.* **1383:** 101–110.

- Di Pietro, A., Cut-Rella, M., Pachlatko, J.P. and Schwinn, F.J. 1992. Role of antibiotics produced by *Chaetomium globosum* in biocontrol of *Pythium ultimum*, a causal agent of damping-off. *Phytopathology.* **82:** 131–135.

- Douglas, L.I. and Deacon, J.W. 1994. Strain variation in tolerance of water stress by *Idriella* (*Microdochium*) *bolleyi*, a biocontrol agent of cereal root and stem base pathogens. *Biocont. Sci. Technol.* **4:** 239–249.

- Duff, J. and Daly, A. 2002. Orchid Diseases in the Northern Territory. *Agnote.* No. I3: ISSN No: 0157-8243. https://transact.nt.gov.au/ebiz/dbird/TechPublications.ns.

- Duffy, B.K. and Defago, G. 1999. Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl. Environ. Microbiol.* **65:** 2429–2438.

- Dugassa, G.D., von Alten, H. and Schonbeck, F. 1996. Effects of arbuscular mycorrhiza (AM) on health of *Linum usitatissimum* L. infected by fungal pathogens. *Plant Soil.* **185:** 173–182.

- Duijff, B.J., Gianinazzi-Pearson, V. and Lemanceau, P. 1997. Involvement of the outer membrane lipopolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. *New Phytol.* **135:** 325–334.

- Duijff, B.J., Meijer, J.W., Bakker, P.A.H.M. and Schippers, B. 1993. Siderophore-mediated competition for iron and induced resistance in the suppression of *Fusarium* wilt of carnation by fluorescent *Pseudomonas* spp. *Neth. J. Plant Pathol.* **99:** 277–289.

- Duijff, B.J., Pouhair, D., Olivain, C., Alabouvette, C. and Lemanceau, P. 1998. Implication of systemic induced resistance in the suppression of *Fusarium* wilt of tomato by *Pseudomonas fluorescens* WCS417r and by non-pathogenic *Fusarium oxysporum* Fo47. *Eur. J. Plant Pathol.* **104:** 903–910.

- Dunne, C., Crowley, J. J., Moenne-Loccoz, Y., Dowling, D. N., de Bruijn, F. J. and O'Gara, F. 1997. Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. *Microbiology.* **143:** 3921-3931.

- Dunne, C., Moenne-Loccoz, Y., McCarthy, J., Higgins, P., Powell, J., Dowling, D. N. & O'Gara, F. 1998. Combining proteolytic and phloroglucinol-producing bacteria for improved biocontrol of *Pythium*-mediated damping-off of sugar beet. *Plant Pathol.* **47:** 299-307.

- DuPuy, D. and Cribb, P. 1988. In: The genus *Cymbidium.* Timber Press, Portland Oregon.

- Elad, Y. 1996. Mechanism involved in the biological control of *Botrytis cinerea* incited diseases, *Eur. J. Plant Pathol.* **102:**719

- El-Tarabily, K.A., Hardy, G.E.StJ., Sivasithamparam, K., Hussein, A.M. and Kurtboke, D.I. 1997. The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. *New Phytol.* **137:** 495–507.

- El-Tarabily, K.A., Sykes, M. L., Kurtboke, I.D., Hardy, G.E.StJ., Barbosa, A.M. and Dekker, R.F.H. 1996. Synergistic effects of a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of *Phytophthora cinnamomi* root rot of *Banksia grandis. Can. J. Bot.* **74:** 618–624.

- Eparvier, A. and Alabouvette, C. 1994. Use of ELISA and GUS-transformed strains to study competition between pathogenic *Fusarium oxysporum* for root colonization. *Biocont. Sci. Technol.* **4:** 35–47.

- Flores, A., Chet, I. and Herrera-Estrella, A. 1996. Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb1*. *Curr. Genet.* **31:** 30–37.

- Fravel, D.R. 1999. Web site for the USDA/ARS biocontrol of plant diseases laboratory http://www.barc.usda.gov/psi/bpdl/bioprod.htm.

- Fravel, D.R. and Roberts, D.P. 1991. *In situ* evidence for the role of glucose oxidase in the biocontrol of *Verticillium* wilt by *Talaromyces flavus*. *Biocont. Sci. Technol.* **1:** 91–99.

- Fuchs, J.G., Moenne-Loccoz, Y. and Defago, G. 1997. Non-pathogenic *Fusarium oxysporum* strain Fo47 induces resistance to *Fusarium* wilt in tomato. *Plant Dis.* **81:** 492–496.

- Fuchs, J.G., Moenne-Loccoz, Y. and Defago, G. 1999. Ability of non-pathogenic *Fusarium oxysporum* Fo47 to protect tomato against *Fusarium* wilt. *Biol. Control.* **14:** 105–110.

- Garbaye, J. 1994. Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol.* **128:** 197–210.

- Gilbert, J.C., Mainland, G.B. and Lohman, M.L. 1950. *Bull. Pac. Orchid Soc.* **8:** 289-293.

- Glick, B.R. 1995. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* **41:** 109–117.

- Govindasamy, V. and Balasubramanium, R. 1989. Biological control of groundnut rust, *Puccinia arachidis* by *Trichoderma harzianum*. *J. Plant Dis. Prot.* **96:** 337-345.

- Graeme-Cook, K.A. and Faull, J.L. 1991. Effect of ultraviolet-induced mutants of *Trichoderma harzianum* with altered production on selected pathogens *in vivo*. Canadian *J. Microbiol.* **37:** 659–664.

- Haas, D. and Defago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads *Nature Rev. Microbiol.* **3:** 307-319

- Handelsman, J. and Stabb, E. V. 1996. Biocontrol of Soilborne Plant Pathogens. *Plant Cell.* **8:** 1855-1869.

- Haran, S., Schickler, H. and Chet, I. 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiology.* **142:** 2321–2331.

- Harman, G.E. and Bjorkman, T. 1998. *Potential and existing uses of Trichoderma and Gliocladium for plant disease control and plant growth enhancement.* In: Trichoderma *and* Gliocladium, Vol. 2. *Enzymes,* biological control and commercial applications. pp. 229-265. eds. Harman, G.E. and Kubicek, C.P. Taylor & Francis, London.

- Harris, A.R. 1999. Plant growth promotion by binucleate *Rhizoctonia* and bacterial isolates in monoxenic cultures. *Microbiol. Res.* **154:** 71–74.

- Harris, A.R. and Lumsden, R. D. 1997. Interactions of *Gliocladium virens* with *Rhizoctonia solani* and *Pythium ultimum* in non-sterile potting medium. *Biocont. Sci. Technol.* **7:** 37–47.

- Herr, L.J. 1995. Biological control of *Rhizoctonia solani* by binucleate *Rhizoctonia* spp. and hypovirulent *R. solani* agents. *Crop Prot.* **14:** 179–186.

- Hervas, A., Trapero-Casas, J.L. and Jimenez-Díaz, R.M. 1995. Induced resistance against *Fusarium* wilt of chickpea by non-pathogenic races of *Fusarium oxysporum* f. sp. *ciceris* and non-pathogenic isolates of *F. oxysporum*. *Plant Dis.* **79:** 1110–1116.

- Hill, C.F. 2004. First report of *Phytophthora multivesiculata* on cymbidium orchids in New Zealand. *Aus. Plant Pathol.* **33:** 603–604.

- Hoffland, E., Pieterse, C.M.J., Bik, L. and Van Pelt, J.A. 1995. Induced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins. Physiological and *Mol. Plant Pathol.* **46:** 309–320.

- Hong, Y., Pasternak, J.J. and Glick, B.R. 1991. Biological consequences of plasmid transformation of the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Can. J. Microbiol.* **37:** 796–799.

- Hooker, J.D. 1890. *Cymbidium and Cyperorchis.* In: Flora of British India 6. pp. 8–15, London.

- Hori, S. 1911. A bacterial disease of tropical orchids. *Centralbl. Bakt. U. Infektionkr. Abt.* **31:** 85-92.

- Howell, C.R. 1998. *The role of antibiosis in biocontrol.* In: Trichoderma and Gliocladium, Vol. 2. Enzymes, biological control and commercial applications. pp. 173–184. eds. Harman, G.E and Kubicek, C.P. Taylor & Francis, London.

- Howell, C.R. 1999. Selective isolation from soil and separation *in vitro* of P and Q strains of *Trichoderma virens* with differential media. *Mycologia.* **91:** 930–934.

- Howell, C.R. and Stipanovic, R.D. 1995. Mechanisms in the biocontrol of *Rhizoctonia solani*-induced cotton seedling disease by *Gliocladium virens*: antibiosis. *Phytopathology.* **85:** 469–472.

- Hu, J. S., Ferreira, S., Wang, M., and Xu, M. Q. 1993. Detection of cymbidium mosaic virus, odontoglossum ringspot virus, tomato spotted wilt virus, and potyviruses infecting orchids in Hawaii. *Plant Dis.* **77**: 464-468.

- Ilieva, E., Man in 't Veld, W.A., Veenbaas-Rijks W. and Pieters, R. 1998. *Phytophthora multivesiculata*, a new species causing rot in *Cymbidium*. *Eur. J. Plant Pathol.* **107:** 677-684.

- Inbar, J. and Chet I. 1994. A newly isolated lectin from the plant pathogenic fungus *Sclerotium rolfsii*: purification, characterization and role in mycoparasitism. *Microbiology*. **140:** 651–657.

- Inbar, J., Abramsky, M., Cohen, D. and Chet, I. 1994. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. *Eur. J. Plant Pathol.* **100:** 337–346.

- Inbar, J., Menendez, A. and Chet, I. 1996. Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. Soil Biology Biochemistry **28:** 757–763.

- Jabaji-Hare, S., Chamberland, H. and Charest, P.M. 1999. Cell wall alterations in hypocotyls of bean seedlings protected from *Rhizoctonia* stem canker by a binucleate *Rhizoctonia* isolate. *Mycol. Res.* **103:** 1035–1043.

- Jain, S.K. 1985. *Orchid wealth (?) of India.* In: Biology, Conservation and Culture of Orchid. pp. 319-322. ed. Vij, S. P. East West Press Pvt. Ltd., New Delhi.

- Janisiewicz, W.J., Tworkoski, T.J. and Sharer. C. 2000. Characterizing the Mechanism of Biological Control of Postharvest Diseases on Fruits with a Simple Method to Study Competition for Nutrients. *Phytopathology*. **90:** 1196-1200

- Jeffries, P. and Young, T.W.K. 1994. Interfungal parasitic relationships. Wallingford, UK: CAB International.

- Jensen, D.D. 1952a. Virus diseases of orchids. I. *Calif. Agric.* **6 (1):** 3,15.

- Jensen, D.D. 1952b. Virus diseases of orchids. II. *Calif. Agric.* **6 (2):** 7, 15, 16

- Kang, Y., Carlson, R., Tharpe, W. and Schell, M.A. 1998. Characterization of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani*. *Appl. Environ. Microbiol.* **64:** 3939–3947.

- Kazunori, I. and Takayuki, A. 2000. New Leaf Spot Disease of Cymbidium Species Caused by Fusarium subglutinans and Fusarium proliferatum. *J. Gen. Plant Pathol.* **66:** 213-218.

- Khmel, I.A., Sorokina, T.A., Lemanova, N.B., Lipasova, V.A., Metlitski, O.Z., Murdeinaya, T.V. and Chernin, L.S. 1998. Biological control of crown gall in grapevine and raspberry by two *Pseudomonas* spp. with a wide spectrum of antagonistic activity. *Biocont. Sci. Tech.* **8:** 45–57.

- Kim, B.S., Moon, S.S. and Hwang, B.K. 1999. Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. *Can. J. Bot.***77:** 850–858.

- Kim, D.S., Weller, D.M. and Cook, R.J. 1997. Population dynamics of *Bacillus* sp. L324–92R_{12} and *Pseudomonas fluorescens* 2–79RN_{10} in the rhizosphere of wheat. *Phytopathology*. **87:** 559–564.

- Kim, K.K., Fravel, D.R. and Papavizas, G.C. 1990. Production, purification and properties of glucose oxidase from the biocontrol fungus *Talaromyces flavus*. *Can. J. Microbiol.* **36:** 199–205.

- Kirk, J.J. and Deacon, J.W. 1987. Control of the take-all fungus *Microdochium bolleyi* and interactions involving *M. bolleyi*, *Phialophora graminicola* and *Periconia macrospinosa* on cereal roots. *Plant Soil.* **98:** 231–237.

- Kleifeld, O. and Chet, I. 1992. *Trichoderma harzianum*—interaction with plants and effect on growth response. *Plant Soil* **144:** 267–272.

- Kloepper, J. W., Leong, J., Teintze, M. and Schroth, M. N. 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. *Nature* (London) **286:** 885-886.

- Kloepper, J., Tuzun, S. and Ku, J. 1992. Proposed definitions related to induced disease resistance. *Biocont. Sci.Technol.* **2:** 347–349.

- Knoester, M., Pieterse, C.M.J., Bol, J.F. and van Loon, L.C. 1999. Systemic resistance in *Arabidopsis* induced by rhizobacteria requires ethylene-dependent signaling at the site of application. *Mol. Plant-Microbe Interact.* **12:** 720–727.

- Kraus, J. and Loper, J.E. 1995. Characterization of a genomic region required for production of the antibiotic pyoluteorin by the biological control agent *Pseudomonas fluorescens* Pf-5. *Appl. Environ. Microbiol.* **61:** 849–854.

- Larkin, R.P. and Fravel, D.R. 1999. Mechanisms of action and dose-response relationships governing biological control of *Fusarium* wilt of tomato by non-pathogenic *Fusarium* spp. *Phytopathology*. **89:** 1152–1161.

- Larkin, R.P., Hopkins, D.L. and Martin, F.N. 1996. Suppression of *Fusarium* wilt of watermelon by non-pathogenic *Fusarium oxysporum* and other microorganisms recovered from a disease-suppressive soil. *Phytopathology*. **86:** 812–819.

- Lascaris, D. and Deacon, J.W. 1994. *In vitro* growth and microcycle conidiation of *Idriella bolleyi*, a biocontrol agent of cereal pathogens. *Mycol. Res.* **98:** 1200–1206.

- Laws, N. 2003. Orchid commerce around the world. In: Flora Culture International. Bell Publishing. United States. http://www.floraculture intl.com/archive/articles /293.asp.

- Leeman, M., den Ouden, F.M., van Pelt, J.A., Cornelissen, C., Matamala-Garros, A., Bakker. P.A.H.M. and Schippers, B. 1996*a*. Suppression of *Fusarium* wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. *Eur. J. Plant Pathol.* **102:** 21–31.

- Leeman, M., den Ouden, F.M., van Pelt, J.A., Dirkx, F.P.M., Steijl, H., Bakker, P.A.H.M. and Schippers, B. 1996*b*. Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. *Phytopathology*. **86:** 149–155.

- Leeman, M., van Pelt, J.A., den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M. and Schippers, B. 1995. Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. *Phytopathology*. **85:** 1021–1027.

- Lewis, W.H. and Elvin-Lewis, M.P.F. 1977. Medical Botany: Plants affecting man's health. Wiley, New York.

- Limber, D. P. and Friedman, B. A. 1943. *Erwinia carotovora*, the cause of a soft rot in in orchids, *Cattleya* sp. *Phytopathology*. **33:**80-82.

- Lo, C.T., Nelson, E.B. and Harman, G.E., 1996. Biological control of turfgrass diseases with a rhizosphere competent strain of *Trichoderma harzianum*. *Plant Dis.* **80:** 736–741.

- Lo, C.T., Nelson, E.B., Hayes, C.K. and Harman, G.E. 1998. Ecological studies of transformed *Trichoderma harzianum* strain 1295–22 in the rhizosphere and on the phylloplane of creeping bentgrass. *Phytopathology*. **88:** 129–136.

- Loper, J.E. and Buyer, J.S. 1991. Siderophores in microbial interactions on plant surfaces. *Mol. Plant-Microbe Interact.* **4:** 5–13.

- Loper, J.E. and Henkels, M.D. 1999. Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl. Environ. Microbiol.* **65:** 5357–5363.

- Lorito, M. 1998. *Chitinolytic enzymes and their genes.* In: *Trichoderma* and *Gliocladium*, Vol. 2. Enzymes, biological control and commercial applications. pp. 73-99. eds. Harman, G.E. and Kubicek, C.P. Taylor & Francis Ltd., London.

- Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S., Woo, S.L., Zeilinger, S., Kullnig, C.M., Lorito, M. and Kubicek, C.P. 1999. Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum* P1) is triggered by different regulatory signals. *Appl. Environ. Microbiol.* **65:** 1858–1863.

- Madi, L., Katan, T., Katan, J. and Henis, Y. 1997. Biological control of *Sclerotium rolfsii* and *Verticillium dahliae* by *Talaromyces flavus* is mediated by different mechanisms. *Phytopathology.* **87:** 1054–1060.

- Madsen, A.M., de Neergaard, E. 1999. Interactions between the mycoparasite *Pythium oligandrum* and sclerotia of the plant pathogen *Sclerotinia sclerotiorum. Eur. J. Plant Pathol.* **105:** 761–768.

- Maloney, A.P., Nelson, E.B. and van Kijk, K. 1994. *Genetic complementation of a biocontrol-negative mutant of Enterobacter cloacae reveals a potential role of pathogen stimulant inactivation in the biological control of Pythium seed rots.* In: Improving plant productivity with rhizosphere bacteria. pp. 135-137. eds Ryder, M.H., Stephens, P.M. and Bowen, G.D. CSIRO, Division of Soil, Adelaide, Australia.

- Mandeel, Q. and Baker, R. 1991. Mechanisms involved in biological control of *Fusarium* wilt on cucumber with strains of non-pathogenic *Fusarium oxysporum. Phytopathology.* **81:** 462–469.

- Marilley, L. and Aragno, M. 1999. Phytogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Appl. Soil Eco.* **13:** 127–136.

- Mathivananm, N., Srinivasan, K. and Chelliah, S. 2000. Field evaluation of *Trichoderma viride* Pers. ex. S. F. Gray and *Pseudomonas fluorescens* Migula against foliar diseases of groundnut and sunflower. *J. Biol. Control.* **14:** 31-34.

- Maurhofer, M., Hase, C., Meuwly, P.H., Metraux, J.P. and Defago G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root colonizing *Pseudomonas fluorescens* strain CHA0: influence of the *gacA* gene and of pyoverdine production. *Phytopathology.* **84:** 139–146.

- McInroy, J.A., Kloepper, J.W. 1995. Population dynamics of endophytic bacteria in field-grown sweet corn and cotton. *Can. J. Microbiol.* **41:** 895–901.

- Mehra, P. N. and Vij, S. P. 1974. Some observations on ecological adaptations and distribution pattern of the east Himalayan orchids. *Am. Orchid Soc. Bull.* **43:** 301-315.

- Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science.* **250:** 1004–1006.

- Milner, J.L., Silo-Suh, L., Lee, J.C., He, H., Clardy, J. and Handelsman, J. 1996. Production of kanosamine by *Bacillus cereus* UW85. *Appl. Environ. Microbiol.* **62:** 3061–3065.

- Mischke, S. 1998. Mycoparasitism of selected sclerotia-forming fungi by *Sporidesmium sclerotivorum. Can. J. Bot.* **76:** 460–466.

- Mitchell, R. and Hurwitz E. 1965. Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. *Phytopathology.* **55,** 156–158.

- Morandi, D. 1996. Occurrence of phytoalexins and phenolic compounds in endomycorrhizal interactions, and their potential role in biological control. *Plant Soil.* **185:** 241–251.

- Morris, R.A.C., Ewing, D., Whipps, J.M. and Coley-Smith, J.R. 1995. Antifungal hydroxymethyl-phenols from the mycoparasite *Verticillium biguttatum. Phytochemistry.* **39:** 1043–1048.

- M'Piga, P., Belanger, R.R., Paulitz, T.C. and Benhamou, N. 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 63–28. *Physiol. Mol. Plant Pathol.* **50:** 301–320.

- Munsi, P.S., Mandal, T. and Rowchowdhury, N. 2004 Performances of different *Cymbidium* spp. and hybrids under polyhouse in Darjeeling hills. *Acta Hort.* (ISHS) **659:** 483-490. http://www.actahort.org/books/659/659_64.htm.

- Nagtzaam, M.P.M. and Bollen, G.J. 1997. Colonization of roots of eggplant and potato by *Talaromyces flavus* from coated seed. *Soil Biol. Biochem.* **29:** 1499–1507.

- Nakayama, T., Homma, Y., Hashidoko, Y., Mizutani, J. and Tahara, S. 1999. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. *Appl. Environ. Microbiol.* **65:** 4334–4339.

- Neeno-Eckwall, E.C. and Schottel, J.L. 1999. Occurrence of antibiotic resistance in the biological control of potato scab disease. *Biol. Control.* **16:** 199–208.

- Neethling, D. and Nevalainen, H. 1996. Mycoparasitic species of *Trichoderma* produce lectins. *Can. J. Microbiol.* **42:** 141–146.

- Nehl, D.B., Allen, S.J., Brown JF. 1996. Deleterious rhizosphere bacteria: an integrating perspective. *Appl. Soil Ecol.* **5:** 1–20.

- Neilands, J. B. 1981. *Microbial iron transport compounds (siderophores) as chelating agent.* In: Development of Iron Chelators for Clinical Use. pp. 39 eds. Martell, A. E., Anderson, W. J. and Badman, D. G. Elseyvier Press, North Hilland Amsterdam.

- Nelson, E.B., Chao, W.L., Norton, J.M., Nash, G.T. and Harman, G.E. 1986. Attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum*: possible role in biological control of *Pythium* pre-emergence damping-off. *Phytopathology.* **76:** 327–335.

- Nielsen, M.N., Sorensen. J., Fels, J and Pedersen, H. C. 1998. Secondary metabolite and endochitinase dependent antagonism toward plant pathogenic microfungi of *Pseudomonas fluorescens* isolated from sugar beet rhizosphere. *Appl. Environ. Microbiol.* 64: 3563-3569.

- Nielsen, T. H., Sorensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M. and Sorensen. J. 2002. Antibiotic and Biosurfactant Properties of Cyclic Lipopeptides Produced by Fluorescent *Pseudomonas* spp. from the Sugar Beet Rhizosphere. *Appl. Environ. Microbiol.* **68:** 3416–3423.

- Nowak-Thompson B, Chaney N, Wing JS, Gould SJ, Loper JE. 1999. Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* **181:** 2166–2174.

- O'Sullivan, D.B. and O'Gara, F. 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiol. Rev.* **56:** 662–676.

- Ongena, M., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T.C., Cornelis, P., Koedam, N. and Belanger, R.R. 1999. Protection of cucumber against *Pythium* root rot by fluorescent pseudomonads: predominant role of induced resistance over siderophores and antibiosis. *Plant Pathol.* **48:** 66–76.

- Pathak, P., Sehgal, R.N., Shekhar, N., Sharma, M and Sood, A. 2001. In: Orchids: Science and Commerce. eds. Dun, D., Singh, B. and Singh, M. P. Vedams eBooks (P) Ltd., New Delhi.

- Peterbauer, C.K., Lorito, M., Hayes, C.K., Harman, G.E. and Kubicek, C.P. 1996. Molecular cloning and expression of the *nag1* gene (*N*-acetyl-ß-D-glucosaminidase-encoding gene) from *Trichoderma harzianum* P1. *Curr. Genet.* **30:** 325–331.

- Pieterse CMJ, Van Wees SCM, Hoffland E, Van Pelt JA, Van Loon LC. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell.* **8:** 1225–1237.

- Pirone, P. P., B. O. Dodge, and H. W. Rickett. 1960. In: Diseases and pests of ornamental plants. pp. 776. The Ronald Press Co., New York.

- Pleban, S., Chernin, L. and Chet, I. 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus. Lett. Appl. Microbiol.* **25:** 284–288.

- Poromarto, S.H., Nelson, B.D., Freeman, T.P. 1998. Association of binucleate *Rhizoctonia* with soybean and mechanism of biocontrol of *Rhizoctonia solani. Phytopathology.* **88:** 1056–1067.

- Postma, J. and Luttikholt, A.J.G. 1996. Colonization of carnation stems by a nonpathogenic isolate of *Fusarium oxysporum* and its effect on *Fusarium oxysporum* f. sp. *dianthi. Can. J. Bot.* **74:** 1841–1851.

- Postma, J. and Rattink H. 1991. Biological control of *Fusarium* wilt of carnation with non-pathogenic *Fusarium* isolates. *Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent.* **56:** 179–183.

- Powell, J.F., Vargas, J.M., Nair, M.G., Detweiler, A.R. and Chandra, A. 2000. Management of dollar spot on creeping bentgrass with metabolites of *Pseudomonas aureofaciens* (TX-1). *Plant Dis.* **84:** 19–24.

- Raaijmakers, J.M., Bonsall, R.F. and Weller, DM. 1999. Effect of population density of *Pseudomonas fluorescens* on production of 2, 4-diacetylphloroglucinol in the rhizosphere of wheat. *Phytopathology.* **89:** 470–475.

- Raaijmakers, J.M., Weller, D.M. Thomashow, L.S. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.* **63:** 881–887.

- Rajan, P.P., Sarma, Y.R. and Anandaraj, M. 2002. Management of foot rot disease of black pepper with *Trichoderma* species. *Indian Phytopathol.* **55:** 17 – 21.

- Rey, P., Behamou, N., Wulff, E. and Tirilly, Y. 1998. Interactions between tomato (*Lycopersicon esculentum*) root tissues and the mycoparasite *Pythium oligandrum. Physiol. Mol. Plant Pathol.* **53:** 105–122.

- Roberts, D.P., Dery, P.D., Yucel, I. and Buyer, J.S. 2000. Importance of *pfkA* for rapid growth of *Enterobacter cloacae* during colonization of crop seeds. *Appl. Environ. Microbiol.* **66:** 87–91.

- Ryan, A.D. and Kinkel, L.L. 1997. Inoculum density and population dynamics of suppressive and pathogenic *Streptomyces* strains and their relationship to biological control of potato scab. *Biol. Control.* **10:** 180–186.

- Ryder, M. 1994. Key issues in the deliberate release of genetically manipulated bacteria. *FEMS Microbiol. Ecol.* **15:** 139–145.

- Shankar, D.I., Kurtboke, D.I., Gillespie-Sasse, L.M.J., Rowland, C.Y. and Sivasithamparam, K. 1994. Possible roles of competition for thiamine, production of inhibitory compounds and hyphal interactions in suppression of the take-all fungus by a sterile red fungus. *Can. J. Microbiol.* **40:** 478–483.

- Sharma, J. 1996. In: Orchids of India: Commercialization and Conservation. Vedams eBooks (P) Ltd., New Delhi.

- Sherpa, A.R., Hallan Vipin, Raja Ram, Vij S.P., Pathak P, Garg I.D. and Zaidi, A.A. 2003. First report of Cymbidium mosaic virus on Cymbidiums in India. *Plant Pathol.* **52:** 788.

- Shishido, M., Breuil, C., Chanway, C.P. 1999. Endophytic colonization of spruce by plant growth-promoting rhizobacteria. *FEMS Microbiol. Ecol.* **29:** 191–196.

- Shivanna, M.B., Meera, M.S. and Hyakumachi, M. 1996. Role of root colonization ability of plant growth promoting fungi in the suppression of take-all and common root rot of wheat. *Crop Prot.* **15:** 497–504.

- Siddiqui, K. and Shaukat, S.S. 2003. Endophytic bacteria: prospects and opportunities for the biological control of plant-parasitic nematodes. *Nematologia Mediterranea.* **31:** 111-120.

- Sivan, A. and Harman, G.E. 1991. Improved rhizosphere competence in a protoplast fusion progeny of *Trichoderma harzianum. J. Gen. Microbiol.* **137:** 23–29.

- Sivan, A. and Chet, I. 1989. The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonizaton. *Phytopathology.* **79:** 198–203.

- Sivasithamparam, K. and Ghisalberti, E.L. 1998. *Secondary metabolism in Trichoderma and Gliocladium.* In: Trichoderma *and* Gliocladium, Vol. 1. Basic biology, taxonomy and genetics. pp.139–191. eds. Kubicek, C.P. and Harman, G.E. Taylor & Francis Ltd., London.

- Srivastava, L.S. 1999. *Glomerella cingulata* (Stonem) Spauld & Schnenk: A potential Fungal Pathogen of Orchids in Sikkim. *J. Hill. Res.* **12:** 148.

- St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., Fortin, J.A. 1997. Inhibition of *Fusarium oxysporum* f.sp. *dianthi* in the non-VAM species *Dianthus caryophyllus* by co-culture with *Tagetes patula* companion plants colonized by *Glomus intraradices*. *Can. J. Bot.* **7:** 998-1005.

- Sticher, L., Mauch-Mani, B., Metraux, J.P. 1997. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35:** 235–270.

- Sturz, A.V., Christie, B.R., Matheson, B.G., Arsenault, W.J. and Buchanan, N.A., 1999. Endophytic bacterial communities in the periderm of potato tubers and their potential to improve resistance to soil-borne plant pathogens. *Plant Pathol.* **48:** 360–369.

- Takahashi, Y., Takahashi, K., Sato, M., Watanabe, K. and Kawano, T. 1997. Bacterial leaf rot of *Odontioda* orchids caused by *Enterobacter cloacae*. *Ann. Phytopatho. Soc. Jap.* **63:** 164-169.

- Tanaka, S., Nishii, H., Ito, S., Kameya-Iwaki, M. and Sommartya, P. 1997. Detection of cymbidium mosaic potexvirus and odontoglossum ringspot tobamovirus from Thai orchids by rapid immunofilter paper assay. *Plant Dis.* **81:** 167-170.

- Thomashow, L.S., Weller, D.M., Bonsall, R.F. and Pierson III, L.S.P. 1990. Production of the antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* species in the rhizosphere of wheat. *Appl. Environ. Microbiol.* **56:** 908–912.

- Thrane, C., Tronsmo, A. and Funck Jensen, D. 1997. Endo-1,3-ß-glucanase and cellulase from *Trichoderma harzianum*: purification and partial characterization, induction of and biological activity against plant pathogenic *Pythium* spp. *Eur. J. Plant Pathol.* **103:** 331–344.

- Tjamos, E.C. and Fravel, D.R. 1997. Distribution and establishment of the biocontrol fungus *Talaromyces flavus* in soil and on roots of solanaceous crops. *Crop Prot.* **16:** 135–139.

- Tombolini, R., van der Gaag, D.J., Gerhardson, B. and Jansson, J.K. 1999. Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA 342 on barley seeds visualized by using green fluorescent protein. *Appl. Environ. Microbiol.* **65:** 3674–3680.

- Troxler, J., Berling, C.H., Moenne-Loccox, Y., Keel, C. and Defago, G. 1997. Interactions between the biocontrol agent *Pseudomonas fluorescens* CHA0 and

Thielaviopsis basicola in tobacco roots observed by immunofluorescence microscopy. *Plant Pathol.* 46: 62–71.

- Valois, D., Fayad, K., Barbasubiye, T., Garon, M., Dery, C., Brzezinski, R. and Beaulieu, C. 1996. Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of rashberry root rot. *Appl. Environ. Microbiol.* 62: 1630–1635.

- van den Boogert, P.H.J.F. and Deacon, J.W. 1994. Biotrophic mycoparasitism by *Verticillum biguttatum* on *Rhizoctonia solani*. *Eur. J. Plant Pathol.* 100: 137–156.

- van den Boogert, P.H.J.F. and Velvis, H. 1992. Population dynamics of the mycoparasite *Verticillium biguttatum* and its host, *Rhizoctonia solani*. *Soil Biol. Biochem.* 24: 157–164.

- van Loon, L.C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. *Eur. J. Plant Pathol.* 103: 753–765.

- van Peer, R., Niemann, G.J. and Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. WCS417r. *Phytopathology.* 81: 728–734.

- van Wees, S.C.M., Pieterse, C.M.J., Trijssenaar, A., Van't Westende, Y., Hartog, F. and van Loon, L.C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* 10: 716–724.

- Varmah, J. C. and Sahni, K. C. 1976. Rare orchids of the north eastern region and their conservation. *Ind. For.* 102: 424-431.

- Vesper, S.J. 1987. Production of pili fimbriae by *Pseudomonas fluorescens* and a correlation with attachment to corn roots. *Appl. Environ. Microbiol.* 53: 1397–1405.

- Vij, S.P., Shekhar N., Kashyap, S.K. and Garg, A.K. 1983. Observations on the orchids of Nainital and adjacent hills in the central Himalayas (ecology and distribution). *Res. Bull. (Sci.) Panj. Univ.* 34: 63-76.

- Vij, S.P., Toor, I.S. and Shekhar, N. 1982. Observations on the orchidaceous flora of Simla and adjacent hills in north western Himalayas (ecology and distribution). *Res. Bull. (Sci.)Panj. Univ.* 33: 163-175.

- Vij, S.P. and Kaur, P. 1985. *Orchid Disease and Pests: Symptoms and Control Measures.* In: Biology, Conservation, and Culture of Orchid. pp. 15-22. ed. Vij, S. P. East-West Press Pvt. Ltd. New Delhi.

- Volpin, H., Phillips, D.A., Okon, Y. and Kapulnik, Y. 1995. Suppression of an isoflavonoid phytoalexin defense response in mycorrhizal alfalfa roots. *Plant Physiol.* **108:** 1449–1454.

- Weller, D.M. and Thomashow, L.S. 1994. *Current challenges in introducing beneficial microorganisms into the rhizosphere.* In: Molecular Ecology of Rhizosphere Microorganisms: Biotechnology and the Release of GMOs pp. 1-18. eds. O'Gara, F., Dowling, D. and Boesten, B. VCH. New York.

- Weller, D.M.1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol.* **26:** 379–407.

- Whipps, J.M. 1997. Developments in the biological control of soil-borne plant pathogens. *Adv. Bot. Res.* **26:** 1–134.

- Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* **52:** 487-511.

- Whipps, J.M. and Davies, K.G. 2000. *Success in biological control of plant pathogens and nematodes by microorganisms.* In: Measures of success in biological control. pp. 231-269. eds. Gurr, G. and Wratten, S.D. Kluwer Academic Publishers, Dordrecht: The Netherlands.

- Whipps, J.M. and Lumsden, R.D. 2001. *Commercial use of fungi as plant disease biological control agents: status and prospects.* In: Fungal biocontrol agents progress, problems and potential. pp. 9-22. eds. Butt, T., Jackson, C. and Magan, N. CAB International, Wallingford.

- Whitelaw, M.A. 2000. Growth promotion of plants inoculated with phosphate-solubilizing fungi. *Adv. Agron.* **69:** 99–151.

- Wilhite, S.E., Lumsden, R.D. and Straney, D.C. 1994. Mutational analysis of gliotoxin production by the biocontrol fungus *Gliocladium virens* in relation to suppression of *Pythium* damping-off. *Phytopathology.* **84:** 816–821.

- Windham, M.T., Elad, Y. and Baker, R. 1986. A mechanism for increased plant growth induced by *Trichoderma* spp. *Phytopathology.* **76:** 518–521.

- Wong, P.T.W. and Southwell, R.J. 1980. Field control of take-all by avirulent fungi. *Ann. Appl. Biol.* **94:** 41–49.

- Wright, J. 1994. *Orchid Pests and Diseases.* In: Growing Orchids in Canberra. pp. 56-62. Canberra, Australia.

- Wu, Y., Liu, Z. J., Luo, H. L., Chen, W. Y., Li, S. and Zhang, J. N. 1997. Study on the orchid disease *Stagonospora curtisssii*. *J. South-Chi-Agricult-Univ.* **18:** 38-41.

- Wulff, E.G., Pham, A.T.H., Cherif, M., Rey, P., Tirilly, Y. and Hockenhull, J. 1998. Inoculation of cucumber roots with zoospores of mycoparasitic and plant pathogenic *Pythium* species: differential zoospore accumulation, colonization ability and plant growth response. *Eur. J. Plant Pathol.* **104:** 69–76.

- Xue, L., Charest, P.M. and Jabaji-Hare, S.H. 1998. Systemic induction of peroxidases, 1,3-ß-glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. *Phytopathology.* **88:** 359–365.

- Yang, C.H. and Crowley, D.E. 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl. Environ. Microbiol.* **66:** 345–351.

- Yedidia, I., Benhamou, N. Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* **65:** 1061–1070.

- Zeilinger, S., Galhaup, C., Payer, K., Woo, S.L., Mach, R.L., Fekete, C., Lorito, M. and Kubicek, C.P. 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet. Biol.* **26:** 131–140.

- Zettler, F. W., Ko, N.J., Wisler, G. C., Elliott, M. S., and Wong, S.-M. 1990. Viruses of orchids and their control. *Plant Dis.* **74**:621-626.

CHAPTER II

Cymbidium Rot: Isolation, characterization and identification of the causal organisms

2.1. Introduction

The *Cymbidium* has originated from the Greek word Kymbion, which means boat shaped, since the largest petal (labellum or lip) resembles boat. It is one of the most popular and desirable orchids in the world because of the beautiful flowers. The genus *Cymbidium* comprises approximately 50 species. Geographic distribution extends from the northwestern Himalaya to Japan and south through Indochina and Malaysia to northern and eastern Australia (Yukawa and Stern, 2002). Cymbidiums exhibit distinctive ecological diversification and occur as terrestrial, epiphytic, and lithophytic life forms. One species, *C. macrorhizon*, once thought to be a parasite (Hooker, 1890), lacks foliage leaves and has a strong mycoparasitic existence.

Swarts (1799) established the genus *Cymbidium* with a broad generic delimitation. Circumscription of the genus has been variously defined by later workers: Lindley (1833), Blume (1848, 1849, 1858), Reichenbach (1852, 1864), Hooker (1890), Schlechter (1924), Hunt (1970), Seth and Cribb (1984), and DuPuy and Cribb (1988).

Cymbidiums make great houseplants, and are also popular in floral arrangements and corsages. They have been cultivated for thousand of years, especially in ancient China. One feature that makes the plant so popular is the fact that it can survive during cold temperatures (as low as 7^0C). Orchid hobbyists in temperate climates appreciate the fact that they can bloom in winter, when few other orchids are blooming. Seedling establishment of the genus is distinguished by protocorm or by rhizome (Shimasaki and Uemoto, 1987). In terrestrial *Cymbidium,* a rhizome needs 1-2 year to differentiate to a plantlet as, for example, *C. ensifolium* (Chung *et al.*, 1985; Lu *et al.*, 1992), *C. forrestii* (Paek and Yeung, 1991), *C. goeringii* (Nagashima, 1982; Duan and Xie, 1983; Shimasaki and Uemoto 1991) and *C. sinense* (Chiou and Wang, 1985; Chang and Chang, 2000).

Asian Cymbidiums are often sold in "growths" (Fitch, 1999). Each growth is an independent pseudobulb that was divided from the back bulb or parent bulb. Each pseudobulbs have an independent root system. The stem of the 'growth' is usually very short, 1 inch (2-3 cm) and is narrow 1/2 inch (1.5 cm). Each growth bears three to twelve leaves in two rows. The stem supports many leaves that branch from it in a grass like fashion. The pseudobulb acts as the main water and nutrient storage device. In comparison to other orchids, the pseudobulb is small and cannot store large amounts of water, necessitating frequent watering during their growth phase (Fitch, 1999). Most species have thick roots which are covered in a spongy white velamen and have only a thin core of vascular tissue. The erect stems are usually short and swollen to form a prominent pseudobulb which is often slightly flattened. Many species produce a new growth annually. In one section of the genus the pseudobulbs grow and flower for several years before a new shoot is produced, and in *C. mastersii*, *C. elongatuni* and *C. suave,* each shoot grows continuously for many years producing an elongated stem rather than a typical pseudobulb.

The inflorescence in *Cymbidium* is unbranched and may be erect, arching or pendulous. Each mature pseudobulb usually produces one or two inflorescences from leaf axils near the base. In *C. eburneum* and its allies the inflorescences arise from the leaf axils near the apex of the pseudobulb, as they do in *C. suave* and *C. elongatum*. The inflorescences bear up to 50 flowers in *C. canaliculatum* and only one in *C. goeringii* and *C. eburneum*. Most species bear 10-20 flowers; the flowers are all immediately recognizable as cymbidiums. They comprise a dorsal sepal, two lateral sepals, two free petals and a three lobed lip which is hinged at the base of the column. There is usually a callus of two distinct ridges along the upper surface of the lip. The anther contains two pollinia or four pollinia fused in two pairs. This brief summary can be applied to all or any of the species. Individually they can be much more precisely defined than this, and some species also vary to quite a large extent.

Though *Cymbidium* flowers are long-lasting, both on the plant as well as when cut, large in size, attractive and existing in a wide range of colours but the plant is attacked by number of pest and diseases. This include fungal diseases like Anthracnose Orchid Spot (Ciferri, 1926); *Cymbidium* wilt (Bose and Yadav, 1989); *Cymbidium* tip burn (Vij and Kaur, 1985); Leaf and Stem rot of *Cymbidium* (Ilieva *et al.*, 1998); Leaf spot diseases of *Cymbidium* (Kazunori and Takayuki, 2000), Leaf blotch (Hill, 2004) etc. Among bacterial diseases Bacterial Brown Rot (Bose and Yadav, 1989) and *Cymbidium mosaic virus* is responsible for viral disease of *Cymbidium* (Jensen, 1959a; 1959b; Sherpa *et al.*, 2003).

In Eastern Himalaya especially in the hills of Darjeeling and its adjoining areas *Cymbidium* has great horticultural value and has been cultivated extensively. Majority of orchid growers in this region mainly at Kalimpong, Kurseong, Mirik, Ging suffered from a huge loss due to an epidemic pseudobulb rot since 1995 during monsoon months.

This chapter deals with the isolation and characterization of the causal agents responsible for the disease '*Cymbidium* pseudobulb rot'.

2.2. Materials and Methods

2.2.1. Field survey and collection of samples

During field survey (2002-2005), different nurseries of Darjeeling hills and of its adjoining area were visited. Areas under survey were different polyhouses (ph) of various localities like Kurseong, Kalimpong, Mirik and Ging (Figure 2.1).

Pseudobulbs and roots of *Cymbidium,* showed symptoms of rotting of different stages were collected from different polyhouses and were wrapped in polythene bags and brought to the plant pathology laboratory for further study.

2.2.2. Symptomological study

Pseudobulbs and roots, showing different degree of disease symptoms were examined morphologically and compared with the healthy bulb.

2.2.3. Histopathological study

Different degrees of rotted bulbs were cut into small pieces with sterile razor blade and the pieces were fixed in formalin-aceto-alcohol solution (FAA No.1- 90 ml of 70% ethanol; 5 ml of formalin and 5 ml of glacial acetic acid) (Johansan, 1940) for 3 days. The samples were dehydrated through an ethanol series. To observe the host penetration, longitudinal and transverse sections were mounted on clean glass slide and stained with lactophenol-cotton blue. Sections were examined with Leica DMSL research microscope and photographed on Kodak plus film with Minolta X 300 camera.

Rotted samples were fixed in FAA for 3 days. The samples were dehydrated through ethanol series. To observe the bacterial invasion within the cell lumen sections were mounted on small glass block coated with gold by HITACHI E-1010 Ion Sputter and examined under HITACHI S-2360N Scanning electron microscope and photographed on Kodak academy 200 plus film with Nikon camera.

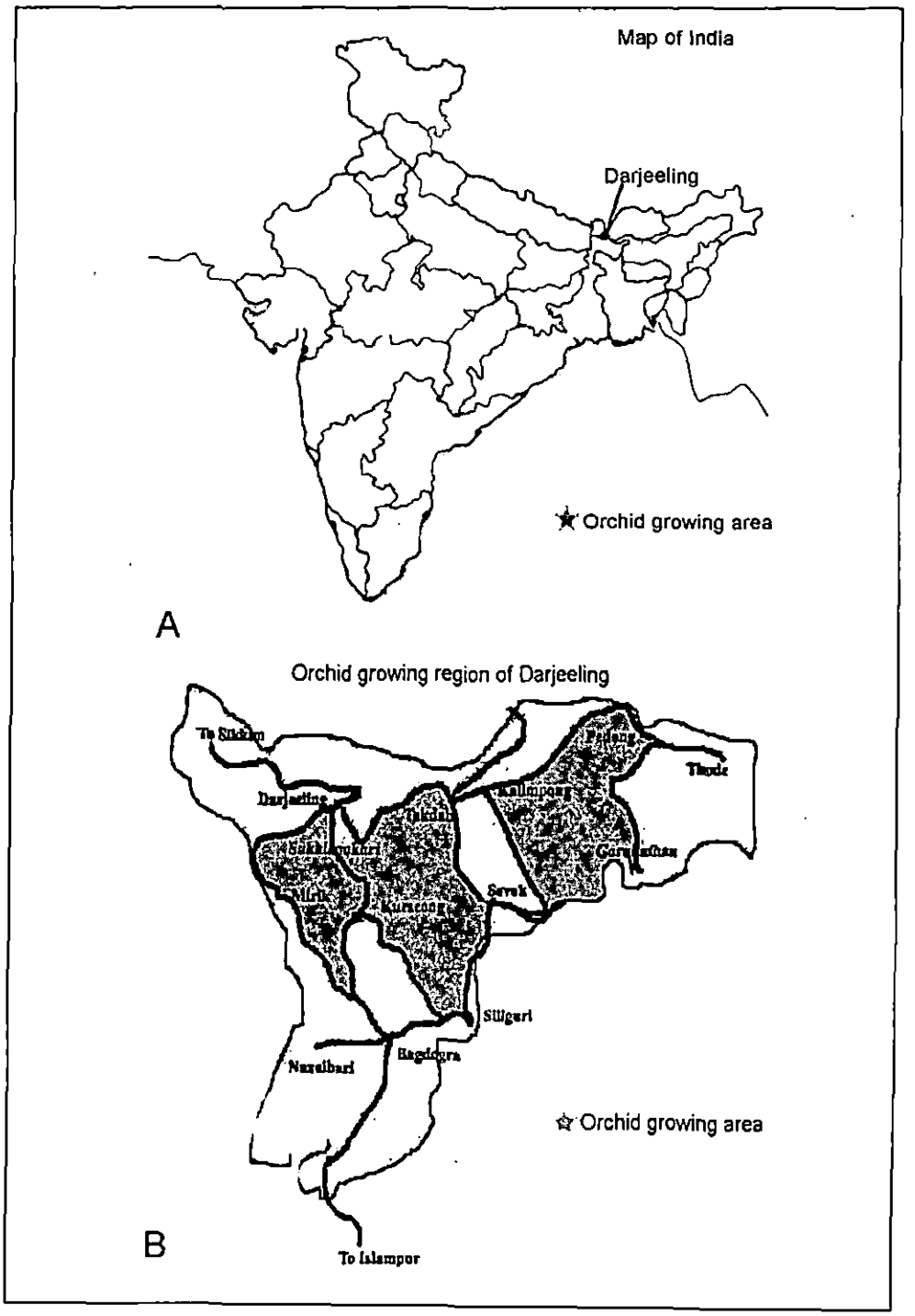


Figure 2.1: Study area: A. Map of India showing the area where the study was undertaken. B. Enlarge view of the area showing different orchid growing zones of Darjeeling and its adjoining area.

2.2.4. *Isolation of pathogens*

The infected samples were rinsed in tap water and rotted portions were cut into small pieces, surface sterilized with 1.5% sodium hypochlorite for 2 min and washed several times with sterile distilled water. Approximately 1 gm of rotted pseudobulb pieces were added to 100 ml of sterile distilled water in flasks and shaken on a rotary shaker at 150 rpm for 30 min. Bulb and root segment as well as a 10 fold dilution series of the resulting water suspension, were plated on various general and selective media (Persson *et al.,* 1997) to recover the *Cymbidium* rot organisms. The plates were then incubated for 4 days at 22^{0}C. Different media were used to isolate the pathogens related to the disease such as Potato Dextrose Agar (PDA) media were used for isolation of fungal pathogen supplemented with antibacterial antibiotic streptomycin (Stevens, 1981); Peptone Glucose Agar (PGA) media were used for isolation of both fungal and bacterial pathogens; Czapex-Dox Agar media for isolation of pathogenic fungi; Malt Extract Agar used for isolation of fungal pathogen (Dreyfusss, 1986; Bills and Polishook, 1994); Nutrient Agar media used for rapid isolation of bacterial pathogen.

2.2.5. *Characterization and Identification of pathogen*

Fungal pathogens were identified through cultural characteristics on agar media and microscopic observation and identified according to Gilman, (1945) and Subramanium (1971). The fungal pathogens were further confirmed by Agharkar Research Institute, Puna, India. These were subcultured and maintained on PDA slant at 4^{0}C for further study.

Cultural, microscopic and biochemical parameters were evaluated to identify bacterial pathogen following Bergy's Manual of Determinative Bacteriology (Holt *et al.,* 1994). The bacterial pathogen was subcultured and maintained on NA slant at 4^{0}C for future reference.

2.2.6. Pathogenicity test

Pathogenicity test was performed by dipping method (Haglund, 1989). Pseudobulbs were surface sterilized with 1.5 percent sodium hypochlorite for 2 minutes and washed by sterile distilled water for several times. Spore / cell suspension were counted on a hemacytometer in order to prepare a concentration of 10^6 cell /spore per milliliter of the suspension. Inoculated bulbs were covered with separate sterilized polythene bags and incubated at 22 $\pm$ 1^0C aseptically upto seven weeks at a relative humidity of 80 percent and disease severity was assayed periodically.

2.3. Results and Discussion

Field trips were done periodically round the year from 2002-2005, at different polyhouses like Kurseong-12 polyhouses; Mirik-3 polyhouses; Kalimpong-4 polyhouses; and Ging-1 polyhouse (Figure 2.2). Disease incidences of *Cymbidium* rot in these polyhouses were recorded (Table-2.1).

Table 2.1. ***Pseudobulbs rot incidence on Cymbidium in different Polyhouses at Growers field at Mirik, Kurseong, Kalimpong, and Ging.***

Location	Poly house	Total number of plants observed	Total number of plants infected	Percentage of infection
Mirik	Poly house 1	300	11	3.6
	Poly house 2	600	495	82.5
	Poly house 3	500	380	76.0
Kurseong	Poly house 1	800	09	1.1
	Poly house 2	700	08	1.1
	Poly house 3	300	300	100
	Poly house 4	1500	1500	100
	Poly house 5	300	300	100
	Poly house 6	250	206	82.4
	Poly house 7	350	03	0.8
	Poly house 8	300	260	86.7
	Poly house 9	600	600	100
	Poly house 10	400	340	85.0
	Poly house 11	300	190	63.3
	Poly house 12	1500	1300	86.6
Kalimpong	Poly house 1	300	170	56.6
	Poly house 2	220	10	4.5
	Poly house 3	1000	800	80
	Poly house 4	1600	1350	84.3
Ging	Poly house 1	300	225	75

From the table it is quite evident that 74% of the visited nurseries showed 50-100% disease severity. In Kurseong cent percent disease incidence were recorded in some polyhouses. The severity of the disease was generally associated with the high humidity, soil moisture and often related to poor drainage system and irrigation water. The disease assumes an epidemic form during wet season and its spread is added by wind and rain. From the table it is also noticeable that some of the polyhouses viz. one from Mirik, three from Kurseong and one from Kalimpong showed less than 50% disease incidence. Even in some polyhouses the rate of disease incidence were negligible. The cause of this insignificant disease incident in such polyhouses has been investigated using various field techniques, such as direct interviews, discussion with the growers and by direct observation. The outcome of such observation is summarized below:-

- These polyhouses are located in isolated places i.e. they are away from other nurseries. No other orchid nurseries were found nearby.
- In these polyhouses proper hygienic conditions were maintained. It has been seen that these houses have proper aeration, light intensity and irrigation system.
- Moreover, growers of these polyhouses propagate the pseudobulb from healthy plants by themselves. They do not procure pseudobulbs from any other sources.

During interviewing with about 25 members of Himalayan Orchid Society of Kurseong a huge loss of *Cymbidium* rot was found to occur. Each member of the society cultivated about 6000 *Cymbidium* clones during the year 1995 and the first appearance of disease occurred after one year of plantation. According to their report, initially the disease symptoms appeared in few plants in the nursery and spreads from one nursery to another during their plant exchange. Disease

Figure 2.2: (a and b). Diseased Cymbidium plants in nurseries; (c and d) Rotted pseudobulbs and roots in view.

severity increases year after year and during 1998 to 2002 it was in its climax when the members lost almost 95-98% of their planted *Cymbidium*.

The severity of the disease stages was assayed symptomatically and considered Disease Severity (DS) as 0 in case of healthy bulbs as 100% when it was completely rotted being hollow, fibrous and dry. Intermediate stages were considered comparing with healthy bulbs. DS was described as earlier middle and later phase.

Earlier phase was considered when bulbs were soft, pulpy followed by oozing of liquid and becomes yellow in colour but there was no apparent change in colour of leaves when compared with the healthy plants.

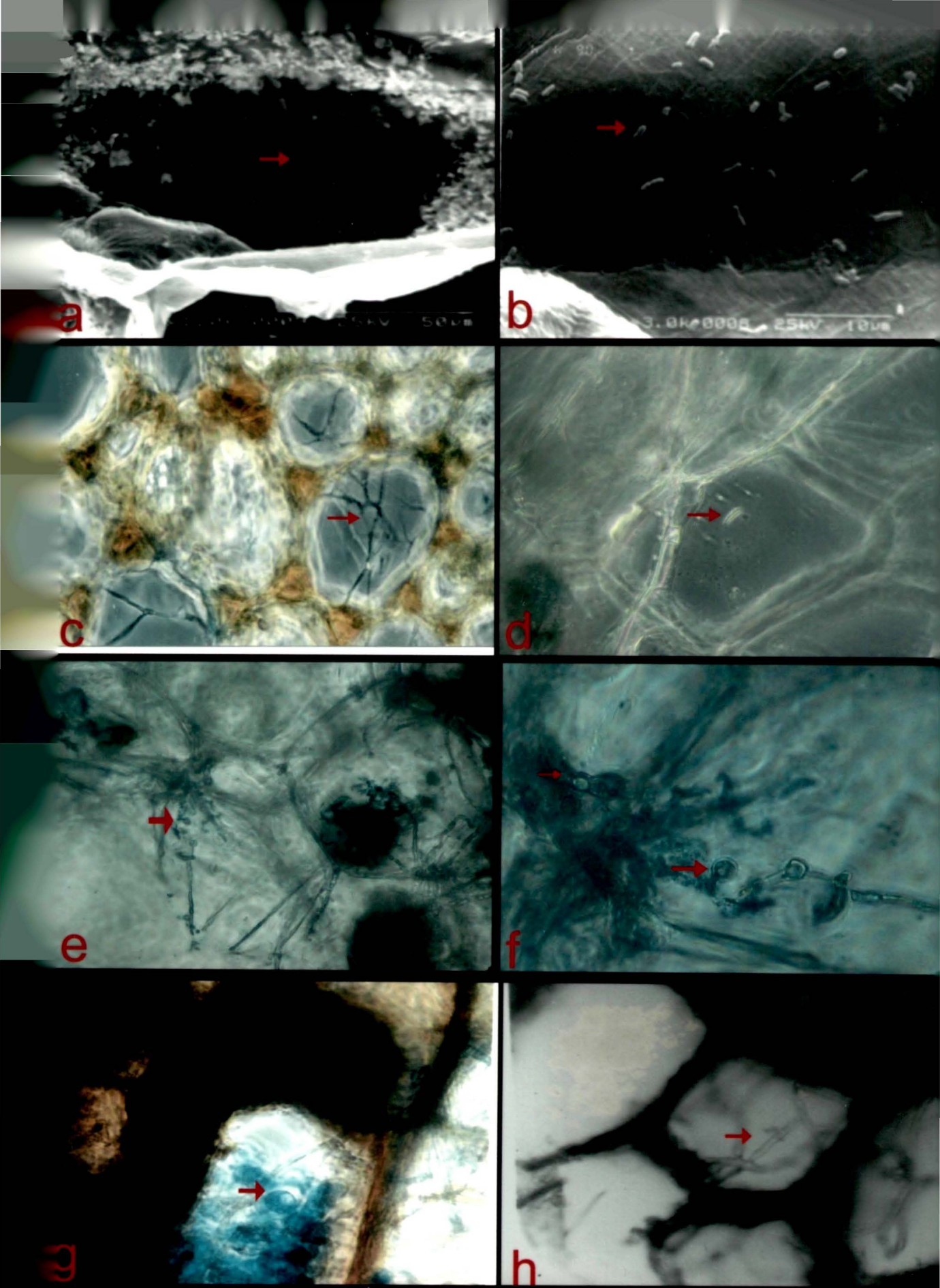
Middle phase was considered when bulbs and roots started to lose weight and disintegration of tissue started infected bulbs become brownish in colour and leaves of the plants become yellow in colour and starts withering.

Later phase was recorded when the internal tissues were completely destroyed, bulbs become hollow, fibrous and dry with dark brown or blackish colour and leaves become dry, black and few in number.

2.3.1. Histological studies

Histological examinations of infected pseudobulb revealed that infection by bacterial and fungal pathogen induced cellular alternations in the epidermal, cortical and vascular parenchymatous tissues.

Scanning electron microscopic study of early stages of rotted pseudobulbs, when the bulbs become soft and pulpy and oozes liquid, it showed the presence of rod shaped, single, sometime paired bacteria, size ranging from 0.9-3.0 × 0.4-0.6 µm within the cortical parenchymatous region (Figure 2.3a and 2.3b). Observation under phase contrast microscope of the same stage of infected bulb showed motile, small, rod shaped bacteria within the parenchymatous ground tissue. The dark brown colour liquid with foul odor which oozes from the



igure 2.3. (a) SEM of early stage of rotted pseudobulb showing abundance of E. carotovora within th ll lumen; (b) Enlarge view of the same; (c) Phase contrast microscopy of septate mycelium of . oxysporum within the cortical region of middle stage of rotted pseudobulb; (d) macroconidia of . oxysporum; (e) Septate mycelium with chlamydospore; (f) Chlamydospore in enlarge view; (g) Phas ntrast microscopy of sporangium of M. hiemalis f. hiemalis within the late stage of rotted pseudobulb nd (h) Coenocytic hyphae of M. hiemalis f. hiemalis within the infected cell.

infected bulb of early stage were also observed under the light microscope and found the abundance of rod shaped, motile gram negative bacteria. This bacterium was isolated and identified as *Erwinia carotovora,* the detail of which is given later in this chapter. Light and phase contrast microscopic observation of transverse and longitudinal section of intermediate stages of infection, when the pseudobulbs lose weight and disintegration of tissue started, it showed that sub-epidermal and cortical parenchymatous tissues were radially colonized by the ramifying intra and intercellular septate hyphae (Figure 2.3c), presence of macroconidia (30-50 µm) (Figure 2.3d) and chlamydospore (6-8 µm) (Figure 2.3e and 2.3f) of the fungi within the sub epidermal and cortical cells which was isolated and identified as *Fusarium oxysporum.* The detailed characterization of this fungus has been discussed later on this chapter. At the later stage of infection when the tissues become more or less dry and fibrous the damaged cortical cells showed coenocytic, hyaline mycelium with globose sporangia within the rotted parenchymatous cells (Figure 2.3g and 2.3h), it was also isolated and identified as *Mucor hiemalis* f. *hiemalis,* the isolation and characterization of the organism discussed later on.

2.3.2. Isolation, characterization and identification of pathogens

Isolation of pathogens were done in different selective and general media as described in detail in materials and methods. At the early stage of infection, a flat, waxy, entire, yellowish bacterial colony found to be present abundantly in Nutrient Agar media. At the intermediate stage of infection a typical white cottony fungal colony found in all selective and general fungal isolation media where as at the later stage of infection a creamy and wooly fungal colony found in abundance both in general and selective fungal isolation media.

At the early stage of infection, an abundance of a bacterium was observed. Different morphological, cultural and biochemical characters were evaluated to identify the bacterium which was as follows:

- **MORPHOLOGICAL**
  - **Size:** 0.9-3.0 × 0.4-0.6µm
  - **Shape:** Rods
  - **Gram Nature:** Gram Negative
  - Aerobic in Nature
- **CULTURAL**

Agar plate character:

Growth: Moderate
Form: Flat
Surface: Smooth and waxy
Elevation: Raised
Edges: Entire
In broth: Turbid with white sediment
Colour: Whitish grey

- **BIOCHEMICAL**

Amylase	: Negative
Pectinase	: Positive
Gelatin hydrolysis	: Positive
Indole Production	: Positive

Acid and Gas formation from different Carbohydrates

Glucose	: Positive
Sucrose	: Positive
Glycerol	: Positive
Manitol	: Positive
Fructose	: Positive

Based on the above-mentioned characters, following Bergy's Manual of Determinative Bacteriology (Holt *et. al.*, 1994) and also by culturing on *Erwinia* specific media (D3 media) which characteristically produced red colouration of the medium (Haskett and Kado, 1970) the bacterium was identified as *Erwinia carotovora* (Figure 2.4a and 2.4d).

At the intermediate stage of infection the infected tissue showed dominance of a fungal pathogen. The pathogen produced typical white cottony colonies on PDA (Figure 2.4e). The asexual reproductive structures consist of conidia and

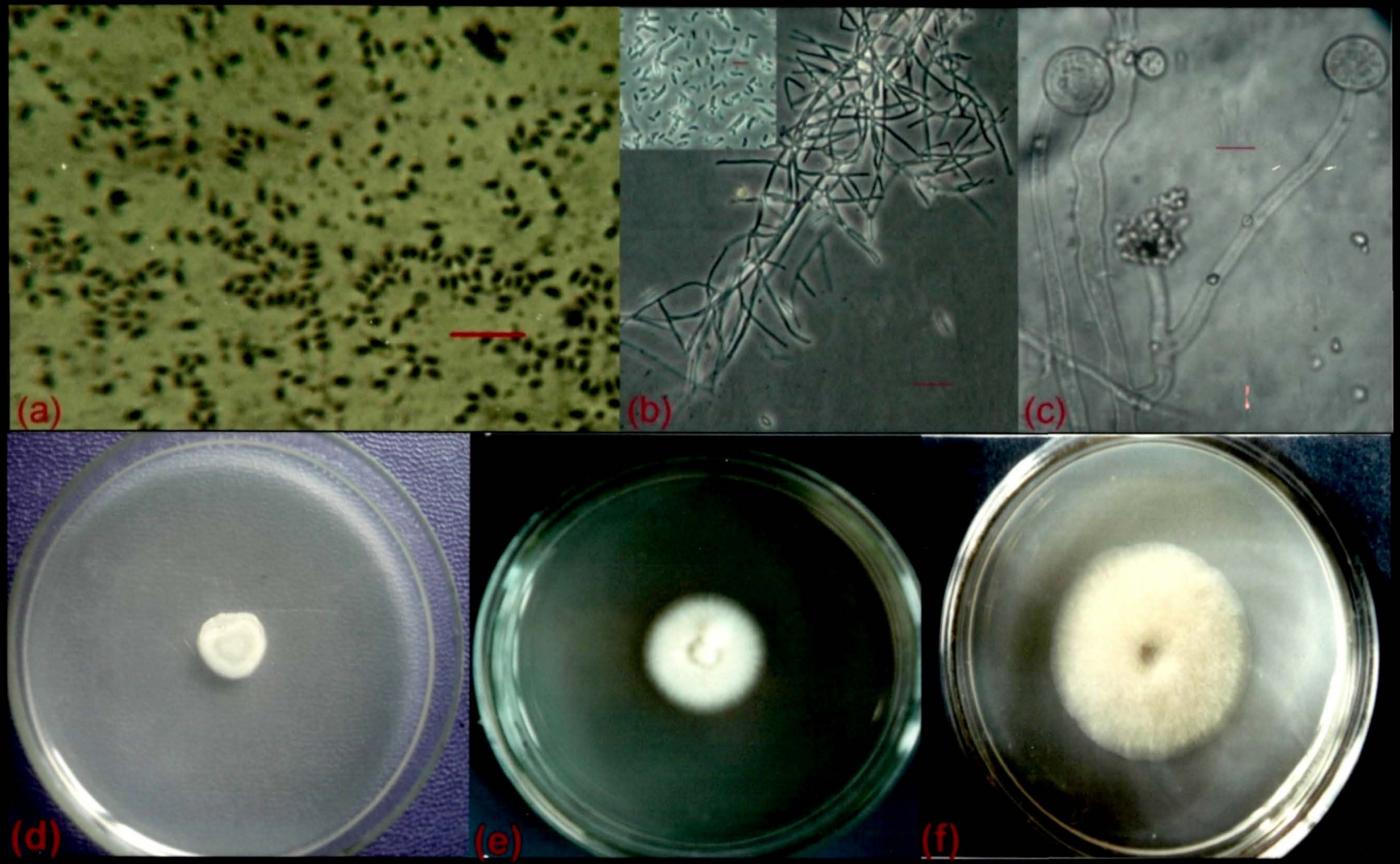


Figure 2.4. Microscopic Photograph of (a) Erwinia carotovora, Bar= 10µm (b) Fusarium oxysporum mycelia with spore in culture, Bar= 50µm (Inset : macro and microconidia, Bar= 30µm), (c) Mucor hiemalis f. hiemalis, Bar= 25µm. (d) Colony of E. carotovora on nutrient agar plate (e) and (f) Colony of F. oxysporum

chlamydospore. Conidia are produced in sporodochia, conidiophore simple; two types of conidia were observed; microconidia and macroconidia (Figure 2.4b). Microconidia mostly one celled with or without septa, hyaline, oval, oblong produced singly from the tip of the phialides, size of microconidia ranges from 4-14 × 2-4 µm.

Macroconidia were long, curved (sickle shaped), cylindrical to sub-cylindrical to fusiform mostly 3-5 septate and pointed at both the ends, size of macroconidia varies from 25-54 × 3-5 µm. Chlamydospores were terminal and intercalary, size of which ranges from 6-10 µm in diameter. Growth rate of the pathogen on PDA plate after five days of incubation ranges from 3.2 to 4 cm. Based on these morphological and cultural characteristics the fungus was identified as *Fusarium oxysporum* and was further confirmed by the Agarkar Research Institute (ARI), Puna, India.

At the later stage of infection, another fungus showed its dominance in the rotted pseudobulbs and roots. A creamy and wooly colony (Figure 2.4f) developed on PDA. Microscopic observation reveled mycelium were slender, coenocytic, sporangiophores unbranched to branched. Sporangia spherical, brownish yellow, visible to necked eye 50-52 µm in diameter (Figure 2.4c). Columella free, spherical 28-48 µm. Spores usually unequal, elongated 7 × 3.2 µm, smooth and hyaline. Growth rate of the fungus on PDA after 72 hours of incubation varies from 7.5 to 7.8 cm. Based on these morphological and cultural characters the fungus was identified as *Mucor hiemalis* f. *hiemalis* and was further confirmed by ARI.

2.3.3. Pathogenicity test

Pathogenicity test was performed by dipping method in two ways; inoculating the pathogens *separately* and *consecutively*. Pseudobulbs were surface sterilized with 1.5 percent sodium hypochlorite for 2 min and washed by sterile distilled water for several times. In the first case sterilized bulbs were dipped separately into

fungal as well as bacterial spore / cell suspension (10^6 cfu / ml) for 1 minute. For control set the healthy bulbs (Figure 2.5a) were dipped in sterilized water. Inoculated and control bulbs were covered with separate sterilized polythene bags and incubated at 20^0C aseptically upto 47 days at a relative humidity of 80 percent and disease severity was assayed periodically.

When the pathogenicity test was performed separately with single pathogen i.e. *E. carotovora, F. oxysporum* and *M. hiemalis* f. *hiemalis* and incubated upto 47 days, *Erwinia carotovora* exhibited maximum tissue disintegration followed by *F. oxysporum* and *M. hiemalis* f. *hiemalis,* but none of the individual pathogens caused 100% tissue disintegration. Maximum of 70% destruction (considered when whole pseudobulbs become dark coloured; soft and pulpy, oozing of liquid with foul odor; epidermal, subepidermal and ground tissue become dark and affected) was recorded in case of *E. carotovora* on 47 days (Figure 2.5d); *F. oxysporum* caused a maximum of 30% tissue disintegration (considered when the bulbs become wrinkled; epidermal and subepidermal cells were affected) on 47 days (Figure 2.5c) and *M. hiemalis* f. *hiemalis* caused 10% destruction (discolouration of pseudobulb) on 47 days (Figure 2.5b).

In the second case i.e. in *consecutive* inoculation, the bulbs were dipped in spore / cell suspension of pathogens according to their natural occurrence. All other parameters were kept as such as in the case of separate inoculation. Bulbs were inoculated firstly by *E. carotovora* and after 12 days of this inoculation (when the symptoms like early phase appear) the same bulbs were inoculated (dipped) with *F. oxysporum* and incubated. After 15 days of second inoculation (when symptoms like middle phase appear) third inoculation was done by *M. hiemalis* f. *hiemalis.* Complete destruction (DS= 100%) was recorded 20 days after third inoculation (Figure 2.5e). Total time period required for complete destruction by consecutive inoculation method was 47 days. When the pathogenicity test was performed in consecutive dipping method symptoms mimicked the natural

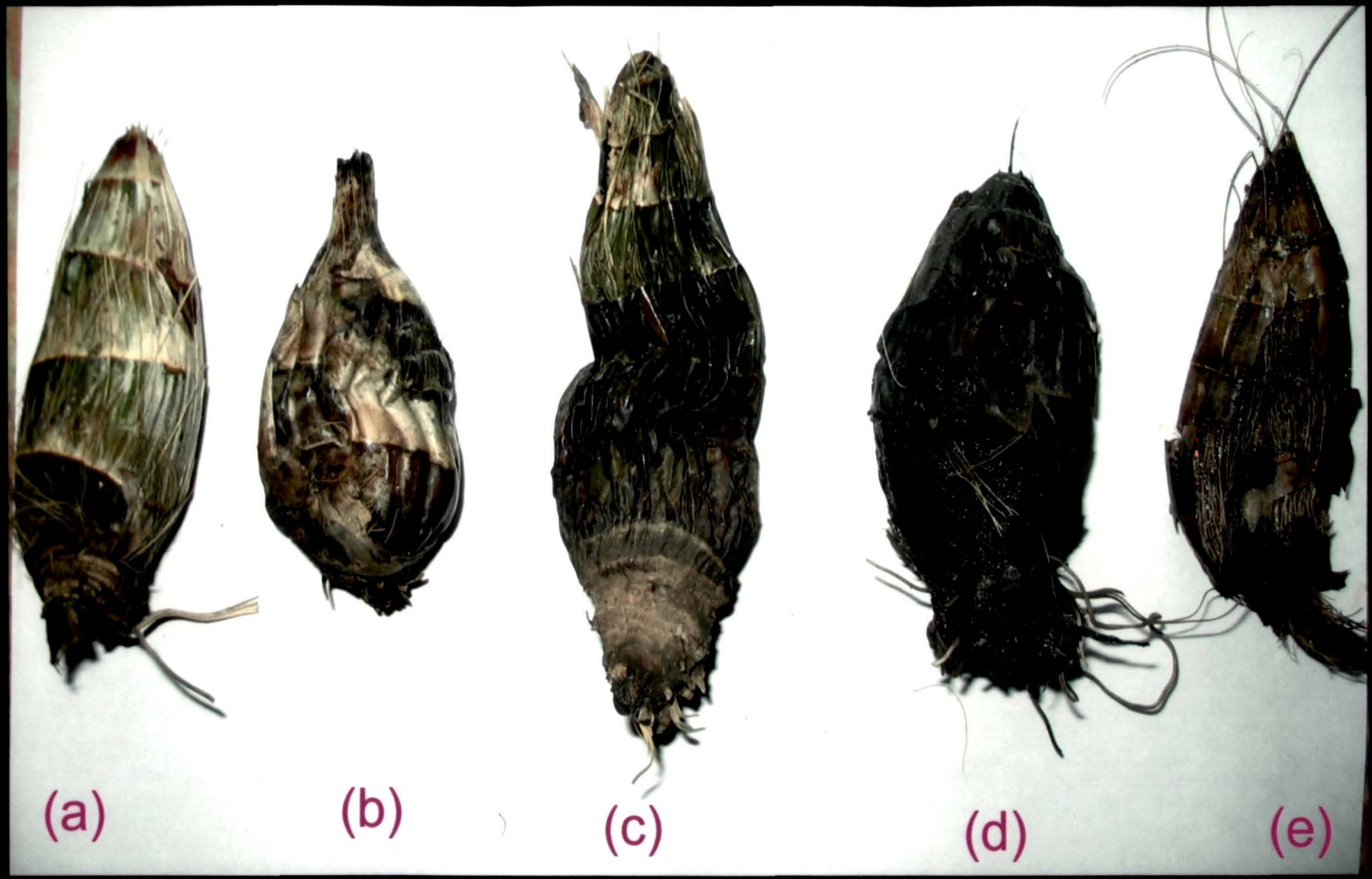


Figure 2.5.Pathogenicity test: (a) Healthy pseudobulb, (b) Inoculated with Mucor hiemalis f. hiemalis.(c) with Fusarium oxysporum , (d) with Erwinia carotovora (e) inoculated all three pathogens.

symptoms found in the field condition. This experiment was done for at least five times and every time they reproduce the same symptoms.

It is an interesting information on host-pathogen combination that three pathogens act synergistically for ultimate demolition of the host. Even from the pathogenicity test it was clear that *Cymbidium* rot is orchestrated by the action of *E. carotovora, F. oxysporum* and *M. hiemalis* f. *hiemalis*. The next step is to isolate a biocontrol agent that can effectively control these three pathogens. Literature survey suggests that bacterial pathogens are unable to control by fungal antagonist or there have been no clear examples regarding the use of fungal antagonist to control bacterial plant pathogens in the rhizosphere or spermosphere (Whipps, 2001) so the objective is to isolate a putative bacterial antagonist that can show strong inhibitory effect against all three pathogen. Although a range of different bacterial genera and species have been studied, the overwhelming number of papers have involved the use of *Pseudomonas* species. Clearly, *Pseudomonas* species must have activity but it begs the question as to the features that make this genus so effective and the choice of so many workers (Whipps, 2001). Pseudomonads are characteristically fast growing, easy to culture and manipulate genetically in the laboratory and are able to utilize a range of easily metabolizable organic compounds, making them amenable to experimentation. In addition, the genus *Pseudomonas* is ubiquitous in nature and can be found readily in ecological habitats as diverse as water, soil, and plant surfaces. (de Weger *et al.*, 1995; Marilley and Aragno, 1999). The next chapter will discuss about the isolation, characterization and screening of potential fluorescent pseudomonads having antagonistic activity against all three pathogens.

2.4. Reference

- Bills, G.F. and Polishook, J.D. 1994. Abundance and diversity of microfungi in leaf litter of a lowland rain forest in Costa Rica. *Mycologia.* **86:** 187-198.
- Blume, C.L. 1848. Orchideae. *Rumphia.* 4: 38–56.
- Blume, C.L. 1849. Cyperorchis. Annales: Museum Botanicum Lugduno-Batavum 1: 48.
- Blume, C.L. 1858. Collection des Orchidees les plus remarquablesde l'archipel Indien et du Japon. Amsterdam.
- Bose, T.K. and Yadav, L.P. 1989. *Orchids.* In: Commercial Flowers. pp. 151-265 eds. Bose, T. K. and Yadav, L.P. Naya Prokash, Calcutta.
- Chang, C. and Chang, W.C. 2000. Effect of thidiazuron on bud development of *Cymbidium sinense* Willd. *in vitro. Plant Growth Regul.* **30:** 171-175.
- Chiou, C.C. and Wang, P.J. 1985. Seed germination and shoot formation of *Cattleya* and *Cymbidium. J. Chinese Soc. Hort. Sci.* **31:** 10-22.
- Chung, J.D., Chun C.K. and Choi, S.O. 1985. Asymbiotic germination of *Cymbidium ensifolium. J. Kor. Soc. Hort. Sci.* **26:** 186-192.
- Ciferri, R. 1926. The pathogenic capacities of fungi causing anthracnose of orchids. *Rivista Pathol. Veg.* **16:** 1-16.
- de Weger, L.A., van der Bij, A.J., Dekkers, L.C., Simons, M., Wijffelman, C.A. and Lugtenberg, B.J.J. 1995. Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiol. Eco.* **17:** 221–228.
- Dreyfuss, M.M. 1986. Neue erkenntnisse aus einem pharmakologischen pilz-screening. *Sydowia.* **39:** 22-36.
- Duan, J.Y. and Xie, Y.H. 1983. Germination of seeds of *Cymbidium goeringii* and the effect of hormones on the differentiation of rhizomes. *Acta Bot. Yunn.* **5:** 197-200.
- DuPuy, D. and Cribb, P. 1988. In: The genus Cymbidium. Timber Press, Portland Oregon.
- Fitch, C.M. 1999. *Asian Cymbidiums: Natural Growing Conditions.* In: American Orchid Society Magazine. http://www.winterview.com/AOS_Article_4.htm.
- Gilman, J.C. 1945. In: A manual of Soil Fungi. The Iowa State College Press. Ames, Iowa.
- Haglund, W.A. 1989. A rapid method for inoculating pea seedlings with *Fusarium oxysporum* f. sp. *pisi. Plant Dis.* **73:** 457-458.

- Hill, C.F. 2004. First report of *Phytophthora multivesiculata* on cymbidium orchids in New Zealand. *Aus. Plant Pathol.* **33:** 603–604.
- Holt, G.J., Krieg, R.N., Sneath, P.H.A., Staley, J.T. and Williams, S.T. 1994. *Bergy's Manual of Determinative Bacteriology*. Willims & Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp.151-168.
- Hooker, J.D. 1890. *Cymbidium and Cyperorchis.* In: Flora of British India 6. pp. 8–15, London.
- Hunt, P.F. 1970. Notes on Asiatic orchids 5. *Kew Bulletin* **24:** 93- 94.
- Ilieva, E., Man in 't Veld, W.A., Veenbaas-Rijks, W. and Pieters, R. 1998. *Phytophthora multivesiculata*, a new species causing rot in *Cymbidium. Eur. J. Plant Pathol.* **107:** 677-684.
- Jensen, D. D. 1952a. Virus diseases of orchids. I. *Calif. Agric.* **6 (1):** 3,15.
- Jensen, D. D. 1952b. Virus diseases of orchids. II. *Calif. Agric.* **6 (2):** 7, 15, 16.
- Johansen, D. A. 1940. Plant microtechnique. Mc Graw Hill Book Co. pp. 523. New York.
- Kado, C.I. and Heskett, M.G. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *xartromonas. Phytopathology*. **60:** 969-976.
- Kazunori, I. and Takayuki, A. 2000. New Leaf Spot Disease of Cymbidium Species Caused by Fusarium subglutinans and Fusarium proliferatum. *J. Gen. Plant Pathol.* **66:** 213-218.
- Lindley, J. 1833. *Cymbidium. The genera and species of orchidaceous plants.* London.
- Lu, J.L., Lee, C.J. and Lee, N. 1992. Effect of medium composition on seed germination in vitro of *Cymbidium ensifolium* var. *misericors. J. Chinese Soc. Hort. Sci.* **38:** 161-169.
- Marilley, L. and Aragno, M. 1999. Phytogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Appl. Soil Eco.* **13:** 127–136.
- Nagashima, T. 1982. Studies on the seed germination and embryogenesis in the *Cymbidium goeringii* Rehb. F. and *Paphiopedilum insigne* var. *sanderae* Rchb. F. *J. Japan. Soc. Hort. Sci.* **51:** 94-105.
- Paek, K.Y. and Yeung, E.C. 1991 The effects of 1-naphthaleneacetic acid and N^6-benzyladenine on the growth of *Cymbidium forrestii* rhizomes in vitro. *Plant Cell Tissue Organ Cult.* **24:** 65-71.
- Persson, L., Bodker, L. and Larsson-Wikstrom, M. 1997. Prevalence and pathogenicity of foot and root rot pathogens of pea in southern Scandinavia. *Plant Dis.* 81:171-174.

- Reichenbach, H.G. 1852. Orchidaceae (*Cymbidium* and *Cyperorchis*). *Annales Botanices Systematicae* **3:** 547–548.
- Reichenbach, H.G. 1864. Orchidaceae. Cymbidium. Annales Botanices Systematicae **6:** 622–626.
- Schlechter, R. 1924. Die Gattungen Cymbidium Sw. und Cyperorchis Bl. Feddes Repertorium Specierum NovarumRegni Vegetabilis. **20:** 96–110.
- Seth, C.J. and Cribb, P.J. 1984. *A reassessment of the sectional limits in the genus Cymbidium.* In: Orchid biology, reviews and perspectives, III. pp. 283–332. ed. Arditti J. Cornell University Press, Ithaca.
- Sherpa, A. R., Hallan Vipin, Raja Ram, Vij S. P, Pathak P, Garg ID and Zaidi A. A. 2003. First report of Cymbidium mosaic virus on Cymbidiums in India. *Plant Pathol.* **52:** 788.
- Shimasaki, K. and Uemoto, S. 1987. Comparative organogenesis between terrestrial and epiphytic cymbidium-spp. *J. Faculty Agri.* Kyushu Univ. **32:** 31-40.
- Shimasaki, K. and Uemoto, S. 1991. Rhizome induction and plantlet regeneration of *Cymbidium goeringii* from flower bud cultures in vitro. *Plant Cell Tissue Organ Cult.* **25:** 49-52.
- Stevens, R. B. 1981. Mycology Guidebook. University of Washington press, Seattle, Washington.
- Subramaniam, C. V. 1971. In: Hyphomycetes. Indian Council of Agricultural Research (ICAR). New Delhi.
- Swartz, O. 1799. Dianome Epidendri generis Linn. *Nova ActaRegiae Societatia Scientiarum Upsaliensia.* **6:** 61–88.
- Vij, S.P. and Kaur, P. 1985. *Orchid Disease and Pests: Symptoms and Control Measures.* In: Biology, Conservation, and Culture of Orchid. pp. 15-22. ed. Vij. S. P. East-West Press Pvt. Ltd. New Delhi.
- Whipps, J.M. 2001. Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot.* **52:** 487-511.
- Yukawa, T. and Stern, W.L. 2002. Comparative vegetative anatomy and systematics of *Cymbidium* (Cymbidieae: Orchidaceae). *Bot. J. Linean Soc.* **138:** 383–419.

CHAPTER III

Isolation, characterization and screening of potential antagonists against causal organisms

3.1. Introduction

Throughout the history of agriculture humans have struggled to reduce the adverse effects of plant disease on their crops. Early agriculturists realized the benefits of cultural practices such as crop rotation and the use of organic soils amendments in promoting plant productivity. Interest in biological control has increased recently fuelled by public concerns over the use of chemicals in the environment in general and the need to find alternatives to the use of chemicals for disease control. The key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in soil and root environments is required to predict the conditions under which biocontrol can be achieved (Deacon, 1994; Whipps, 1997). Management of diseases are difficult in developing countries, where fungicides are very expensive, where as biocontrol agents are cheap, effective and can be used without much skill or additional infrastructure (Jayalashmi *et al.*, 2003). Isolation of rhizospheric bacteria are easy because they are ubiquitous but selection is difficult since only few of them will be disease suppressor (Cook, 1981; Vidhyasekaran, 1998).

Antagonist is an umbrella term for parasites, predators, pathogens, competitors and other organisms that repel, inhibit or kill plant parasites. Microorganisms that can grow in the rhizosphere are ideal to exploit as a biocontrol agents, since the rhizosphere provides the front line of defense for roots against pathogenic attack. Pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection as well as in secondary infection (Weller, 1988). Representative of a range of bacterial (*Pseudomonas, Burkholderia, Bacillus, Serratia, Actinomycetes*) and fungal (*Trichoderma, Penicillium, Gliocladium, Sporodesmium,* non pathogenic *Fusarium* spp.) groups have been identified as antagonists of soil borne plant pathogen. The mechanism by which these microorganisms make soil suppressive can be divided into several categories; nutrient competition, microbial antagonism, parasitism and systemic induced resistance. The potential strains of *Pseudomonas* sp. for suppression of plant

disease in agriculture has been demonstrated in hundreds if not thousands, of experiments world wide, since they represent not only a dominant bacterial group in the rhizosphere ecosystem but are also metabolically and functionally most versatile (Lutenberg and Dekkers, 1999). The worldwide interest in this group of bacteria was sparked by studies initiated at the University of California, Barkley, during the 1970s (Weller, 1988). In 1978 Burr *et al.* reported that strain of *Pseudomonas fluorescens* and *P. putida* applied to seed pieces, improved the growth of potatoes. These strain and similar strains were given the name plant growth promoting rhizobacteria (PGPR). The term rhizobacteria was coined for those bacteria which have the ability to colonize roots aggressively (Schroth and Hancock, 1981). Numerous examples of plant growth stimulation by fluorescent *Pseudomonas* species have been reported (Kloepper *et al.*, 1980b; Howell and Stipanovic, 1980; Suslow and Schroth.1982; Gardner *et al.*, 1984; Bashan, 1986; Weller and Cook, 1986 a; Keel *et al.*1989; Preston, 2004). Summarizing the results from field test, Schroth and Hancock (1982) reported that the fluorescent pseudomonads increased the yield of potato 5-33%, of sugarbeet 4-8 tones per hectors and root weight of radish 60-144%. Van Peer and Schippers (1988) documented increases in root and shoot fresh weight for tomato, cucumber, lettuce, and potato as a result of bacterization with *Pseudomonas* strains. These bacteria are ideally suited as soil inoculants because of their potential for rapid and aggressive colonization. This feature alone was suggested as a disease control mechanism by preventing the invasion of detrimental soil microorganisms onto the root surface (Lucy, 2004). Several antibiotic producing *Pseudomonas* sp. were isolated from soil suppressive to disease such as take-all of wheat, black rot of tobacco (Keel *et al.*, 1989), and fusarium wilt (Kloepper *et al.*, 1980 a; Scher and Baker, 1982; Sneh *et al.*, 1984). Fluorescent *Pseudomonas* spp. have also been implicated in the control of *Phytophthora* root rot of soybean (Lifshitz *et al.*, 1986), potato seed decay due to *Erwinia carotovora* (Xu and Gross 1989), and fungal diseases of orange and lemon citrus roots (Gardner *et al.*1984) and ornamental plants (Yuen and Schroth, 1986). The control of various

Pythium fungi by specific fluorescent *Pseudomonas* strains has also been documented. Howell and Stipanovic (1980) isolated a fluorescent pseudomonad which could inhibit *Pythium ultimum,* an important pathogen of cotton seedlings (Gaber *et al.,* 1979; Johnson *et al.*, 1978).

3.2. Selection of efficacious organism

In vitro screening is frequently used to select prospective antagonists, even though *in vitro* results may not be related to biocontrol in the field. Because of the time and expense required for field testing, both methods are needed to select potential field effective strains (Fravel, 1988). Rhizosphere bacteria with the ability to provide biological control appear to comprise less than 10% of the total population of bacteria in the rhizoplane (Scworth and Hancock, 1981; 1982; Weller and Cook, 1986 b). There is no shortage of methods and techniques for isolating and testing putative antagonists of plant pathogens (Merriman and Russell 1990). *In vitro* tests, such as bioassays conducted on nutritive media in petiplates that identify zones of antagonistic activity by microbials against plant pathogens, remains popular as at least a preliminary screening method to identify potential antagonists of pathogens, this is despite the fact that studies have demonstrated that the test medium greatly sways the results of *in vitro* antagonism bioassays (Schisler *et al.,* 1997) and that correlations between *in vitro* results and the level of biological control observed *in situ* are frequently inconsistent (Reddy *et al.,* 1993). Conversely, a bioassay of putative antagonists that involves testing microbial agents immediately after isolation using a whole plant system is enormously time consuming. It can also result in settling for mediocre biocontrol performance due to limitation on number of strains that can be processed in this manner.

There are several reports of correlation between assays *in vitro* and biocontrol *in vivo.* Production of the antibiotic chetomin by *Chaetomium globosum in vitro* was positively correlated with antagonism of *Venturia inequalis* on apple seedlings in a growth chamber (Cullen and Andrews, 1984). Similarly, the presence of zones of inhibition *in vitro* was good predictors of field performance of *Agrobacterium radiobacter* in controlling *A.*

tumifaciens on peaches (Alconero, 1980). *In vitro* and *in vivo* tests showed that antagonism to *Drechslera dictyoides* by bacteria isolated from the phylloplane was related to the *in vitro* production of inhibitory metabolites by these bacteria (Austin *et al.*, 1977).

Many published studies employ *in vitro* tests in which the pathogen and the antagonist are inoculated at opposing sides of petri-dishes filled with nutrient-rich laboratory medium, and inhibition of pathogen growth is measured. Most laboratory media are not representative of natural, nutrient-limited substrates. Such *in vitro* interaction have been criticised for over 30 years (Huber and Watson, 1966). Unfortunately, despite of modern reiterations of the warning (Sandys-Winsch *et al.*, 1994; Whipps, 1987), *in vitro* interactions are still widely used in the selection of biocontrol agents. This chapter deals with isolation, characterization of a large number of fluorescent pseudomonads and study were undertaken to identify the antagonistic activity of the isolates by *in vitro* screening against *Erwinia carotovora, Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* and finally selection of one putative biocontrol agent by comparing its level of antagonism with other screened fluorescent pseudomonads.

3.3. Materials and Methods

3.3.1. Isolation of fluorescent pseudomonads

Fluorescent pseudomonads were isolated from potato rhizosphere and healthy *Cymbidium* rhizosphere during 2003-2004 from five potato fields, viz., Darjeeling, Rimbik, Srikhola, Bijonbari and Singtam and five orchid nurseries such as Darjeeling, Kurseong, Kalimpong, Mirik and Ging. Each field samples consisted of roots of the plants and soils from the surrounding plants were taken in polythene bags and brought to the plant pathology laboratory. Roots of both potato and *Cymbidium* were shaken vigorously to remove loosely adhering soil prior to bacterial isolation. Roots and soil (1 gm) were washed in 10 fold (w/v) quantity of sterile phosphate buffer (pH 7.1) by agitation for 30 min on a rotary shaker at 150 rpm (Xu and Gross, 1986). Aliquots from appropriate serial dilutions were plated in duplicate on King's medium B agar (King, *et al.*, 1954). After incubation at 28°C for 48 hours, counts were made of total fluorescent colonies under UV light on King's B medium.

3.3.2. Characterization of fluorescent pseudomonads

3.3.2.1. Morphological characters

Young cultures (18 to 24 hours) were used for morphological characterization and the following morphological characters were evaluated.

3.3.2.1.1. ***Gram morphology:*** - A drop of bacterial suspension was placed on a clear grease free slide and made smear, dried and then heat fixed, slides were flooded with crystal violet solution for 30-60 sec. Poured off strain and applied iodine solution to washed away the crystal violet . Then slides were covered with fresh iodine solution for 30-60 sec. Washes the iodine solution with absolute alcohol until colour ceases to come out of preparation .Then saffranin was put on the slide for 1-2 min, washed with water and dried with blotting paper (Skerman, 1967).

3.3.2.1.2. ***Fluorescence and Motility:*** - Bacterial cultures were inoculated on King's B medium and incubated at 28°C for 48 hours. The bacterial colony appears on the plate were seen under UV light to check for fluorescence (Shinde and Lukezie, 1974).

Motility was checked by making hanging drop preparation of young broth and agar cultures (Skerman, 1967)

3.3.2.1.3. **Diffusible pigment:** - Pigment productions by fluorescent pseudomonads were determined by streak inoculation on King's B medium. Cultures were examined for pigmentation after 1, 2, 3 and 6 days of incubation at 28°C. Ultraviolet light used to check for fluorescence (Shinde and Lukezie, 1974).

3.3.2.1.4. **Size and Shape:** Leica DMSL research microscope was used to determine the size and shape of the fluorescent pseudomonads native isolates.

3.3.2.2. Cultural characteristics

Different cultural parameters like growth, form, elevation, colour, surface edges etc. were tested in King's B slant and plates after incubation at 28°C for 48-72 hours (Seeley and Vandenask, 1962).

3.3.2.2.1. ***Slant characters:*** - Fluorescent pseudomonads were grown on King's B slants and incubated at 28°C and there cultural characteristics were recorded as;

Growth: Scanty, moderate, abundant

Form: Beaded, arborescent, effuse, echinulate, rhizoid, filiform

Elevation: Flat or raised

3.3.2.2.2. ***Plate characters:*** - Fluorescent pseudomonads were point inoculated on King's B plate and incubated at 28°C and their cultural characteristics were recorded as;

Growth: Slow, moderate, rapid

Form: Punctiform, Circular, rhizoid, irregular

Surface: Smooth, rough, dry, moist, dull, glistening

Elevation: Flat, raised, convex, umbonate, pulvinate

Edges: Entire, undulate, lobate.

3.3.2.3. Biochemical characteristics

3.3.2.3.1. ***Levan production*****:** -Plates of Difco nutrient agar containing 5% sucrose was streaked with the isolates. Two days after inoculation, isolates that produced large, white, domed mucoid colonies were considered to be levan producers (Lelliott *et al.*, 1966; Misaghi *et al.*, 1969).

3.3.2.3.2. ***Oxidase production*****:** - Kovac's method (Kovacs, 1956) was used to test for the presence of oxidase. Loopful of bacteria from 48 hours old cultures grown on nutrient agar and King's B medium was smeared on filter paper previously soaked with 1% (w/v) aqueous solution of N,N-dimethyl-p-phenylene-diamine with a platinum needle. Production of dark purple colour in 10 sec indicated the presence of oxidase.

3.3.2.3.3. ***Arginine dihydrolase production*****:** - About 10 ml of Thornley's arginine medium 2A (Shinde and Lukezie, 1974) in screw capped test tubes were sterilized by autoclaving. The pH was adjusted to 7.2 with NaOH. Duplicate tubes were stab inoculated with 48 hours old cultures. One was sealed with sterile, melted and cooled vaseline to a depth of 5 mm. The screw cap of the other tube was left loose. The anaerobic formation of alkali from arginine was detected by a change in colour of the indicator after 4 days. This change suggested the presence of all or part of the arginine dihydrolase system (Lelliott *et al.*, 1966; Misaghi *et al.*, 1969).

3.3.2.3.4. ***Amylase production*****:** - Plates of nutrient agar containing 1% soluble starch were streaked once and incubated at 28°C. After 48 hours iodine solution were poured in the plate. The presence of a clear area around the bacterial growth indicated starch hydrolysis (Shinde and Lukezic ,1974).

3.3.2.3.5. ***Protease production*:** - Gelatin liquifaction was tested by stabbing tubes of Difco nutrient gelatin and checking 1,5,10 and 14 days after inoculation. The cultures were placed at 4°C for 30 min before recording the results. Liquifaction was positive if the medium flowed readily when tubes were tilted (Lelliott *et al.*, 1966; Misaghi *et al.*, 1969).

Gelatin hydrolysis was confirmed by streaking duplicate plates of nutrient agar with 0.4% gelatin with each isolate and testing with an $HgCl_2$/Tri Chloro Acetic Acid (TCA) (Skerman, 1967).

Protease activity was also performed by inoculating the isolates on 5X minimal media supplemented with 5% of skim milk and 1% agar; incubate at 28°C for 24-48 hours and testing with TCA solution. Presence of clear zone around the colonies indicated protease activity (Gaur *et al.*, 2004).

3.3.2.3.6. ***Pectinase production*:** - Plates of 5X minimal media supplemented with 1% pectin were inoculated with the isolates. After 2-4 days plates were flowed with cetrimide solution. The presence of clear zone around the colonies indicated pectin hydrolysis (Gaur *et al.*, 2004).

3.3.2.3.7. ***Cellulase production*:** - Plates of 5X minimal media supplemented with 1% carboxy methyl cellulose (CMC) were inoculated with the isolates. After 2-4 days plates were flowed with cetrimide solution. The presence of clear zone around the colonies indicated cellulose hydrolysis (Gaur *et al.*, 2004).

3.3.2.3.8. ***Catalase activity*:** - Colony of bacteria from a 24 hours old nutrient agar slants were transfer on a glass slide and few drops of H_2O_2 (3% w/v) were poured over the culture. The release of bubble within 20 sec indicates positive catalase activity (Skerman, 1967).

3.3.2.3.9. ***Indole production*:** - Tubes of 1% tryptone were inoculated with one drop of bacterial suspension. Strips of dry filter paper previously soaked in a saturated solution of oxalic acid were inserted with the cotton plugs in such a position that they did not touch the surface of the medium and incubate it

at 28°C for 48 hours. The presence of indole was indicated by the development of pink colour on the filter paper strip (Shaffer, 1967).

3.3.2.3.10. ***Nitrate reduction*****:** -Tube of Nitrate broth (KNO_3-0.1%; peptone-1%; beef extract-0.3%) were inoculated with bacterial culture and incubated at 28°C for 48 hours. Three drops of reagent (Zink chloride-20 gm; starch -4 gm; potassium iodide-2 gm; powdered zinc metal; water-1 lit) and 1 drop of sulphuric acid were poured to a drop of 48 hours old bacterial culture. Appearance of blue colour indicates nitrate production (Shaffer, 1967).

Nitrate reduction was also performed by adding few drops of solution A (sulphanilic acid) and same amount of solution B (dimethyl-alpha-napthyalamine) in 48 hours old nitrate broth culture. Appearance of red colour indicates the nitrate reduction (Dubey and Musheshwari, 2002).

3.3.2.3.11. ***Acid and gas formation from carbohydrates*****:** - To detect acid and gas formation from carbohydrates, thirteen different types of carbohydrates (Glucose, sucrose, glycerol, maltose, manitol, rhaffinose, xylose, ribose, mannose, galactose, arabinose, rhamnose and fructose) were used. Fermentation broth (peptone- 10 gm; Carbohydrate- 5 gm; NaCl- 5 gm; Phenol red- 0.018 gm, water – 1 lit.) of thirteen different sugars were taken in fermentation tube and inoculated with fluorescent pseudomonad isolates, whereas uninoculated tubes were served as control. All the inoculated and uninoculated tubes were incubated at 28°C for 24 to 48 hours. Change in colour (due to production of acid) or change in colour and appearance of gas (due to production of acid from gas) were recorded by comparing with the uninoculated (control) tube (Dubey and Musheshwari, 2002).

3.3.2.3.12. ***Methyl red (MR) test*** **:-** The ability of different isolates to produce acid from glucose in amount sufficient to reduce the pH to 4.2 or less

and to maintain this low pH for at least 4 days was determined by using the glucose-phosphate-peptone-water method outlined by Skerman (Skerman, 1967). Cultures were tested for acid production 2 and 4 days after inoculation by adding a few drops of methyl red solution. The presence of red colour was taken as a positive indication and yellow colour as a negative indication.

3.3.2.3.13. *Voges-Proskaner (VP) test*: - The ability of different isolates to produce acid from glucose and subsequently to convert it to acetylmethylcarbinol or 2:3-butylene glycol, both neutral substances, was determined by the method outlined by Skerman (Skerman, 1967). Cultures grown in glucose-phosphate-peptone-water were tested for the presence of a neutral substance with 1 ml of 10% KOH solution, after 2 and 4 days of incubation. The presence of pink colour was scored positive and no colouration as negative.

3.3.3. Preliminary screening of antagonists

All the rhizospheric fluorescent pseudomonads were preliminary screened for antagonistic activity by using the petriplate assay as described by Anderson et al. (1980). *In vitro* antagonistic activities of the isolated fluorescent pseudomonads were performed against both the fungal pathogens i.e., *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* as well as against bacterial pathogen i.e. *Erwinia carotovora* by inoculating four isolates 2-3 cm apart from each other on Peptone Glucose and Agar (PGA) medium. Mycelial plugs of 5 mm in diameter of the fungal pathogens were transferred at the centre of the four inoculated fluorescent pseudomonads on PGA plate whereas for bacterial pathogen two fluorescent pseudomonads were streaked straight to an nutrient agar plate and *E. carotovora* was streaked aseptically perpendicular to the two streaks in such a way that they do not touch each other. Plates were replicated and repeated thrice. They were incubated at

30°C and antagonistic activities of different isolates were recorded after 72 hours. Isolates, which showed antagonism, were subcultured and maintained on King's B slant at 4°C for further use.

3.3.4. *Interaction of potential (screened) fluorescent pseudomonads against Cymbidium rot pathogens by dual culture technique*

3.3.4.1. Dual plate culture technique

The antagonistic effects of screened fluorescent pseudomonads were tested against fungal pathogens on Peptone Glucose Agar (PGA) plate by following the method of Skidmore and Dikinson (1970). This was done by streaking the test fluorescent *Pseudomonas* from 24 hours old culture (containing 10^7 cfu/ml) in a circle / O and semicircle / U shaped pattern, where as fungal pathogens (*Fusarium oxysporum* and *Mucor hiemalis* separately) were point inoculated at the centre of the plate. Uninoculated sets were served as control. The plates were incubated at 30°C for 5 days and inhibitions of colony growth were measured.

To test the inhibitory effect of screened fluorescent pseudomonads against the bacterial pathogen (*Erwinia carotovora*), the pathogen was streaked straight to an agar plate and incubate it at 30°C for 24 hours. After incubation and appearance of growth, the test fluorescent *Pseudomonas* was streaked perpendicular to the pathogenic bacteria in such a way that they do not touch each other. Then inoculated plates were again incubated at 30°C.

3.3.4.2. Dual liquid culture technique:

To test the antifungal activity of the screened fluorescent pseudomonads, dual liquid culture method was employed (Basha and Ulaganathan, 2002). One ml of freshly grown (24 hours old) fluorescent pseudomonad culture (containing 10^7 cfu / ml) and an agar block (5mm diameter) of fungal pathogen was inoculated onto 50 ml of peptone, glucose medium in 250 ml conical flask. The

culture was incubated on a rotary shaker at 30°C for 72 hours. For the control experiment the fungal pathogens were inoculated alone in the peptone glucose broth. Experiments were performed thrice taking triplicate for every set.

The difference in dry weights between the fungal culture grown with the test isolates or the control culture grown without any bacterium were recorded. For this 72 hours old dual liquid culture were passed through the pre weighed Whatman No 1 filter paper. It was dried for 24 hours at 70°C and weights were measured.

3.4. Results and Discussion

A total of fifty-four fluorescent pseudomonad strains were isolated from rhizosphere of potato and *Cymbidium*. Of which thirty-six strains were isolated from potato rhizosphere. Among them Darjeeling contributes - 6, Rimbik- 3, Srikhola- 3, Bijonbari- 9 and Singtam- 15. Eighteen strains were isolated from healthy *Cymbidum* rhizosphere, of which, from Darjeeling- 5; Kurseong- 8 and Kalimpong -5. Morphological, cultural, physiological and biochemical parameters of these native isolates were further evaluated.

3.4.1. Morphological characterization of fluorescent pseudomonads

All the isolates of fluorescent pseudomonads were non spore forming, fluoresces under UV light, gram negative, medium size rods, size ranges from 0.5 to 0.8 × 1 to 3 μm, with round to slightly pointed ends, motile in hanging drop preparation. On King's B medium they produce greenish-yellow to yellow fluorescent pigment when seen under UV light.

3.4.2. Biochemical and physiological characterization of fluorescent pseudomonads

All the fluorescent pseudomonad native isolates were aerobic as they grew in the upper layer and on the medium surface. They were levan positive (Table 3.1). The isolates were oxidase positive on nutrient agar and King's B medium. All the isolates were able to show reduction of nitrate after 48 hours of incubation. Most of the isolates were catalase positive, strong liquefiers of gelatin. A clear zone of 1 to 12 mm around each colony within 48 to 72 hours of incubation, indicating rapid hydrolysis of gelatin by the isolates (Table 3.1). The organisms did not produce arginine dihydrolase, indole production negative but few isolates showed faint reaction which was indicated by the development of pink colouration on saturated oxlic acid strip. In most of the cases methyl red (MR) test were negative as well as Voges-Proskauer (VP) test results were negative till after 72 hours of incubation. There were no cellulase

activity in most of the cases but protease, pectinase, amylase activities of the isolates were recorded as moderate to wide clear zone around several colonies (Table 3.1).

Table 3.1. Biochemical characterization of fifty-four fluorescent pseudomonads

Strains	Amylase	Gelatin hydrolysis	Cellulase	Pectinase	Protease	Catalase	Oxidase	Nitrate Reduction	Arginine Dihydrolase	Indole Production	MR test	VP test-	Levan production
BRL-1	-	+	-	-	+	++	+	+	-	+	-	-	+
BRL-2	-	++	-	+	-	+	+	+	-	-	-	-	+
BRL-3	-	-	-	+	+	++	+	+	-	-	-	-	+
BRL-4	+	+	-	-	-	+++	+	+	-	-	-	-	+
BRL-5	-	-	-	-	-	+	+	+	-	-	-	-	+
BRL-6	++	+++	+	-	-	++	+	+	-	-	-	-	+
BRL-PR-1	-	+	++	-	-	-	+	+	-	-	-	-	+
BRL-PR-2	+	+	++	-	++	+	+	+	-	-	-	-	+
BRL-PR-3	-	+	++	+	+	-	+	+	-	+	-	-	+
BRL-Slg-1	+	++	++	+++	+	++	+	+	-	+	-	-	+
BRL-Slg-2	+	++	++	++	-	-	+	+	-	-	-	-	+
BRL-Slg-3	++	++	-	++	-	-	+	+	-	-	-	-	+
BRl-Slg-4	+	-	-	+++	-	+	+	+	-	-	-	-	+
BRl-Slg-5	++	++	-	+	++	++	+	+	-	-	-	-	+
BRl-Slg-6	+	-	-	+++	+	++	+	+	-	-	-	-	+
BRl-Slg-7	+++	-	-	-	-	+++	+	+	-	-	-	-	+
BRL-Slg-8	+++	++	-	-	-	+	+	+	-	-	-	-	+
BRL-Dj1	-	+++	-	-	+++	+	+	+	-	-	-	-	+
BRL-DJ2	-	+	-	+	-	+++	+	+	-	-	-	-	+
BRL-Dj3	-	++	-	+	-	+	+	+	-	-	-	-	+
BRL-DJ4	+	++	-	+	-	+	+	+	-	-	-	-	+
BRL-DJ5	+	++	-	-	-	+++	+	+	-	-	-	-	+
BRL-Rim-1	-	+	-	-	+	+	+	+	-	+	-	-	+
BRL-Rim-2	-	++	-	-	-	++	+	+	-	-	-	-	+
BRL-Rim-3	-	++	-	+	+	+	+	+	-	-	-	-	+
BRL-BB1	+	++	-	+	++	-	+	+	-	-	-	-	+
BRL-BB2	-	+	-	-	-	-	+	+	-	-	+	-	+
BRL-BB3	+	++	-	+	+++	-	+	+	-	-	-	-	+
BLR-BB4	-	-	-	+	-	++	+	+	-	+	-	-	+
BRL-BB5	+	+	+	+	-	+++	+	+	-	-	-	-	+

Strains	Amylase	Gelatin hydrolysis	Cellulase	Pectinase	Protease	Catalase	Oxidase	Nitrate Reduction	Arginine Dihydrolase	Indole Production	MR test	VP test-	Levan production
BRL-BB6	+	++	-	+++	+	-	+	+	-	-	-	-	+
BRL-BB7	+	++	-	+	-	+	+	+	-	+	-	-	+
BRL-BB8	+	+	+	-	-	++	+	+	-	-	-	-	+
BRL-BB9	++	+	-	-	-	++	+	+	-	+	-	-	+
BRL-Sg-1	+	+	-	+	-	+	+	+	-	+	-	-	+
BRL-Sg-2	+	+	-	+	+	++	+	+	-	-	-	--	+
BRL-Sg-3	-	-	-	+	-	+++	+	+	-	-	-	-	+
BRL-Sg-4	-	-	-	-	-	-	+	+	-	-	-	-	+
BRL-Sg-5	+	+	-	-	-	++	+	+	-	+	-	-	+
BRL-Sili1	-	-	-	-	-	+	+	+	-	+	+	-	+
BRL-Sili2	+++	-	-	+	+	-	+	+	-	-	+	-	+
BRL-Sili3	++	+	-	++	++	++	+	+	-	+	+	-	+
BRL-Sili4	-	+	-	-	+	++	+	+	-	+	-	-	+
BRL-Sili5	-	+++	+	-	++	+	+	+	-	-	+	-	+
BRL-Sili6	-	+++	-	-	+++	+	+	+	-	-	+	-	+
BRL-Sili7	-	++	-	-	+++	-	+	+	-	-	+	-	+
BRL-Sili8	-	+++	-	-	++	-	+	+	-	-	+	-	+
BRL-Sili9	++	+	-	+	+	++	+	+	-	-	+	-	+
BRL-Sili10	-	-	+	-	-	-	+	+	-	-	+	-	+
BRL-Sili11	++	+	-	+	+	+	+	+	-	-	+	-	+
BRL-Sili12	-	+++	-	-	++	-	+	+	-	-	+	-	+
BRL-Sili13	-	+++	+	-	+++	++	+	+	-	-	+	-	+
BRL-Sili14	+++	++	-	+	+	+++	+	+	-	+	+	-	+
BRL-Sili15	++	+	-	++	++	++	+	+	-	+	-	-	+

The columns containing '+++', '++', '+' and '-' denoting High, Moderate, Low and Negative activity respectively. The columns containing '+' and/or '-' denoting positive and negative activity against that biochemical test.

Acid and gas formation by the isolates against thirteen different carbohydrate sources showed a wide variation on the level of their acid production during growth after 48 hours of incubation. Gas formations by the isolates from different carbohydrate sources were recorded as negative (Table 3.2).

Table 3.2. Acid and gas formation by the isolates using thirteen different carbohydrate sources.

Strains	Glucose	Sucrose	Glycerol	Maltose	Manitol	Rhaffinose	Xylose	Ribose	Mannose	Galactose	Arabinose	Rhamnose	Fructose
BRL-1	+++	+++	++	+	++	+	+	++	+++	+	-	-	+++
BRL-2	++	+	+	+	-	-	-	+	-	-	-	+	+++
BRL-3	+++	+	+	+	-	+	+	+	+	+	+	+	+++
BRL-4	+++	+	+	+	++	+	+	+	++	-	-	+	+++
BRL-5	++	+++	++	++	++	++	++	+	+++	+++	++	+	+++
BRL-6	+++	+++	++	+	+	-	-	+	++	++	-	-	+++
BRL-PR-1	++	+	++	++	++	+	-	+	+	+	-	+	+++
BRL-PR-2	+++	++	+	++	+	+	+	+	-	++	-	++	++
BRL-PR-3	+++	+++	+	++	++	++	+	++	++	++	-	+	+++
BRL-Slg-1	++	+	-	-	-	+	-	+	-	-	+	+	+++
BRL-Slg-2	++	+	-	-	+	+	-	+	-	-	+	+	++
BRL-Slg-3	++	+++	+	++	++	++	+	++	+	+	+	+	+++
BRl-Slg-4	+++	++	+	+	-	+	++	+	-	+	++	+	+++
BRl-Slg-5	++	+	-	+	-	+	+	++	+	+	+	+	+++
BRl-Slg-6	+	++	-	+	+	++	+	++	+	-	-	-	++
BRl-Slg-7	+++	+++	+	+	-	+	+	+	++	+	+	+	+++
BRL-Slg-8	+++	++	+	++	+	+	+	+	+	+	-	+	++
BRL-Dj1	+++	+++	+	++	+	++	-	++	+	+	+	+	+++
BRL-DJ2	+++	++	+	++	++	+++	+	++	+	+	+	+	++
BRL-Dj3	+++	+++	+	+	+	-	+	+	+++	+	+	+	+
BRL-DJ4	++	-	-	+	-	+	-	+	-	-	-	-	+
BRL-DJ5	+++	+++	++	+	+++	+	++	++	+++	+	+	+	++
BRL-Rim1	++	-	-	++	-	-	-	-	+	+	-	-	++
BRL-Rim2	+++	++	-	+	+	+	+	+	+++	+	-	-	++
BRL-Rim3	+++	++	-	+	+	+	+	+	+++	+	+	+	+++
BRL-BB1	+	+++	-	-	++	-	-	-	++	-	-	+	++
BRL-BB2	+++	++	-	-	+	-	-	-	+	-	-	-	+++
BRL-BB3	+++	+++	+	+	+	+	+	+	++	+	+	+	+++
BRL-BB4	+++	+++	+	+	++	+	+	+	+++	+	+	+	+
BRL-BB5	++	+	-	-	-	+	-	-	+	-	+	-	++
BRL-BB6	+++	+	+	++	-	+	++	+	++	-	+	+	+++
BRL-BB7	+++	+++	++	+	+	+	+	+	+	+	+	++	+++
BRL-BB8	+	+++	-	+	-	-	-	-	++	-	-	+	+
BRL-BB9	+++	+++	+	++	++	+	-	+	+++	+	-	++	+++
BRL-Sg-1	+++	+++	+	+	+++	+	+	++	+++	+	+	++	+++
BRL-Sg-2	+++	+++	-	-	+	+	-	+	+	-	-	-	++
BRL-Sg-3	+++	+	-	-	-	+	-	+	+	-	+	-	++
BRL-Sg-4	+++	++	+	++	++	+	++	+	++	+	-	-	++
BRL-Sg-5	+++	+++	+	++	+++	+	++	+	+++	+	-	+	+++
BRL-Sili1	++	+	-	-	-	-	+	-	+	-	-	-	+++

Strains	Glucose	Sucrose	Glycerol	Maltose	Manitol	Rhaffinose	Xylose	Ribose	Mannose	Galactose	Arabinose	Rhamnose	Fructose
BRL-Sili2	+++	++	+	++	+	++	+	+	+	+	+	+	+++
BRL-Sili3	++	+	+	++	+	+	++	++	++	+	++	+	++
BRL-Sili4	+	+	+	+	+	+	+	+	++	++	+	+	+
BRL-Sili5	+++	+	+	++	+	+	-	+	-	-	+	-	+++
BRL-Sili6	+++	+	+	+	+	+	+	++	++	+	++	+	+++
BRL-Sili7	+++	+++	+	+++	+	-	-	+++	+++	+	+	+	+++
BRL-Sili8	+++	+	+	+	+	+	+	++	+	+	+	+	+++
BRL-Sili9	+++	+++	+	+	+	-	+	++	+++	+	+	+	+++
BRL-Sili10	+++	+++	+	++	+	-	-	++	++	+	+	+	+++
BRL-Sili11	+++	+++	+	+++	+	-	+	+++	+++	+	+	+	+++
BRL-Sili12	+++	+	++	++	+	+	+	++	++	-	+	+	+++
BRL-Sili13	+++	+	+	+++	+	+	+	+++	+++	+	+	+	+++
BRL-Sili14	+++	+	+	++	+	+	+	+++	+++	-	+	+	+++
BRL-Sili15	+++	+++	+	+++	+	-	+	+	+++	+	+	+	+++

'-' = *No change*
'+' = *Minor change (reddish yellow)*
'++' = *Moderate change (Yellowish red)*
'+++' = *High (Yellow)*

3.4.3. Cultural characteristics

Different cultural parameters were tested in King's B slant and plate after incubation at 30°C for 48-72 hours and their mode of growth on plate as well as in slants were recorded (Table 3.3).

Table 3.3. Cultural Characteristics of fifty four fluorescent pseudomonads

	Slant				Plate				
Strain	**Growth**	**Form**	**Elevation**	**Colour**	**Growth**	**Form**	**Surface**	**Elevation**	**Edges**
BRL-1	Abundant	Echinulate	Raised	Whitish	Rapid	Irregular	Smooth	Raised	Undulate
BRL-2	Moderate	Beaded	Flat	Whitish	Moderate	Irregular	Rough	Raised	Undulate
BRL-3	Moderate	Filiform	Flat	Yellowish	Rapid	Circular	Smooth, Glistening	Flat	Entire
BRL-4	Abundant	Echinulate	Raised	Whitish	Rapid	Irregular	Rough, Dry	Raised	Entire
BRL-5	Scanty	Beaded	Raised	Whitish	Slow	Circular	Smooth, Glistening	Raised	Undulate
BRL-6	Abundant	Filiform	Flat	Whitish	Moderate	Irregular	Rough, Dull	Flat	Lobate
BRL-PR-1	Abundant	Effuse	Raised	Orange	Rapid	Irregular	Glistening	Raised	Undulate
BRL-PR-2	Abundant	Beaded	Raised	Whitish	Rapid	Circular	Smooth	Raised	Entire
BRL-PR-3	Abundant	Echinulate	Raised	Orange	Rapid	Circular	Smooth, Glistening	Raised	Circular
BRL-Slg-1	Moderate	Beaded	Flat	Whitish	Rapid	Circular	Smooth	Raised	Entire
BRL-Slg-2	Scanty	Filiform	Flat	Whitish	Moderate	Irregular	Rough, Dry	Flat	Entire
BRL-Slg-3	Abundant	Echinulate	Raised	Whitish	Rapid	Circular	Smooth	Raised	Entire
BRL-Slg-4	Abundant	Filiform	Flat	Whitish	Rapid	Punctiform	Smooth, Dry	Raised	Undulate
BRL-Slg-5	Moderate	Echinulate	Flat	Whitish	Rapid	Circular	Rough	Flat	Entire
BRL-Slg-6	Abundant	Echinulate	Flat	Whitish	Moderate	Circular	Smooth, Glistening	Convex	Entire
BRL-Slg-7	Abundant	Beaded	Raised	Whitish	Rapid	Irregular	Rough	Raised	Undulate
BRL-Slg-8	Abundant	Effuse	Raised	Whitish	Rapid	Circular	Smooth, Glistening	Flat	Entire
BRL-Dj-1	Abundant	Echinulate	Flat	Whitish	Rapid	Irregular	Rough	Raised	Lobate
BRL-Dj-2	Scanty	Beaded	Raised	Whitish	Moderate	Circular	Smooth, Glistening	Raised	Entire
BRL-Dj-3	Moderate	Beaded	Flat	Whitish	Moderate	Circular	Smooth	Raised	Entire
BRL-Dj-4	Abundant	Beaded	Raised	Whitish	Rapid	Irregular	Rough	Raised	Entire
BRL-Dj-5	Moderate	Filiform	Flat	Whitish	Rapid	Irregular	Rough	Raised	Undulate
BRL-Rim-1	Moderate	Effuse	Raised	Orange	Moderate	Circular	Smooth, Glistening	Raised	Undulate
BRL-Rim-2	Abundant	Echinulate	Flat	Yellowish	Moderate	Circular	Smooth, Glistening	Raised	Entire
BRL-Rim-3	Moderate	Filiform	Flat	Whitish	Rapid	Circular	Rough, Dry	Raised	Undulate
BRL-BB1	Abundant	Arbores cent	Raised	Whitish	Moderate	Circular	Glistening	Convex	Entire
BRL-BB2	Abundant	Beaded	Raised	Reddish	Rapid	Irregular	Rough	Umbonate	Undulate
BRL-BB3	Scanty	Effuse	Flat	Whitish	Slow	Punctiform	Rough	Flat	Undulate

	Slant				Plate				
BRL-BB4	Abundant	Effuse	Flat	Whitish	Moderate	Irregular	Rough	Flat	Undulate
BRL-BB5	Moderate	Beaded	Raised	Yellowish	Moderate	Circular	Smooth	Flat	Entire
BRL-BB6	Abundant	Beaded	Raised	Whitish	Moderate	Irregular	Rough	Flat	Undulate
BRL-BB7	Abundant	Beaded	Raised	Grayish	Moderate	Irregular	Rough	Flat	Undulate
BRL-BB8	Scanty	Arbores cent	Flat	Whitish	Slow	Rhizoid	Rough, Moist	Flat	Lobate
BRL-BB9	Abundant	Echinulate	Flat	Whitish	Moderate	Irregular	Smooth, Glistening	Raised	Undulate
BRL-Sg1	Moderate	Beaded	Raised	Yellowish	Slow	Circular	Dry, Rough	Flat	Undulate
BRL-Sg2	Moderate	Echinulate	Flat	Yellowish	Slow	Circular	Smooth, Glistening	Raised	Entire
BRL-Sg3	Abundant	Echinulate	Flat	Whitish	Moderate	Circular	Smooth	Flat	Entire
BRL-Sg4	Scanty	Filiform	Flat	Whitish	Rapid	Irregular	Rough	Raised	Lobate
BRL-Sg-5	Abundant	Beaded	Flat	Whitish	Moderate	Irregular	Rough	Raised	Lobate
BRL-Sili-1	Abundant	Effuse	Flat	Whitish	Moderate	Circular	Smooth, Dry	Flat	Entire
BRL-Sili-2	Abundant	Filiform	Raised	Whitish	Rapid	Circular	Smooth, Glistening	Raised	Entire
BRL-Sili-3	Moderate	Effuse	Raised	Whitish	Rapid	Circular	Smooth, Glistening	Raised	Entire
BRL-Sili-4	Moderate	Echinulate	Raised	Yellowish	Slow	Punctiform	Rough, Dull	Flat	Entire
BRL-Sili-5	Abundant	Filiform	Flat	Whitish	Rapid	Circular	Smooth, Glistening	Raised	Undulate
BRL-Sili-6	Abundant	Echinulate	Raised	Whitish	Rapid	Irregular	Rough	Flat	Undulate
BRL-Sili-7	Abundant	Echinulate	Raised	Whitish	Rapid	Circular	Glistening	Flat	Undulate
BRL-Sili-8	Abundant	Filiform	Raised	Whitish	Moderate	Punctiform	Rough, Dull	Flat	Undulate
BRL-Sili-9	Abundant	Filiform	Raised	Whitish	Rapid	Circular	Smooth, Glistening	Raised	Entire
BRL-Sili-10	Scanty	Beaded	Flat	Whitish	Slow	Punctiform	Rough, Dry	Flat	Entire
BRL-Sili-11	Scanty	Echinulate	Flat	Whitish	Rapid	Circular	Smooth, Glistening	Convex	Entire
BRL-Sili-12	Scanty	Filiform	Flat	Whitish	Moderate	Irregular	Dry, Rough	Flat	Undulate
BRL-Sili-13	Scanty	Beaded	Flat	Whitish	Rapid	Circular	Smooth, Glistening	Raised	Entire
BRL-Sili-14	Scanty	Filiform	Flat	Whitish	Moderate	Circular	Smooth	Raised	Entire
BRL-Sili-15	Abundant	Beaded	Flat	Whitish	Moderate	Irregular	Rough	Raised	Undulate

3.4.4. Preliminary screening of potential antagonists

During preliminary screening of potential antagonist it has been found eleven strains out of fifty-four native isolates showed antagonistic activity, but considering their effectiveness only six isolates viz., BRL-1, BRL-Dj5, BRL-Sg-5, BRL-BB-9, BRL-Sg-1 and BRL-Sili-15 showed antagonism against all three pathogens (Table 3.4). All the fluorescent pseudomonad isolates tested, reacted similarly in almost all the physiological and biochemical tests to which they were subjected. Slight variations in reactions to a few of the tests are not unusual, since these isolates were collected from various localities and at different times. Similar variation among the isolates of other bacteria has been reported by others (Dye, 1968; Lelliott *et al.*, 1966; Misaghi and Grogan 1969; Sands *et al.*, 1970).

Among the fifty four fluorescent pseudomonad native isolates, though there were similarities in their physiological and biochemical parameters, but only six of them have been found to be the most potent antagonists. *In vitro* assay of these six screened antagonists were evaluated against *Erwinia carotovora, Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* by dual plate and dual liquid culture technique.

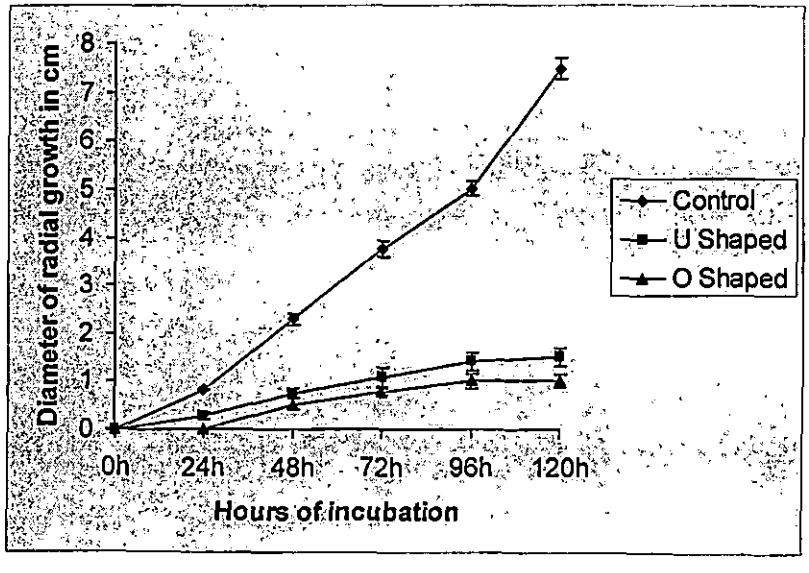
Table 3.4: Screening of fifty-four fluorescent pseudomonads against three pathogens of *Cymbidium* rot

Strains	*Erwinia carotovora*	*Fusarium oxysporum*	*Mucor hiemalis* f. *hiemalis*
BRL-1	+	+	+
BRL-2	-	-	-
BRL-3	-	-	-
BRL-4	-	-	-
BRL-5	-	-	-
BRL-6	-	-	-
BRL-PR-1	-	-	-
BRL-PR-2	-	-	-
BRL-PR-3	-	+	+
BRL-Slg-1	-	-	-
BRL-Slg-2	-	-	-
BRL-Slg-3	-	-	-
BRl-Slg-4	-	-	-
BRl-Slg-5	-	-	-
BRl-Slg-6	-	+	-
BRl-Slg-7	-	-	-
BRL-Slg-8	-	-	-
BRL-Dj1	-	-	-
BRL-DJ2	-	-	-
BRL-Dj3	-	-	-
BRL-DJ4	-	-	-
BRL-DJ5	+	+	+
BRL-Rim-1	-	-	-
BRL-Rim-2	-	-	-
BRL-Rim-3	-	-	-
BRL-BB1	-	-	-
BRL-BB2	-	-	-

Strains	*Erwinia carotovora*	*Fusarium oxysporum*	*Mucor hiemalis f. hiemalis*
BRL-BB3	-	-	-
BLR-BB4	-	-	-
BRL-BB5	-	-	-
BRL-BB6	-	-	-
BRL-BB7	-	-	-
BRL-BB8	-	-	-
BRL-BB9	+	+	+
BRL-Sg-1	+	+	+
BRL-Sg-2	-	-	-
BRL-Sg-3	-	-	-
BRL-Sg-4	-	-	-
BRL-Sg-5	+	+	+
BRL-Sili1	-	-	-
BRL-Sili2	-	-	-
BRL-Sili3	-	-	-
BRL-Sili4	+	-	-
BRL-Sili5	-	-	-
BRL-Sili6	-	-	-
BRL-Sili7	-	-	+
BRL-Sili8	-	-	-
BRL-Sili9	-	-	-
BRL-Sili10	+	-	-
BRL-Sili11	-	-	-
BRL-Sili12	-	-	-
BRL-Sili13	-	-	-
BRL-Sili14	-	-	-
BRL-Sili15	+	+	+

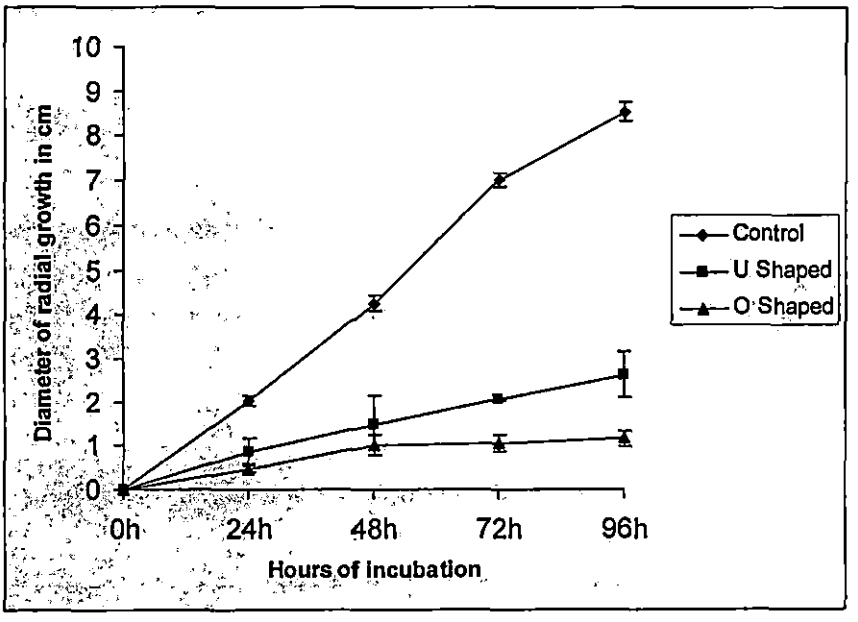
3.4.5. *In vitro antagonistic activity of fluorescent Pseudomonas BRL-1 against Fusarium oxysporum, Mucor hiemalis* f. *hiemalis and Erwinia carotovora*

In dual culture, significant growth inhibition of the fungal pathogens i.e. *F. oxysporum* and *M. hiemalis* f. *hiemalis* occurred due to antagonistic effect of fluorescent *Pseudomonas* BRL-1, which was evident from inhibition of colony diameter. The results are presented in Figure 3.1(A and B). At 48 hours of incubation, pathogens were strongly inhibited by fluorescent *Pseudomonas* BRL-1 and simultaneous increase in incubation time corresponded escalation in percent growth inhibition (Figure 3.2). There was more than 68% and 60% reduction in dry weight of the *F. oxysporum* and *M. hiemalis* f. *hiemalis* culture respectively grown with fluorescent *Pseudomonas* BRL-1 strain when compared to the control. In nutrient agar plate, the growth inhibition of the bacterial pathogen i.e. *Erwinia carotovora* was clearly seen after 48 hours of incubation which shows no growth in the interacting zone where as luxuriant growth away from the interaction (Figure 3.13a).



A

Figure 3.1A. In vitro inhibitory effect of fluorescent Pseudomonas BRL-1 cultured as circular and semicircular streak, around Fusarium oxysporum, the fungal pathogen was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.



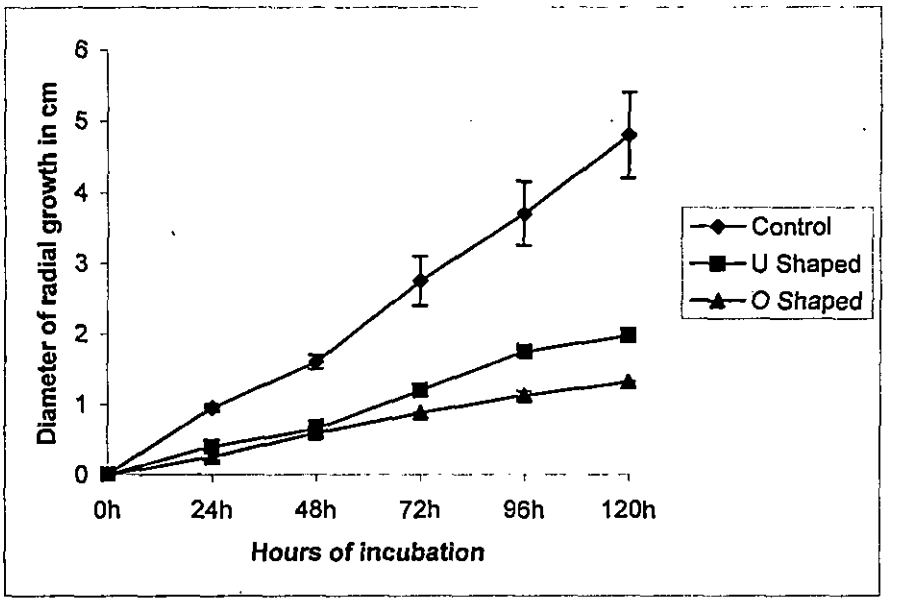
B

Figure 3.1B: In vitro inhibitory effect of fluorescent Pseudomonas BRL-1 cultured as circular and semicircular streak around Mucor hiemalis f. hiemalis, the fungal pathogen was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.

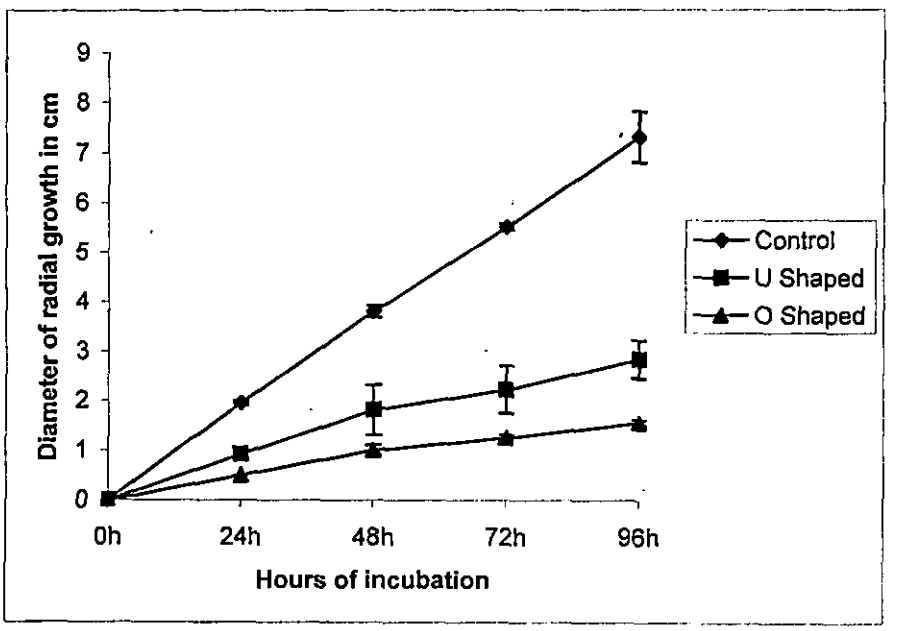
3.4.6. In vitro antagonistic activity of fluorescent Pseudomonas Dj-5 against Fusarium oxysporum, Mucor hiemalis f. hiemalis and Erwinia carotovora:

Fluorescent *Pseudomonas* Dj-5 is also a potent organism, shows satisfactory growth inhibition of the fungal pathogens which was evident in dual plate culture. The results are presented in Figure 3.3 (A and B). At 48 hours of incubation, pathogens were well inhibited by fluorescent *Pseudomonas* Dj-5 and the percent growth inhibitions were increased with rise in incubation time (Figure 3.4). It was shown that there were more than 62 and 57 percent reduction in dry weight of the *F. oxysporum* and *M. hiemalis* f. *hiemalis* culture respectively. The growth inhibition of the bacterial pathogen was also observed after 48 hours of incubation indicates inadequate growth at the interacting zone (Figure 3.13b).

Figure 3.2. Inhibitory effect of fluorescent Pseudomonas BRL-1 on F. oxysporum and M. hiemalis f. hiemalis: (A and D) Control set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; (B and E) Colony showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing restricted growth due to the antagonist inoculated as a circular streak



A



B

Figure 3.3: In vitro inhibitory effect of fluorescent Pseudomonas Dj-5 cultured as circular and semicircular streak, around A. Fusarium oxysporum and B. Mucor hiemalis f. *hiemalis, where as the fungal pathogens were point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.*

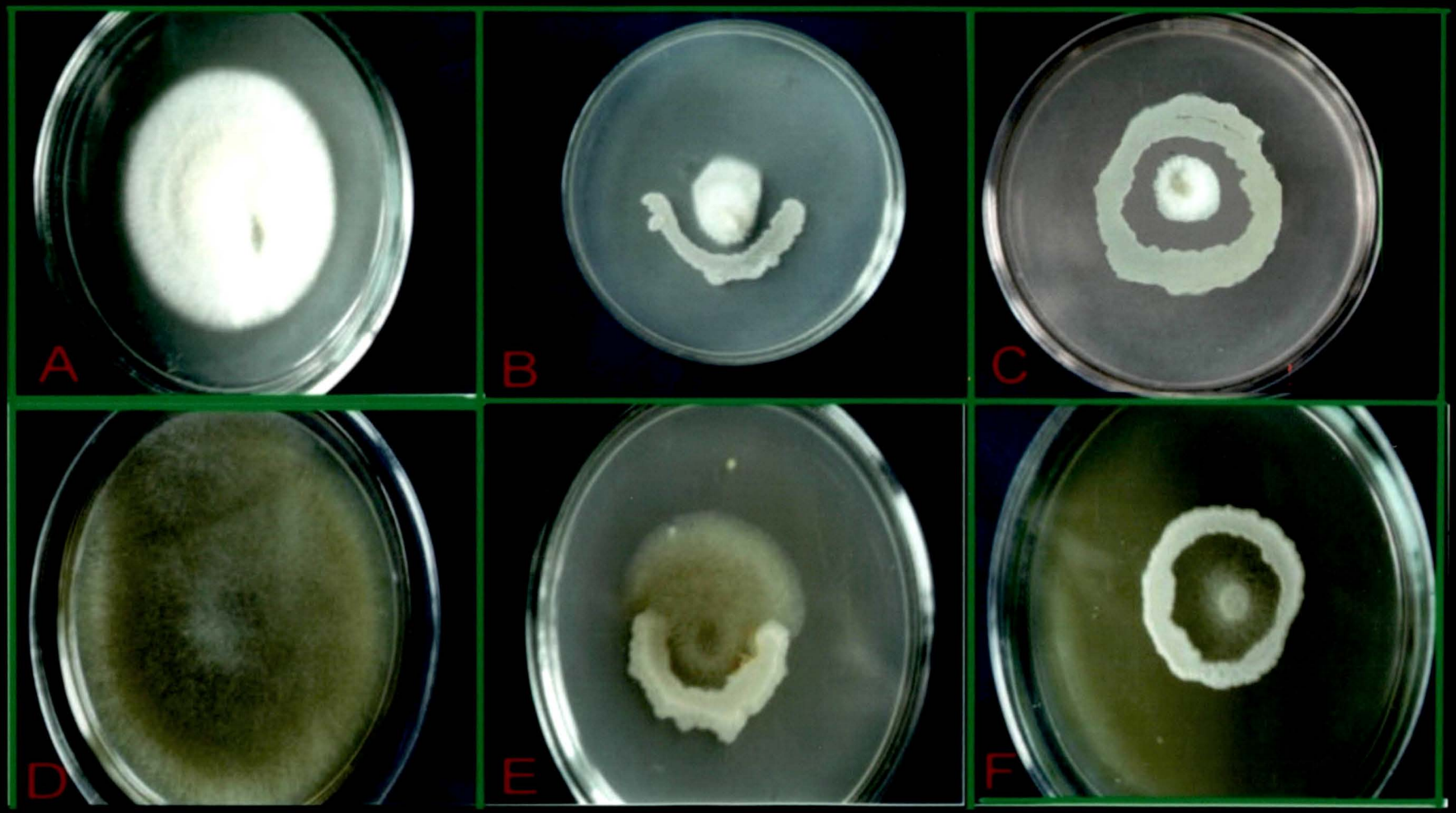
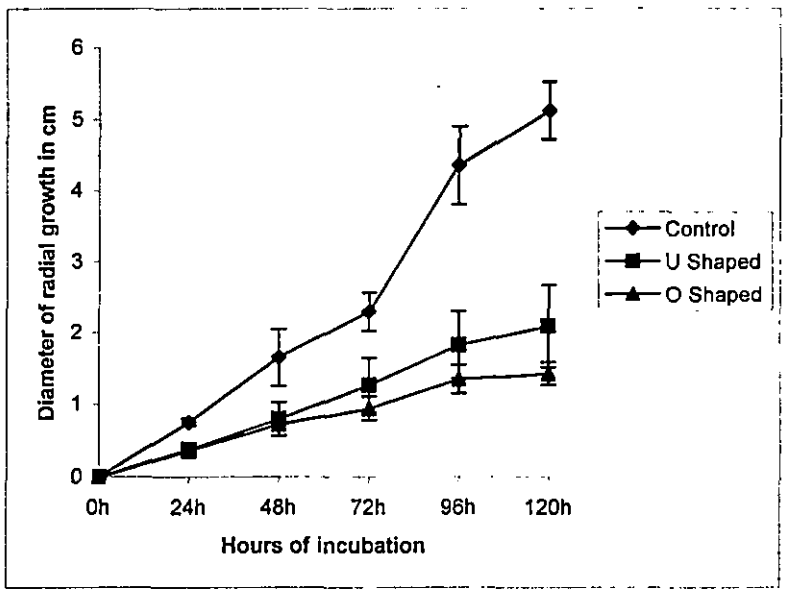


Figure 3.4. Inhibitory effect of fluorescent Pseudomonas Dj-5 on F. oxysporum and M. hiemalis f. hiemalis: (A and D) Control set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; (B and E) Colony showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing restricted growth due to the antagonist inoculated as a circular streak.

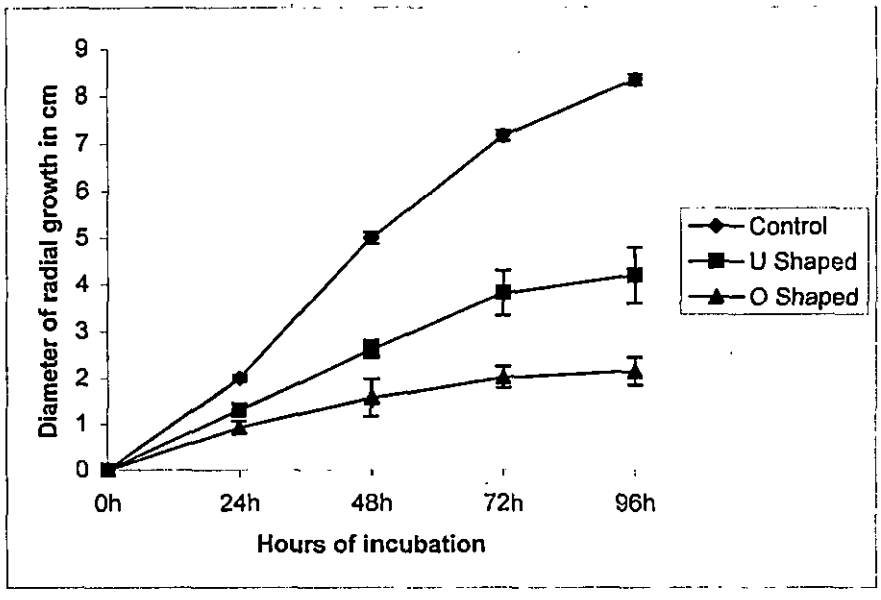
3.4.7. In vitro antagonistic activity of fluorescent Pseudomonas BB-9 against Fusarium oxysporum, Mucor hiemalis f. *hiemalis and Erwinia carotovora:*

From dual culture it was quite visible that the isolate fluorescent *Pseudomonas* BB-9 was also responsible for considerable growth inhibition of the fungal pathogens as was visualized with clear inhibition of colony diameter (Figure 3.5 A and B). The fungal pathogens were inhibited by fluorescent *Pseudomonas* BB-9 and with the simultaneous increase in incubation time, the percent growth inhibition increased respectively (Figure 3.6). Reduction in dry weights of about 59 percent in *F. oxysporum* and 58 percent in case of *M. hiemalis* f. *hiemalis* was noted when compared to the control. The isolate showed marked antagonistic activity against *Erwinia carotovora* at 48 hours of incubation, the growth of *Erwinia carotovora* was restricted in the interacting zone where as abundant growth away from the interaction (Figure 3.13c).



A

Figure 3.5A: In vitro inhibitory effect of fluorescent Pseudomonas BB-9 cultured as circular and semicircular streak, around Fusarium oxysporum, the fungal pathogen was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.



B

Figure 3.5B: In vitro inhibitory effect of fluorescent Pseudomonas BB-9 cultured as circular and semicircular streak, around Mucor hiemalis f. hiemalis, the fungal pathogens was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.

3.4.8. In vitro antagonistic activity of fluorescent Pseudomonas Sg-5 against Fusarium oxysporum, Mucor hiemalis f. hiemalis and Erwinia carotovora:

The antagonist strain Sg-5 of fluorescent *Pseudomonas* showed moderate inhibitory effect against both fungal and bacterial pathogen. The results were shown in Figure 3.7 (A and B). The growths of the fungal pathogens were moderately inhibited after 48 hours of incubation. The percent growth inhibitions were also increased with increase in time when compared to control (Figure 3.8). There were more than 54 percent reduction in dry weight of *F. oxysporum* and about 57 percent in case of *M. hiemalis* f. *hiemalis* were recorded. *Erwinia carotovora* was also inhibited by the antagonist. It is clear from the figure (3.13d) that the growth of the pathogen was checked at the interacting zone and ample growth away from the interaction.

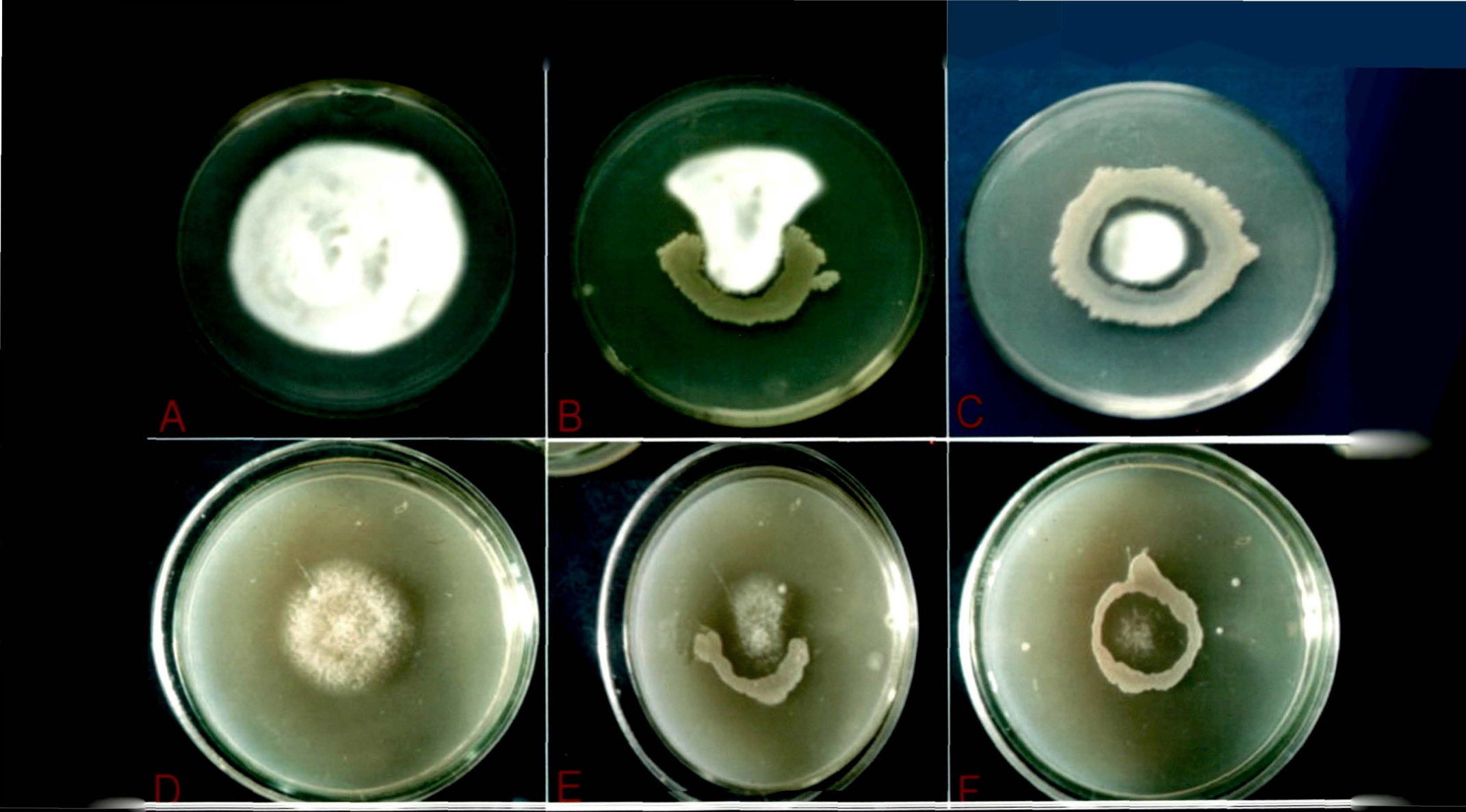
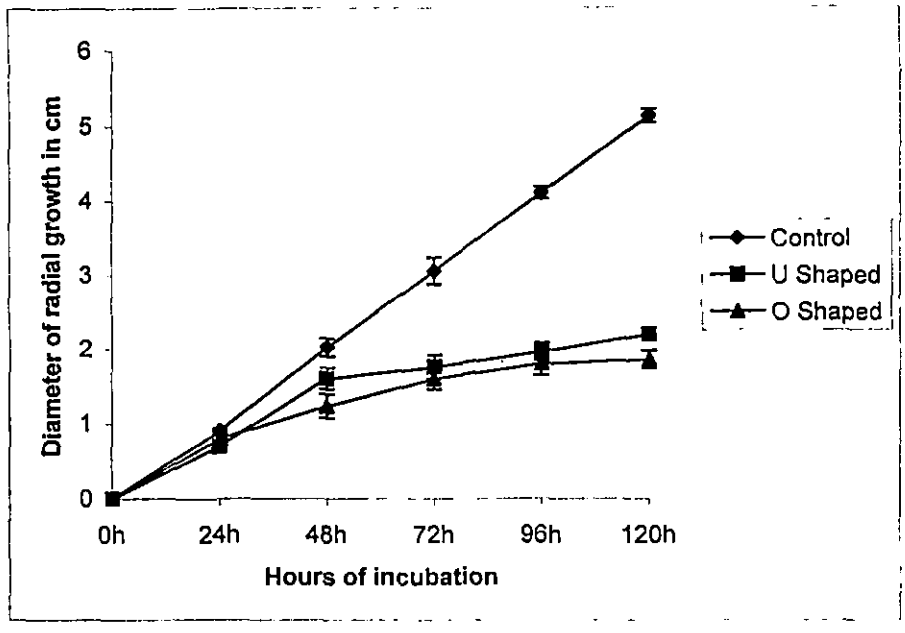
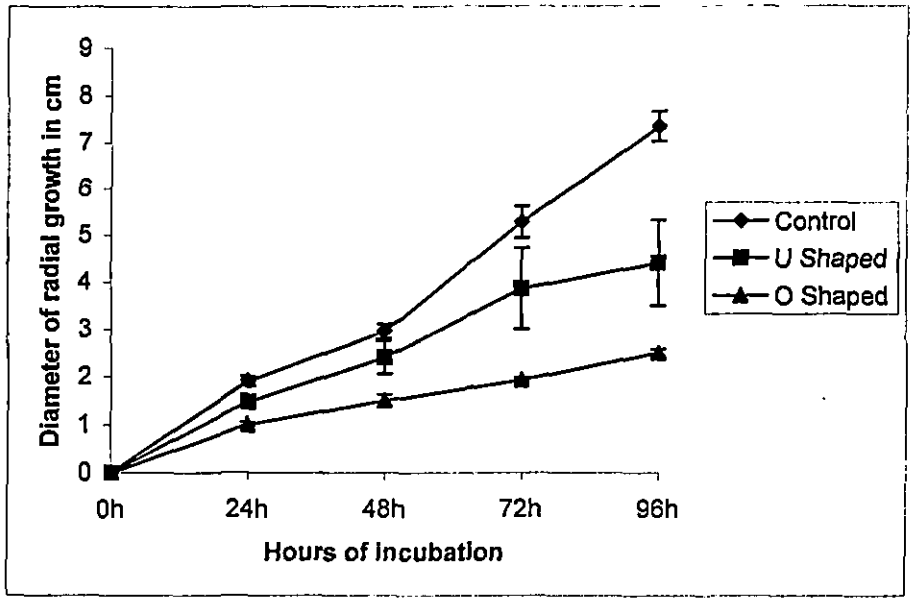


Figure 3.6. Inhibitory effect of fluorescent Pseudomonas BB-9 on F. oxysporum and M. hiemalis f. hiemalis: (A and D) Control set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; (B and E) Colony showing restricted growth, but growing freely towards the top end away from the U shape streak culture of the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing

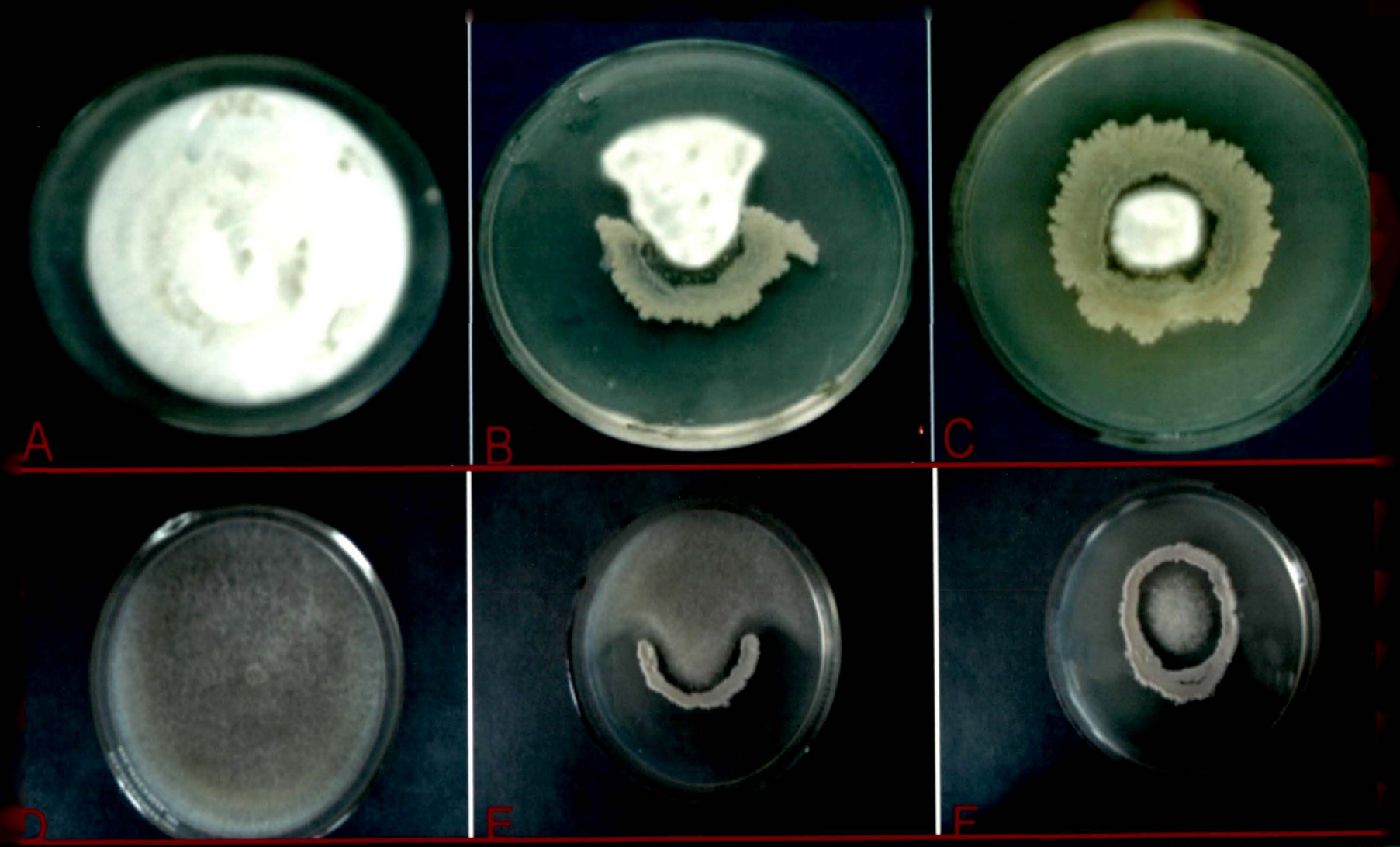


A



B

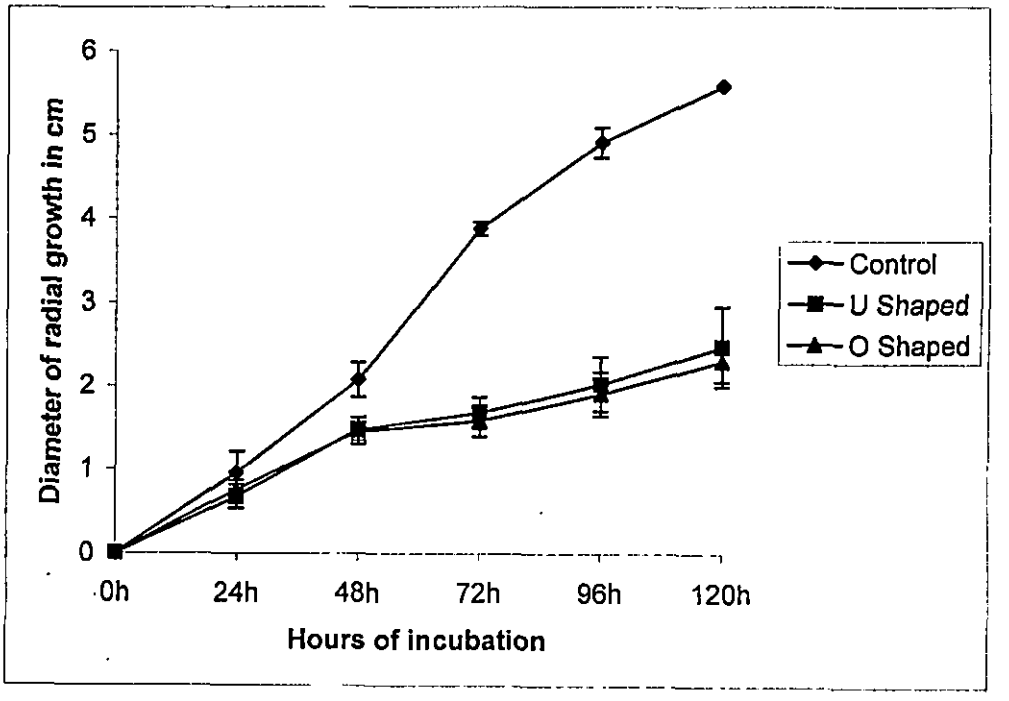
Figure 3.7: In vitro inhibitory effect of fluorescent Pseudomonas Sg-5 cultured as circular and semicircular streak, around A. Fusarium oxysporum and B. Mucor hiemalis f. hiemalis, where as the fungal pathogens were point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.



gure 3.8. Inhibitory effect of fluorescent Pseudomonas Sg-5 on F. oxysporum and M. hiemalis f. hiemalis: (A and ntrol set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; and E) Colony showing restricted growth, but growing freely towards the top end away from the U shaped streak c the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing restricted grov

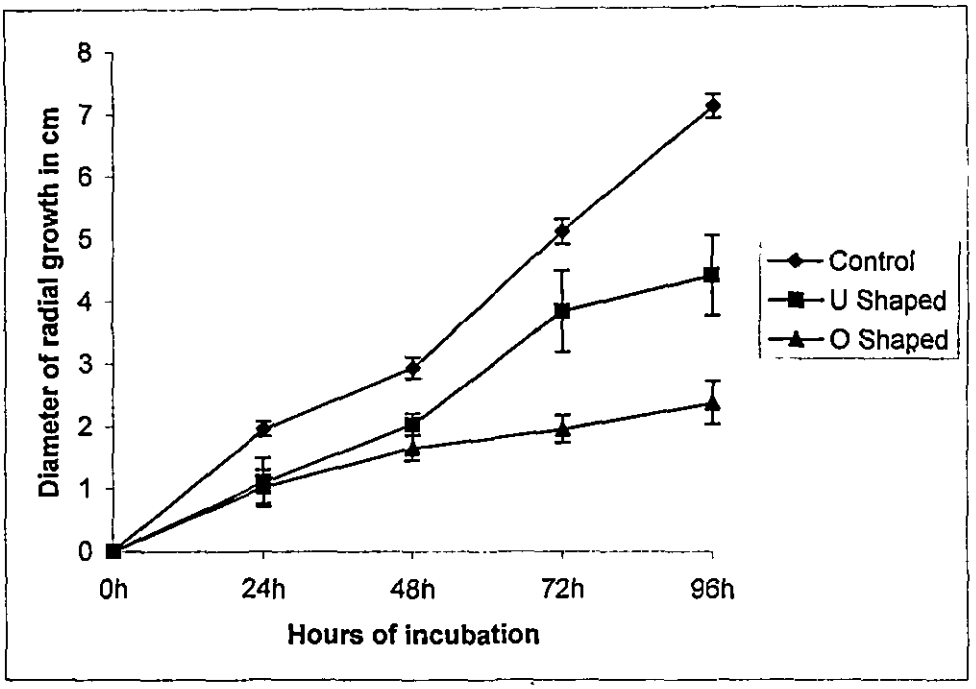
3.4.9. In vitro antagonistic activity of fluorescent Pseudomonas Sg-1 against Fusarium oxysporum, Mucor hiemalis f. hiemalis and Erwinia carotovora:

Inhibitory effect of Sg-1 was also found to be moderate against the fungal pathogens. The results were depicted in Figure 3.9 (A and B). After 48 hours of incubation pathogens were inhibited by the antagonistic strain (Figure 3.10). There were more than 54 and 56 percent reduction in dry weight of *F. oxysporum* and *M. hiemalis* f. *hiemalis* culture respectively grown with strain Sg-1 when compared to the control. The antagonist shows moderate growth inhibition of the bacterial pathogen which was visualized form the figure (3.13e).



A

Figure 3.9A: In vitro inhibitory effect of fluorescent Pseudomonas Sg-1 cultured as circular and semicircular streak, around Fusarium oxysporum, the fungal pathogen was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.



B

Figure 3.9B: In vitro inhibitory effect of fluorescent Pseudomonas Sg-1 cultured as circular and semicircular streak, around Mucor hiemalis f. hiemalis, the fungal pathogen was point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.

3.4.10. In vitro antagonistic activity of fluorescent Pseudomonas Sili-15 against Fusarium oxysporum, Mucor hiemalis f. hiemalis and Erwinia carotovora:

In dual culture fluorescent *Pseudomonas* Sili-15 showed meager antagonistic effect against the fungal as well as the bacterial pathogen. Results were furnished in the Figure 3.11 (A and B). Increasing incubation period moderately inhibit the percent growth inhibition (Figure 3.12). More than 53 and 55 percent reduction in dry weights were recorded for *F. oxysporum* and *M. hiemalis* f. *hiemalis* respectively. Bacterial pathogen was also moderately inhibited by this antagonist (Figure 3.13f).

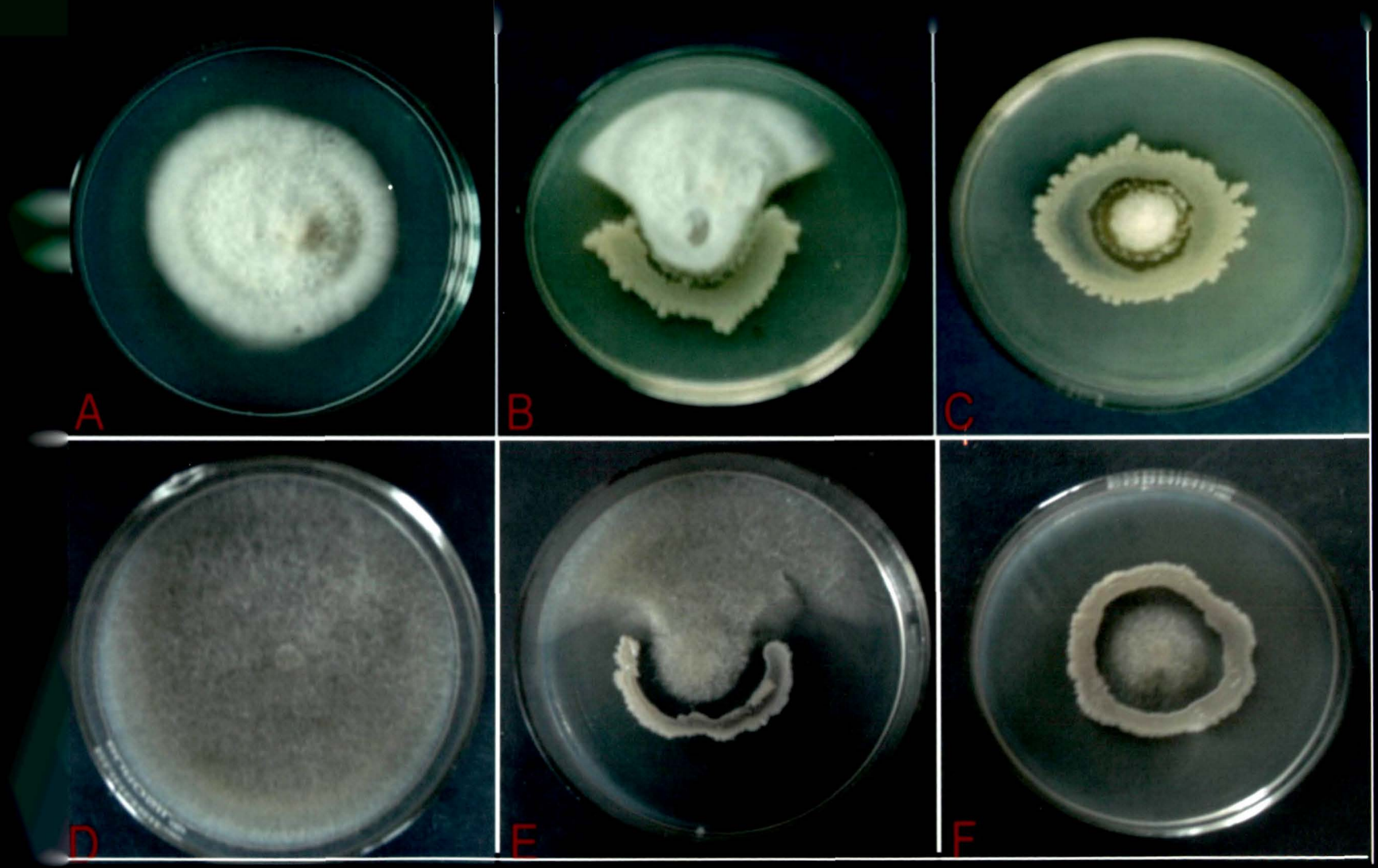
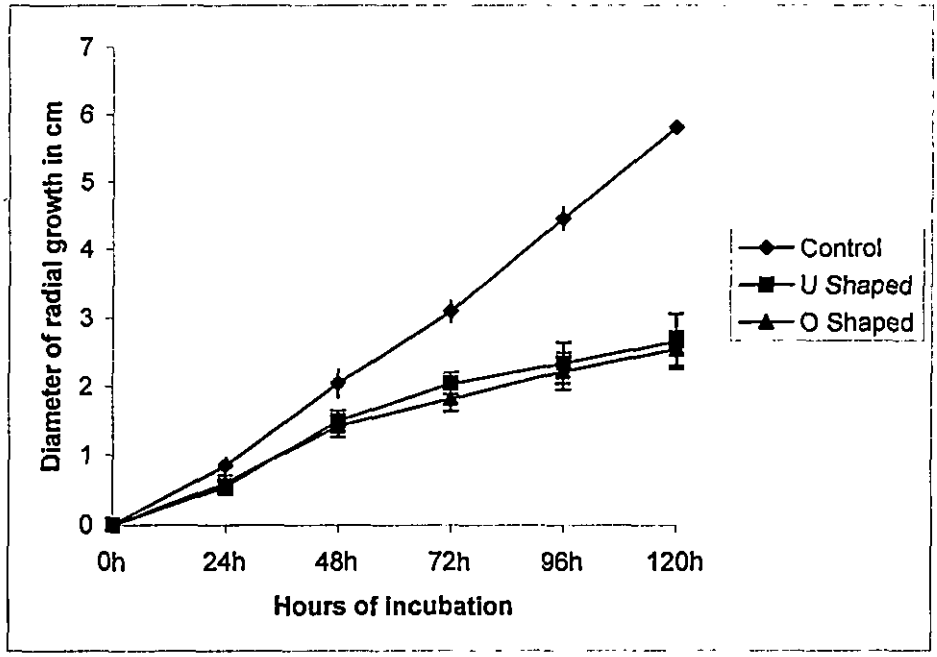
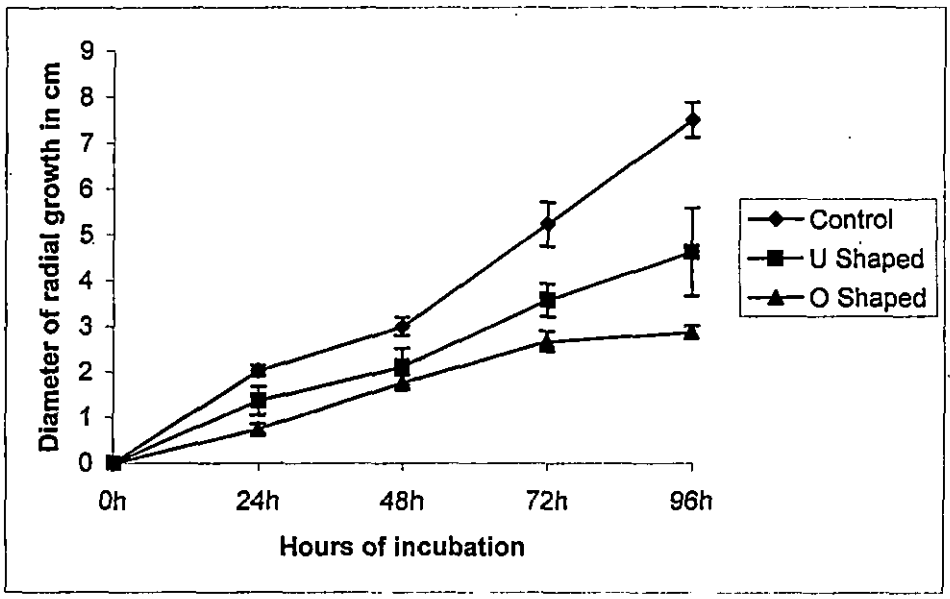


Figure 3.10. Inhibitory effect of fluorescent Pseudomonas Sg-1 on F. oxysporum and M. hiemalis f. hiemalis: (A and D) Control set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; (B and E) Colony showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing restricted growth due to the antagonist inoculated as a circular streak



A



B

Figure 3.11: In vitro inhibitory effect of fluorescent Pseudomonas Sili-15 cultured as circular and semicircular streak, around A. Fusarium oxysporum and B. Mucor hiemalis f. hiemalis, where as the fungal pathogens were point inoculated at the center of PGA plates, incubated as 30°C. Data represents the mean of triplicate sets of experiments.

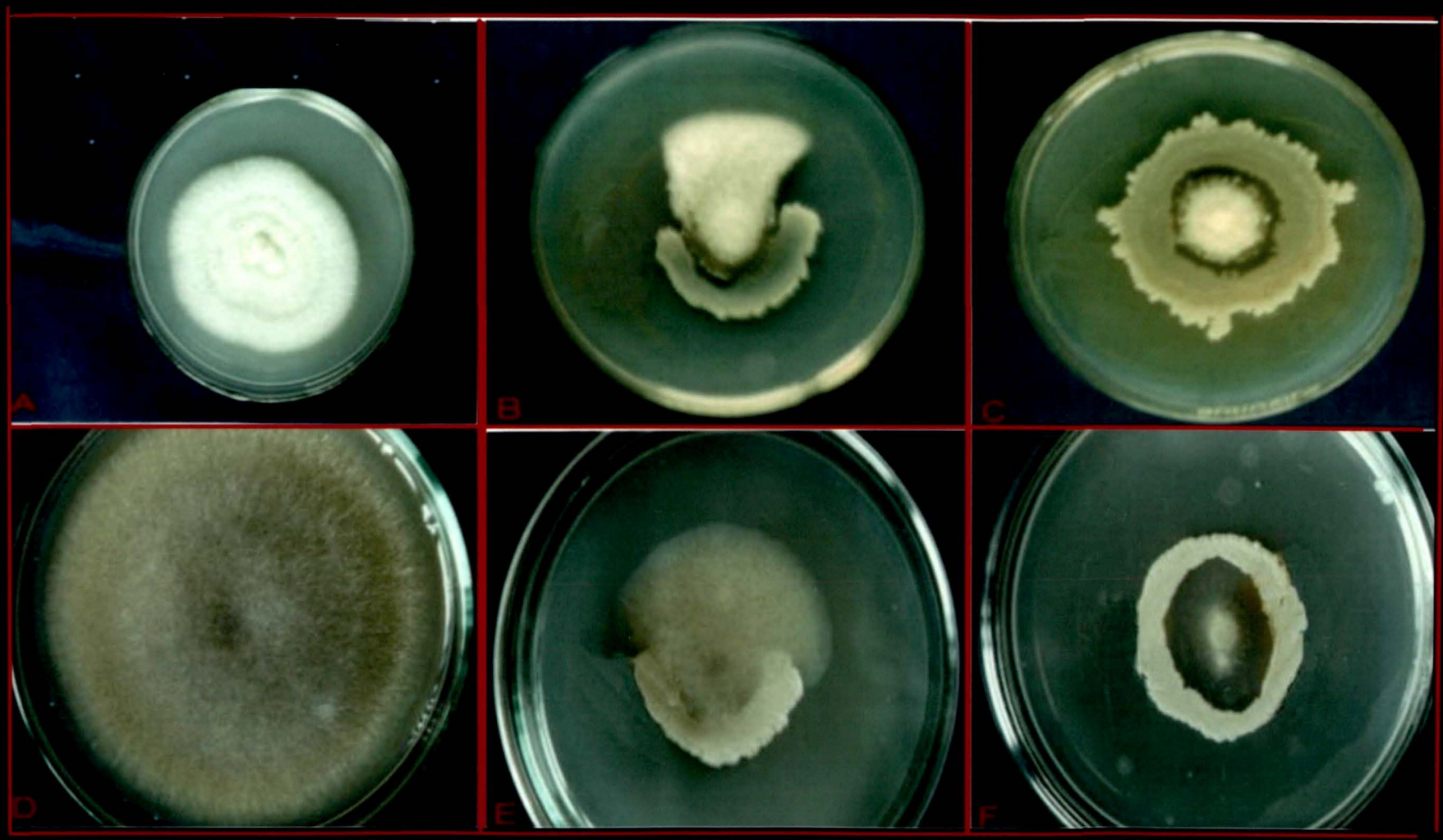


Figure 3.12. Inhibitory effect of fluorescent Pseudomonas Sili-15 on F. oxysporum and M. hiemalis f. hiemalis: (A and D) Control set (A) F. oxysporum and (D) M. hiemalis f. hiemalis colony in pure culture showing more rapid growth; (B and E) Colony showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist; and (C and F) Colony of (C) F. oxysporum and (F) M. hiemalis f. hiemalis showing restricted growth due to the antagonist inoculated as a circular streak.

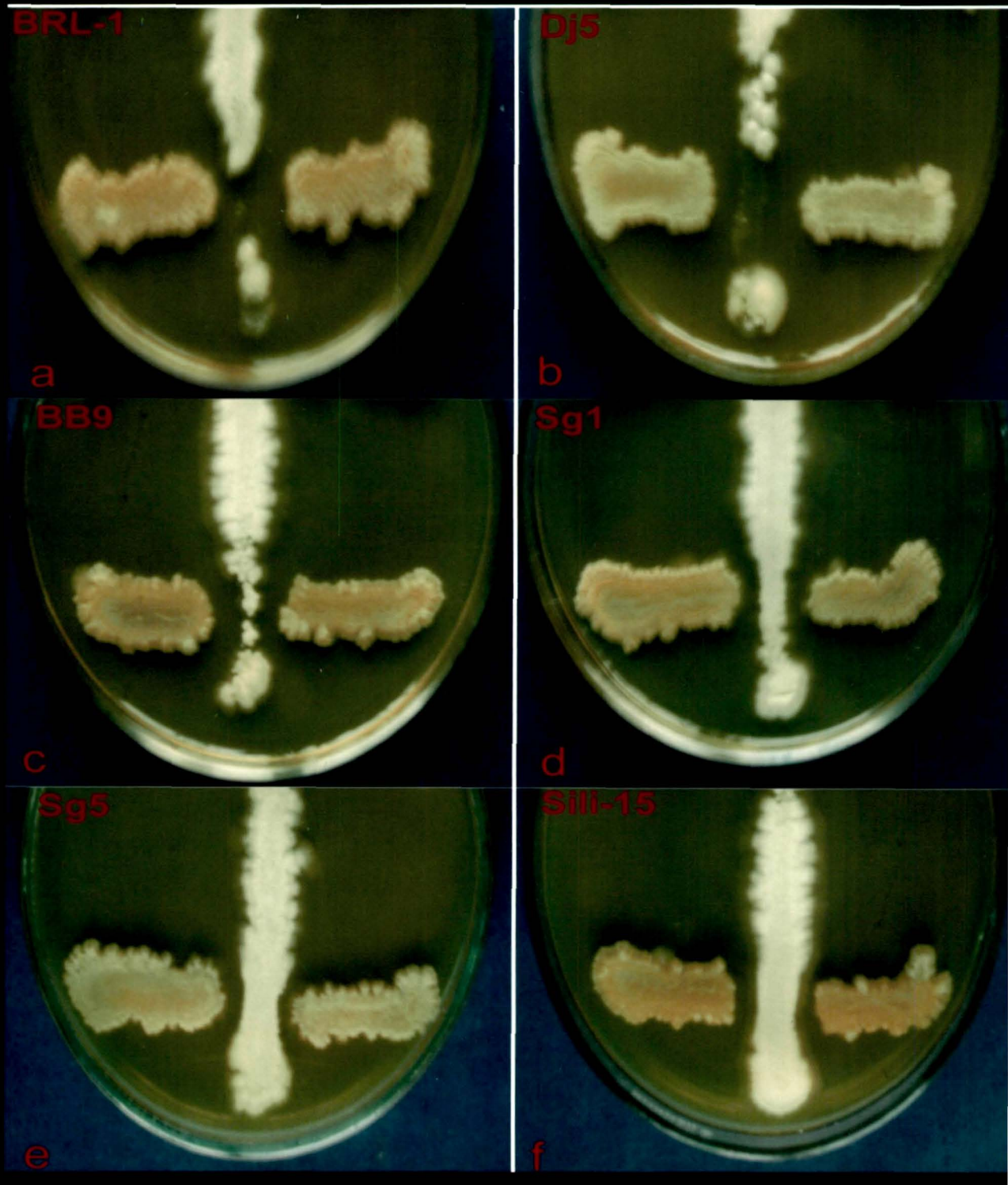


Figure 3.13: Growth inhibition of Erwinia carotovora by the six screened fluorescent pseudomonads. (a) Growth in inhibition by fluorescent Pseudomonas BRL-1 showing no growth of the pathogen in the interacting zone; (b) Inhibition by Dj-5 where faint growth of the pathogen was visualized; (c and d) Moderate growth inhibition by the isolates BB-9 and Sg-1; and (e and f) Meager growth inhibition by Sg-5 and Sili-15.

In dual culture, growth inhibition of *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* were variably inhibited by all the six strains which were evident with clear inhibition of colony diameter. In all cases it was observed that after 72 hours of incubation the mycelia growing towards the interaction zone, which was prominently evident in semicircular/ U-shaped streak stopped, and the mycelia gradually lost vigor. Among these six screened isolates three strains viz., BRL-1, Dj-5 and BB-9 showed effective antagonism as compared to other, but the effect was more significant in case of fluorescent *Pseudomonas* BRL-1, than that of any other strain investigated (Figure 3.14). Maximum reduction of mycelial dry weight in dual liquid culture after 72 hours of incubation was furnished by strain BRL-1 which was 68% and 61% in case of *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* respectively (Table 3.5).

Table 3.5: Percent reduction in dry weight of *Fusarium oxysporum* and *Mucor hiemalis* by the six screened fluorescent *Pseudomonas*

Name of the Screened Isolates	Fungal Pathogen (s)	% reduction in dry weight
Fluorescent *Pseudomonas* BRL-1	*Fusarium oxysporum*	68.8
	Mucor hiemalis	60.72
Fluorescent *Pseudomonas* Dj-5	*Fusarium oxysporum*	62.3
	Mucor hiemalis	57.4
Fluorescent *Pseudomonas* BB-9	*Fusarium oxysporum*	59.71
	Mucor hiemalis	58.62
Fluorescent *Pseudomonas* Sg-5	*Fusarium oxysporum*	54.23
	Mucor hiemalis	57.2
Fluorescent *Pseudomonas* Sg-1	*Fusarium oxysporum*	54.0
	Mucor hiemalis	56.25
Fluorescent *Pseudomonas* Sili-15	*Fusarium oxysporum*	53.4
	Mucor hiemalis	55.5

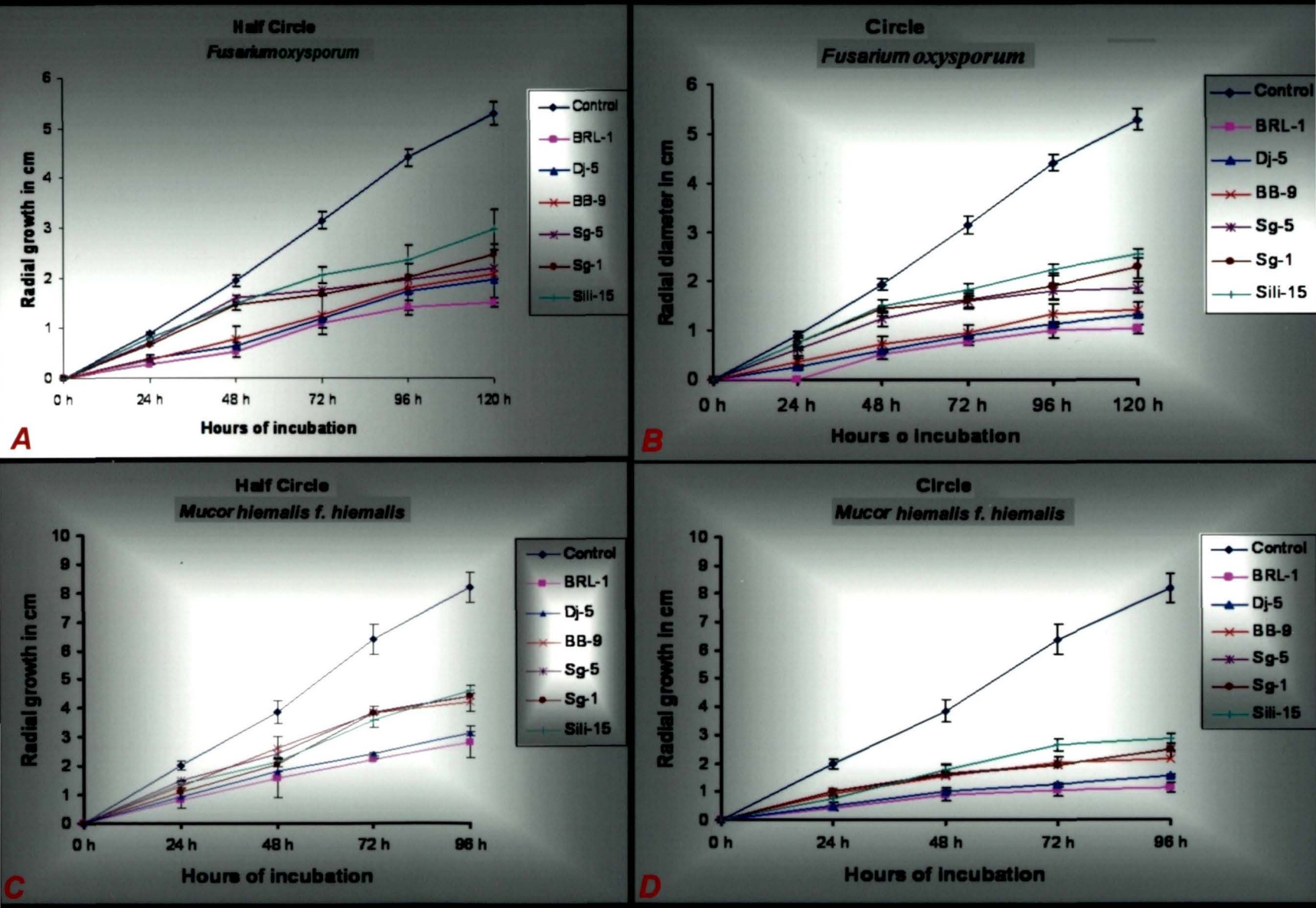


Figure 3.14: Comparative analysis of antagonistic activity of six fluorescent Pseudomonas strains A and B showing the inhibitory effect of all six strains against Fusarium oxysporum and C and D showing inhibitory effect of all six strains against Mucor hiemalis f. hiemalis.

The growth inhibition of the bacterial pathogen (*Erwinia carotovora*) was clearly seen after 48 hours of incubation (Figure 3.13). All the six isolates showed positive result to check the growth of the pathogen, but it is more prominent in case of fluorescent *Pseudomonas* BRL-1 strain which shows no growth in the interacting zone and abundant growth away from the interaction.

The next chapter of this thesis will investigate the possible mechanism behind the antagonistic activity of fluorescent *Pseudomonas* BRL-1.

3.5. References

- Alconero, R. 1980. Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. *Plant Dis.* **64:** 835-838.
- Anderson, R.C., Liberta, A.E. and Neville, M.E. 1980. Inhibition of selected fungi by bacterial isolates from *Tripsacum dactyloides* L. *Plant Soil.* **56:** 149-152.
- Austin, B., Dickinson, C.H. and Goodfellow, M. 1977. Antagonistic interactions of phylloplance bacteria with *Drechslera dictyoides* (Drechsler) Shoemaker. *Can. J. Microbiol.* **23:** 710-715.
- Basha, S. and Ulagnathan, K. 2002. Antagonism of *Bacillus* species (strain BC121) toward *Curvularia lunata. Curr. Sci.* **82:** 1457-1463.
- Bashan, Y. 1986. Migration of rhizosphere bacteria Azospiril-lum brasilense and Pseudomonas fluorescens towards wheat roots in the soil. *J. Gen. Microbiol.* **132:**3407-3414.
- Burr, T.J., Schroth, M.N. and Suslow, T. 1978. Increased potato yields by treatment of seed pieces with specific strains of *Pseudomonas fluorescens* and *P. putida. Phytopathology.* **68:** 1377.
- Cook, R.J. 1981. *Biological control of plant pathogens: Overview.* In: *Biological control in crop production.* pp. 23-44. ed. Papavizas, G. C. Osmun Publishers, Allanheld, Totowa.
- Cullen, D. and Andrews, J. W. 1984. Evidence for the role of antibiosis in the antagonism of *Chaetomium globosum* to the apple scab pathogen, *Venturai inaequalis. Can. J. Bot.* **62:** 1819-1823.
- Deacon, J.W. 1994. *Rhizosphere constraints affecting biocontrol organisms applied to seeds.* In: Seed treatment: prospects and progress. BCPC Monograph 57. Thornton Heath: pp. 315–327. British Crop Protection Council.
- Dubey, R.C. and Maheshwari, D.K. 2002. In: Practical Microbiology. SS. Chand and Company Ltd. New delhi.
- Dye, D.W. 1968. A taxonomic study of the genus Erwinia. The 'Amylovora' group. *N.Z.J. Sci,* **11:** 590-607.
- Fravel, D.R. 1988. Role of Antibiosis in the biocontrol of Plant Diseases. *Annu. Rev. Phytopathology.* **26:** 75-91.
- Gaber, R.H., DeVay, J. E. Weinhold, A.R. and Matheron, D. 1979. Relationship of pathogen inoculum to cotton seedling disease control with fungicides. *Plant Dis. Rep.* **63:**246-250.
- Gardner, J.M., Chandler, J.L. and Feldman, A.W. 1984. Growth promotion and inhibition by antibiotic-producing fluorescent pseudomonads on citrus roots. *Plant Soil.* **77:** 103-113.

- Gaur, R., Shani, N., Johri, B.N., Rossi, P. and Aragno, M. 2004. Diacetylphloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Curr Sci.* **86:** 453-457.
- Hawes, M.C. 1991. *Living plant cells released from the root cap: A regulator of microbial populations in the rhizosphere?* In: The Rhizosphere and Plant Growth. pp. 51-59. eds. Keister D .L. and Cregan, P.B. Kluwer Academic Publishers, Boston, MA.
- Howell, C.R. and Stipanovic. R.D. 1980. Suppression of *Pythium ultimum*-induced damping off of cotton seedlings by Pseudomonas fluorescens and its antibiotic pyoluteorin. *Phytopathology.* **70:** 712-715.
- Huber, D.M. and Watson, R.D. 1966. How valid is the agar plate inhibition test for determining antagonism between soil micro-organisms? *Phytopathology.* **56:** 882.
- Jayalakshmi, V., Sivaprakasam, K. and Deetharaman, K. 2003. Effect of *Pseudomonas fluorescens* (Migula) on *Rhizoctronia solani* Kuhn and on the growth of rice plant. *J. Biol. Control.* **17:** 189-192.
- Johnson, H.F., Baird, D.D., Chambers, A.Y. and Shamiyeh, N.B. 1978. Fungi associated with postemergence seedling disease of cotton in three soils. *Phytopathology.* **68:**117-120.
- Keel, C., Voisard, C., Berling, C.H., Kadr, G. and Defago, G. 1989. Iron sufficiency, a prerequisite for the suppression of tobacco root rot by *Pseudomonas fluorescens* strain CHAO under gnotobiotic conditions. *Phytopathology.* **79:**584-589.
- King, E.O., Ward, M. K. and Raney, D.E. 1954. Two simple media for demonstration of pyocyanin and fluorescin. *J. Lab Clin Med.* 44: 301-307.
- Kloepper, J.W., Leong, J., Teintze, M. and Schroth. M.N.1980a. *Pseudomonas siderophores:* a mechanism explaining disease suppressive soils. *Curr. Microbiol.* **4:** 317-320.
- Kloepper, J.W., Schroth, M.N. and Miller. T.D. 1980b. Effects of rhizosphere colonization by plant growth-promoting rhizobacteria on potato plant development and yield. *Phytopathology.* **70:** 1078-1082.
- Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. *Nature.* **178:** 703.
- Lelliott, R.A., Billing, E. and Hayward, A.C. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. *J. Appl. Bacteriol.* **29:** 470-489.
- Lifshitz, R., Simonson, C. Scher, F.M. Kloepper, J.W. Rodrick-Semple, C. and Zaleska, I. 1986. Effect of rhizobacteria on the severity of *Phytophthora* root rot of soybean. *Can. J. Plant Pathol.* **8:** 102-106.
- Lucy, M., Reed, E. and Glick, B.R. 2004. Applications of free living plant growth-promoting rhizobacteria. *Antonie van Leeuwenhoek* 86: 1–25.

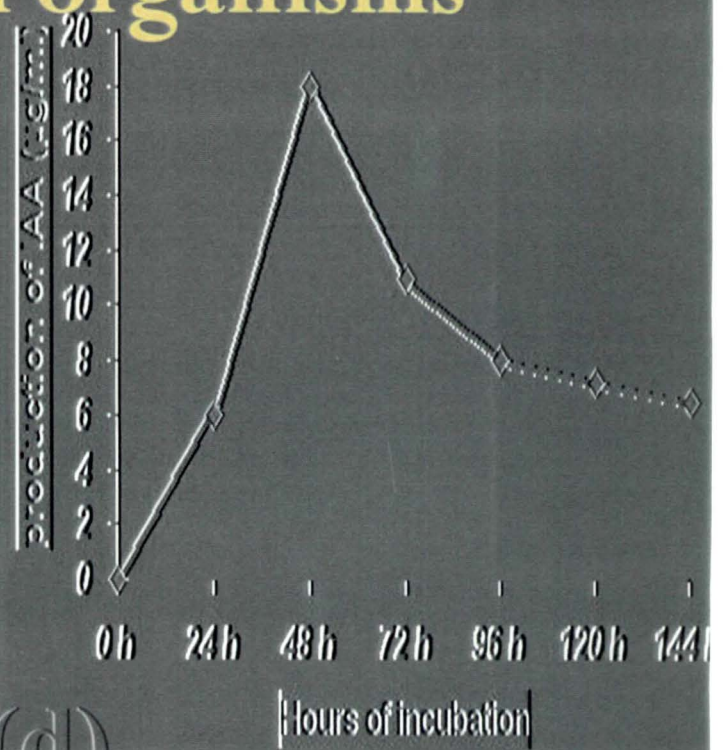
- Lugtenberg, B.J.J. and Dekkers, L.C. 1999. What makes *Pseudomonas* bacteria rhizosphere competent? *Environ. Microbiol.* 1: 9-13.
- Maurhofer, M., Hase, C., Meuwly, P., Metraux, J.P. and Defago, G. 1994a. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing Pseudomonas fluorescens Strain CHAO: Influence of the gacA gene and pyoverdine production. *Phytopathology*. 84: 139-146.
- Maurhofer, M., Keel, C., Haas, D. and Defago, G. 1994b. Pyoluteorin production by Pseudomonas fluorescens strain CHAO is involved in the suppression of Pythium damping-off of cress but not of cucumber. *Eur. J. Plant Pathol.* 100: 221-232.
- Merriman, P. and Russell, K. 1990. *Screening strategies for biological control.* In: Biological Control of Soilborne Plant Pathogens. pp. 427-435. ed. Hornby, D. CAB International, Redwood Press Limited, UK.
- Misaghi, L. and Grogan, R.G. 1969. Nutritional and biochemical comparisons of plant-pathogenic pseudomonads. *Phytopathology*. 59: 1436-1450.
- Preston, G.M. 2004. Plant perceptions of plant growth-promoting *Pseudomonas*. *Phil. Trans. R. Soc. Lond. B* 359, 907–918.
- Reddy, M.S., Hynes R.K. and Lazarovits, G. 1993. Relationship between *in vitro* growth inhibition of pathogens and suppression of preemergence damping off and postemergence root rot of white bean seedlings in the greenhouse by bacteria. *Can. J. Microbiol.* 40: 113-119.
- Sands, D.C., Schroth, M.N. and Hildebrand, D.C. 1970. Taxomomy of phytopathogenic pseumonads. *J. Bacteriol,* 101: 9-23.
- Sandy-Winsch, D.C., Whipps, J.M., Fenlon J.S. and Lynch, J.M. 1994. The validity of *in vitro* screening methods in the search for fungal antagonists of *Sclerotinia sclerotiorum* causing wilt of sunflower. *Biocont. Sci.Technol.* 4: 269-277.
- Scher, F.M. and Baker, R. 1982. Effect of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium* wilt pathogens. *Phytopathology*. 72: 1567-1573.
- Schisler, D.A., Slininger P.J. and Bothast, R.J. 1997. Effects of antagonist cell concentration and two strain mixture on biological control of *Fusarium* dry rot of potatoes. *Phytopathology*. 87: 177-183.
- Schroth, M.N. and Hancock, J.G. 1981. Disease suppressive soil and root colonizing bacteria. *Science* 216: 1376-1381.
- Schroth, M.N. and Hancock, J.G. 1982. Selected topics in biological control. *Ann. Rev. Microbiol.* 35: 453-476.
- Seeley, H.W. and Vandemark, P.J. 1972. In: Microbs in action. A laboratory manual of Microbiology. eds. Kennedy, W.H. and Park, R.B. Freeman and Company. Sanfrancisco.

- Shaffer, W.H.Jr, 1967. *Procedure for the identification of bacteria plant pathogens* In: Proc. 1st Workshop on Phytobacteriology. pp. 68-71. ed. Goodman, R.N. Univ. of Missouri, Columbia.
- Shinde, P.A. and Lukezie, F.L. 1974. Isolation, Pathogenicity and Characterization of Fluorescent Pseudomonads Associated with Discolored Alfalfa Root. *Phytopathology,* 64: 865-871.
- Skerman, V.B.D. 1967. In: A guide to the identification of the genera of bacteria. pp. 303. Williams and Willims Co., Baltimore.
- Skidmore, A.M. and Dickinson, C.H. 1970. Colony interaction and hyphal interference between *Septoria nodorum* and phyloplane fungi. *Trans. Br. Mycol. Soc.* **66:** 57-64.
- Sneh, B., Dupler, M., Elad, Y. and Baker, R. 1984. Chlamydos-pore germination of *Fusarium oxysporum f. sp. cucumennum* as affected by fluorescent and lytic bacteria from *Fusanum* suppressive soils. *Phytopathology.* **74:** 1115-1124.
- Suslow, T.V. and Schroth, M.N. 1982. Rhizobacteria on sugar beets: effects of seed application and root colonization on yield. *Phytopathology.* **72:** 199-206.
- Van Peer, R., and B. Schippers. 1988. Plant growth responses to bacterization with selected *Pseudomonas* spp. strains and rhizosphere microbial development in hydroponic cultures. *Can. J. Microbiol.* **35:** 456-463.
- Vidhyesekaran, P. 1998. *Biological suppression of major diseases of field crops using bacterial antagonists.* In: Biological Suppression of Plant Diseases, Phytoparasitic Nematodes and Weeds. pp. 81-95. eds. Singh, S.P. and Hussaini, S.S. Project of Biological Control, Bangalore, India.
- Weller, D.M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria, *Ann. Rev. Phyopathol.* **26:** 379-407.
- Weller, D.M. and Cook, R.J. 1986a. Increased growth of wheat by seed treatments with fluorescent pseudomonads and implications of *Pythium* control. *Can. J. Plant Pathol.* 8:328-334.
- Weller, D.M. and Cook, R.J. 1986b. Suppression of root diseases of wheat by fluorescent *Pseudomonas* and mechanism of action. In: Iron, Siderophore and plant diseases. pp. 99-197. ed. Susburne, T. R. Plenum, New York.
- Weller, D.M., Raaijmakers, J.M., Gardener B.B.M. and Thomashow, L.S. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40: 309–348.
- Whipps, J.M. 1987. Effect of media on growth and interaction between a range of soilborne glasshouse pathogens and antagonistic fungi. *New Phytologist.* **107:** 127-142.
- Whipps, J.M. 1997. Developments in the biological control of soil-borne plant pathogens. *Adv. Bot. Res.* **26:** 1–134.

- Xu, G.W. and Gross, D.C. 1986. Selection of fluorescent pseudomonads antagonistic to *Erwinia carotovora* and suppressive of potato seed piece decay. *Phytopathology*. **76**:414-422.
- Yuen, G.Y. and Schroth, M.N. 1986. Interactions of *Pseudomonas fluorescens* strain E6 with ornamental plants and its effect on the composition of root-colonizing microflora. *Phytopathology*. **76**:176-180.

CHAPTER IV

Isolation, characterization and screening of potential antagonists against causal organisms



4.1 Introduction

One of the most interesting aspects of the science of biological control is the study of the mechanisms employed by biocontrol agents to affect disease control (Howell, 2003). The mechanisms of biological control of plant pathogens by antagonistic bacteria and fungi have been a subject of many studies in the past two decades. Most of these studies dealt with antagonists controlling soil borne and to a lesser extent, foliar pathogens. Mechanisms of biological control included antibiosis, parasitism, induced resistance and competition for space and limited resources (Janisiewicz *et al., 2000*). Knowledge of mechanisms involved in biocontrol is important for estimating and predicting its reliability and selection of better strains. Besides other criteria, the choice of an antagonist with its characteristic mechanisms depends on the stage of the life cycle of the pathogen the antagonist is aimed at. Allowable interaction times and niche characteristics determine the suitability of certain modes-of-action during different developmental stages of the pathogen.

4.2. Plant Disease Suppression Mechanisms

4.2.1. Siderophore mediated suppression

The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Because almost all living organisms require iron for growth, survival in a heterogenous environment such as the rhizosphere depends largely on the ability to scavenge sufficient iron from a limiting pool. To date, the only known exceptions to this rule are certain lactobacilli, which are devoid of heme proteins and hence have no iron requirement (Archibald, 1983.). The unavailability of iron for growth is surprising, since it is the fourth most abundant element in the Earth's crust (Crichton and Charioteaux-Wauters, 1987). However, it is largely insoluble and thus is unavailable for direct microbial assimilation. In highly oxidized and aerated soils, the predominant form of iron is the ferric form (Matzanke, 1987), which is soluble in water (pH 7.4) at about 10^{-18}M (Nailands *et al.*, 1987). This is too low to support the growth of microorganisms, which

generally need concentrations approaching 10^{-6}M for normal growth. Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganism. Most known siderophores can be grouped into hydroxamate and phenolate/catecholate type structures and have different affinities for ferric iron. Soil pseudomonads generally produce fluorescent, yellow-green, water soluble siderophores with both a hydroxamate and phenolate group; these siderophores have been classified as either pyoverdins or pseudobactins. Fluorescent siderophores, which have a very high affinity for ferric iron, are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer cell membrane (Buyer and Leong, 1986). In this way, fluorescent *Pseudomonas* strains may restrict the growth of deleterious bacteria and fungi at the plant root; this has been reviewed by Loper and Buyer in 1991. These iron starvation conditions may also prevent the germination of fungal spores. A direct correlation has been observed *in vitro* between siderophore synthesis in fluorescent pseudomonads and their capacity to inhibit germination of chlamydospores of *Fusarium oxysporum* (Elad and Baker, 1985a; Elad and Baker, 1985b; Sneh *et al.*, 1984). This efficient iron uptake mechanism may also be a significant contributing factor to the ability of these strains to aggressively colonize plant roots, thus aiding the physical displacement of deleterious organisms.

4.2.2. Antibiotic mediated suppression

Biocontrol is often attributed to antibiosis. In many biocontrol systems that have been studied, one or more antibiotics have been shown to play a role in disease suppression. The diversity in the type of antibiotics produced by different strains is only now being fully realized. Compounds such as

phenazines (Thomashow and Weller, 1988.), pyoluteorin (Howell and Stipanovic, 1980), pyrrolnitrin (Howell and Stipanovic, 1979), tropolone (Lindberg, 1981), pyocyanin (Dahiya *et al.*, 1988), and 2,4-diacetylphloroglucinol (DAPG) (Keel *et al.*, 1990) have been isolated from soil fluorescent pseudomonads. The first antibiotics clearly implicated in biocontrol by fluorescent pseudomonads were the phenazine derivatives that contribute to disease suppression by *Pseudomonas fluorescens* strain 2-79 and *P. aureofaciens* strain 30-84, which control take-all of wheat (Weller and Cook, 1983; Brisbane and Rovira, 1988). Genetic analyses have been particularly informative in determining the role of antibiotics in biocontrol, in part because mutants can be screened easily *in vitro* for changes in antibiotic accumulation, providing the means to conduct thorough genetic analyses. Genetic evidence indicated that DAPG could protect sugar beet roots from infection by *Pythium* fungi, since a Tn5-induced DAPG mutant had lost this ability. The same antibiotic compound produced by another fluorescent pseudomonad was found by Keel et al. (1990) to suppress black rot in tobacco and take-all of wheat.

Hydrogen cyanide (HCN) is a broad-spectrum antimicrobial compound involved in biological control of root diseases by many plant-associated fluorescent pseudomonads. Direct inhibition of the fungi by HCN is thought to be the main mechanism of action (Blumer and Haas, 2000), in which case, the effect of the bacterium would be comparable to the HCN-mediated plant defense mechanism (Luckner, 1990). HCN inhibits the terminal cytochrome-c oxidase in the respiratory chain (Knowles, 1976) and binds to metalloenzymes (Blumer and Haas, 2000). However, apart from its role in plant protection, a deleterious effect of microbial HCN on several plants has also been reported (Bakker and Schippers, 1987; Alstrom and Burns, 1989), and indeed, HCN production appears to represent a virulence factor in *P. aeruginosa* (Gallagher and Manoil, 2001). Deleterious HCN-producing strains have even been studied for biological control of weeds (Kremer and Souissi, 2001).

4.2.3. Parasitism

If fungal cells are lysed and cell walls are degraded then it is generally assumed that cell wall-degrading enzymes produced by the bacteria are responsible, even though antibiotics may be produced at the same time. Considerable effort has gone into identifying cell wall-degrading enzymes produced by biocontrol strains of bacteria even though relatively little direct evidence for their presence and activity in the rhizosphere has been obtained. For example protease and chitinase activity has also been reported among fluorescent pseudomonads (Nielsen *et al.*, 1998; Nielsen, *et al.*, 2002).

4.2.4. Colonization

It seems logical that a biocontrol agent should grow and persist, or "colonize," the surface of the plant it protects, and colonization is widely believed to be essential for biocontrol (Weller, 1983; de Weger *et al.*, 1987; Parke, 1991). However, colonization, or even the initial population size of the biocontrol agent, has been shown to be significantly correlated with disease suppression in only a few instances (Paulitz and Baker, 1987; Parke, 1990; Bull *et al.*, 1991). Suppression of take-all of wheat is correlated with colonization of roots by *P. fluorescens* strain 2-79 (Bull *et al.*, 1991). However, even in interactions that require colonization for disease suppression, the biocontrol agent may not be required at high population density.

4.2.5. Induced Resistance

Some biocontrol agents induce a sustained change in the plant, increasing its tolerance to infection by a pathogen, a phenomenon known as induced resistance. In some cases, it is clear that induced resistance by biocontrol agents involves the same suite of genes and gene products involved in the well documented plant response known as systemic acquired resistance (SAR), but this is not always the case. SAR is typically a response to a localized infection or an attenuated pathogen, which is manifested in subsequent resistance to a broad range of other pathogens (Ross, 1961; Uknes *et al.*, 1992; Ryals *et al.*, 1996) in

this issue. It has been shown that the biocontrol agent *P. fluorescens* strain CHAO (Maurhofer *et al.*, 1994) induces SAR-associated proteins, confers systemic resistance to a viral pathogen, and induces accumulation of salicylic acid, which plays a role in signal transduction in SAR (Gaffney *et al.*, 1993; Ryals *et al.*, 1996). Mutants of CHAO that do not produce the siderophore pyoverdin do not induce SAR, suggesting a novel role for bacterial metabolites in disease suppression (Maurhofer *et al.*, 1994). Another fluorescent pseudomonad, *P. putida*, induces expression of the gene encoding PRIa, which is associated with the classical SAR response (Zdor and Anderson, 1992). Other strains of *P. fluorescens* do not induce expression of the gene products associated with the classic SAR response but appear instead of inducing a functionally analogous response (Hoffland *et al.*, 1995).

This chapter will focus on the mechanism of action of fluorescent *Pseudomonas* BRL-1 against the pathogens responsible for *Cymbidium* rot.

4.3. Materials and Methods

4.3.1. Microscopic study

To study the morphological changes of the mycelium of *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* a small amount of fungal culture from the interacting zone of the dual plate culture as well as a drop of fungal culture from the dual liquid culture (discussed in the preceding chapter) were taken in a clean glass slide and stained with lactophenol and cotton blue and observed under Leica DMLS Research microscope. Mycelial morphology was compared with those of the control sets.

4.3.2. Effect of non-volatile substances

The production of non-volatile compounds by fluorescent *Pseudomonas* BRL-1 was tested by an 'agar layer technique' (Dennis and Webster, 1971a). A single sterile cellophane paper was placed aseptically on the solidified Peptone Glucose Agar (PGA) plates. At the centre of the plate 0.5 ml of fluorescent *Pseudomonas* BRL-1 was inoculated at a concentration of 10^7 cfu /ml and these plates were incubated at 30^0C for 48 hours. Then cellophane paper along with the grown bacterial isolate was removed carefully. An agar block (5 mm diameter) of four days old culture of *F. oxysporum* and *M. hiemalis* f. *hiemalis* were placed separately on the centre of these plate whereas point inoculation at the center of the plate was done for *E. carotovora* on nutrient agar (NA) at an concentration of 10^6 cfu /ml. Plates with cellophane paper inoculated with sterile distilled water served as control. Each treatment was replicated thrice. After three days of incubation at 30^0C colony diameter of the test fungi were recorded as well as morphological changes of the bacterial pathogen was documented by comparing with the control sets.

4.3.3. Effect of volatile substance

To investigate the inhibitory effect on fluorescent *Pseudomonas* BRL-1 for the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971b). An agar block (5 mm diameter) of four days old culture of

F. oxysporum and *M. hiemalis* f. *hiemalis* were placed separately on the centre of the petri plate containing 20 ml of PGA media and for bacterial pathogen NA plates were streaked with 24 hours old culture of *E. carotovora* containing 10^6 cfu /ml. A loopful of 24 hours old fluorescent *Pseudomonas* BRL-1 culture (10^7 cfu /ml) was streaked on petriplate having 20 ml of PGA as well as NA then inverted over the plates inoculated with the fungal pathogens and bacterial pathogens. Two plates were sealed together (mouth to mouth) with parafilm, control plates consist of only fungal and bacterial pathogen inverted over uninoculated PGA and NA plate respectively. These plates were then incubated at 30^0C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the test fungal pathogens was measured, as well as morphological changes of the colonies of the bacterial pathogen was compared with the control.

4.3.4. HCN production

Production of hydrogen cyanide was tested qualitatively according the method of Wei et al. (1991). The fluorescent *Pseudomonas* BRL-1 was inoculated in Tryptone Soya Agar (TSA) medium supplemented with amino acid glycine (4.4 gm/lit. of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30^0C for 4 days. A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded.

4.3.5. IAA production

To test the production of IAA, active culture of fluorescent *Pseudomonas* BRL-1 was inoculated in 10 ml of minimal salt media (5X: $(NH_4)_2SO_4$ –5 gm; KH_2PO_4-22.5 gm; K_2HPO_4-52.5 gm; Na-citrate-2.5 gm; distilled water-1 lit.) supplemented with 100 µg/ml of tryptophan, and incubated at 30^0C under shaking for 48 hours. Culture broth was centrifuged at 7500 rpm for 10 min. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and contents

incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (Gaur *et al.*, 2004).

4.3.6. Chitinase production

To test the chitinolytic property of fluorescent *Pseudomonas* BRL-1 it was inoculated on Luria Bertani (LB) medium (Gunasekaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicates chitinase activity of the strain (Basha and Ulagnathan, 2002).

4.3.7. Production of hydrolytic enzyme

Production of hydrolytic enzyme was qualitatively assayed in 5X minimal media containing gelatin (0.4%), starch (1%), pectin (1%), Carboxy methyl cellulose (1%), for protease, amylase, pectinase, cellulase respectively. Plates were incubated for 72 hours at 30°C and formation of clear zone around bacterial colony was read as positive. (Gaur *et al.*, 2004).

4.3.8. Siderophore production

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect the siderophore production. CAS agar (blue agar) was inoculated with 24 hours old fluorescent *Pseudomonas* BRL-1 at the center of the plate and kept for incubation at 30°C for 72 hours. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Chemical assay was performed to detect the nature of siderophore-

Tetrazolium test (Snow, 1954) was performed to detect the hydroxamate nature. Triphenyltrtrazolium chloride (about 1mg) was dissolved in a drop of culture supernatant of fluorescent *Pseudomonas* BRL-1, on a tile and a drop of 2 N of sodium hydroxide (NaOH) added. Immediate development of deep red colour was taken as a positive reaction.

Arnow's test (Arnow, 1937) was performed to detect catecholate siderophore. The cell free filtrate of fluorescent *Pseudomonas* BRL-1 was used as a source of

catechol. Catechol (Sigma Co.) was used as standard. Culture filtrate and standard catechol each in a volume of 1 ml were taken separately in two test tubes graduated at 5 ml. To each test tube was added 1 ml of 0.5 N hydrochloric acid, 1 ml of nitrate molybdate reagent (10 gm of sodium nitrate and 10 gm of sodium molybdate in 100 ml of distilled water), 1 ml of 1 N NaOH and enough distilled water to make the volume of 5 ml. Development of yellow colour of solution following the addition of nitrate molybdate reagent and its subsequent intensification of deep orange colour upon addition to NaOH indicated the presence of catechol.

4.4. Results and Discussion

Microscopic observation of the mycelium from the interacting zone showed hyphal shriveling, mycelial deformities, swelling, fragmentation, short branching and granulation of cytoplasm ultimately resulting into lysis (Figure 4.1). Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the antagonist. Further study was made to screen for the production of different secondary metabolites including siderophore, hydrogencyanide, non-volatile and volatile compound, hydrolytic enzymes, IAA investigated for their effect on fungal as well as bacterial pathogens (Table 4.1).

Table 4.1: Showing different secondary metabolites production and enzymatic activity of fluorescent Pseudomonas BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Antibiotic Substances	-
HCN production	-
Volatile substances	-
Chitinase activity	+
Protease activity	++
IAA production	+++

'+++' Stronger production; '++' Moderate production; '+' Low production; '-' No production.

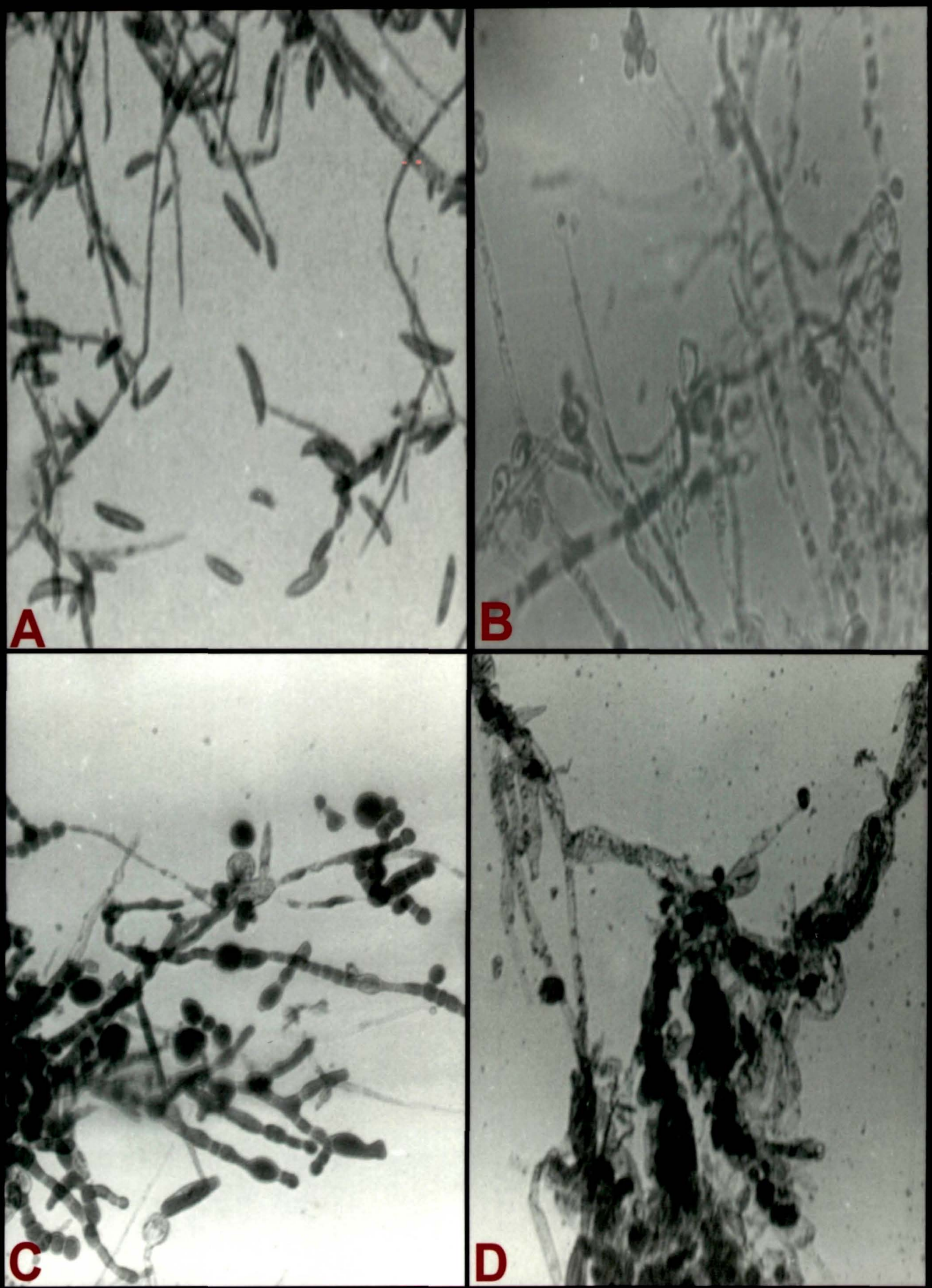


Figure 4.1. Microscopic observations of mycelium inhibited by fluorescent Pseudomonas BRL-1 strain: (A) Mycelium of F. oxysporum grown in PGA media (Control); (B) Present in the inhibition zone, when grown with fluorescent Pseudomonas BRL-1 on PGA; (C) Mycelium of M. hiemalis f. hiemalis grown on PGA media (Control); and (D) Present in the inhibition zone when grown with fluorescent Pseudomonas BRL-1 on PGA.

The effect of non-volatile and volatile substances production by antagonist fluorescent *Pseudomonas* BRL-1 was tested against *Fusarium oxysporum, Mucor hiemalis* f. *hiemalis* and *Erwinia carotovora* as described in materials and methods. Even after 96 hours of incubation there were no significant reductions in colony diameter of the fungal pathogens as well as no morphological changes of the colony of *Erwinia carotovora* was observed.

HCN production by fluorescent *Pseudomonas* BRL-1 was tested and found that the there was no change in colour of the picric acid saturated filter paper strip and results was recorded as negative. In fact it was reported that production of HCN is deleterious to plant (Bakker and Schippers, 1987; Alstrom and Burns, 1989).

In general, Indole acetic acid is universally accepted as a plant growth promoter. Other than that, it has been reported by Chirst and Mosinger (1989) that this phytohormone could also induce resistance through PR protein production. Again in the year 2004, Sharaf and Farrag reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by soil pathogens. The present study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1 after 48 hours of incubation (Figure 4.2A).

Lorito et al. (1994) and Dunne et al. (1997) reported that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease could result in the degradation of the structural matrix of the fungal cell wall. Fluorescent *Pseudomonas* BRL-1 has been found to produce chitinase and protease, when they were grown in chitin and gelatin media respectively, as a sole carbon source (Figure 4.2B and 4.2C). As the fungal cell wall contains chitin, as the major component and chitinase are well known to lyse the cell walls of both live and dead fungi (Ueno *et al.*, 1990), the reduction in dry weight of the fungal pathogen may be due to the proteolytic and chitinolytic activity of fluorescent *Pseudomonas* BRL-1.

CAS agar (blue agar) plate assay indicate siderophore production by fluorescent *Pseudomonas* BRL-1. The strain forms colonies with an orange halo. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye

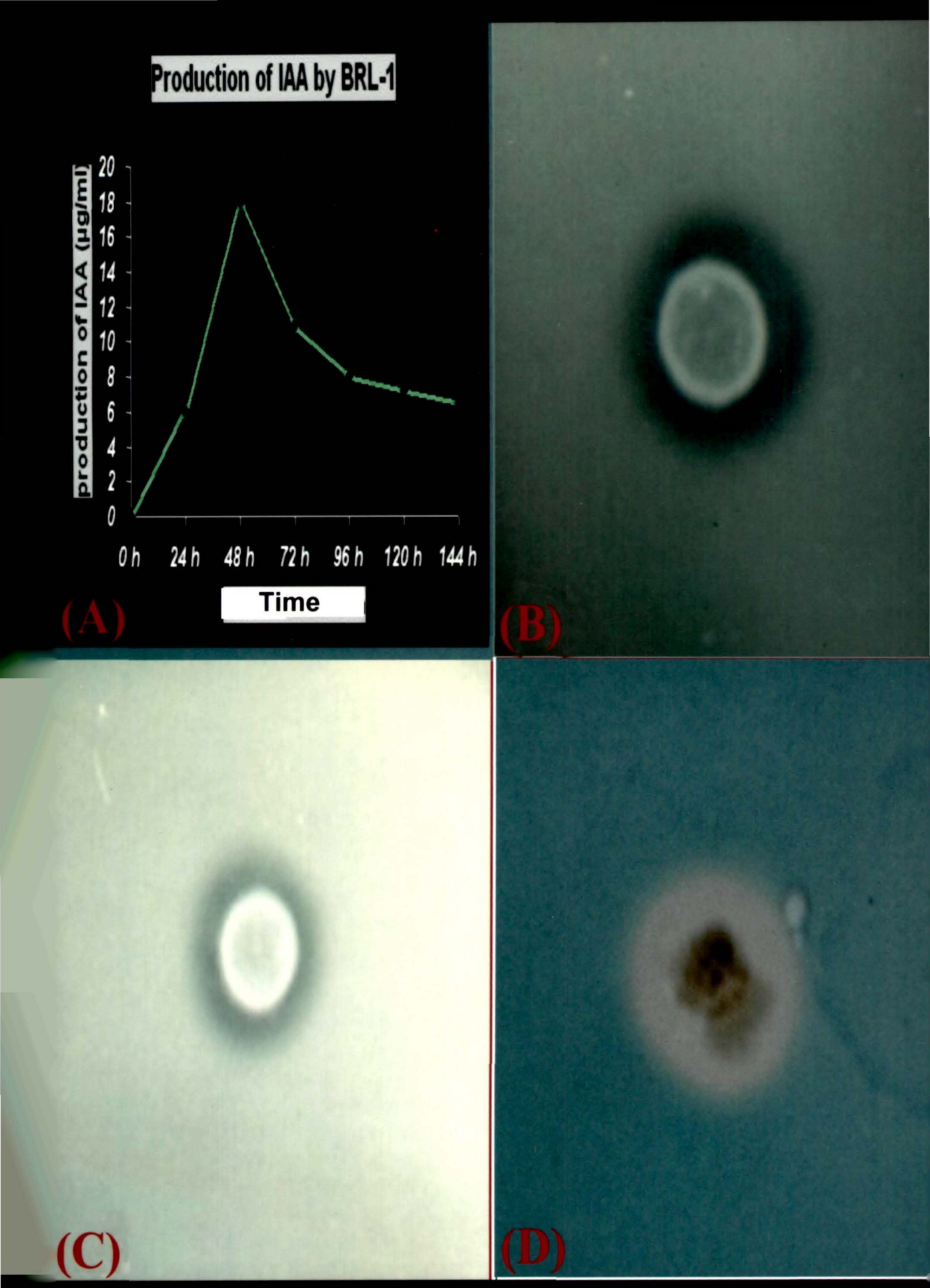


Figure 4.2: (A) Production of IAA by the isolate on minimal media supplemented with tryptophan; (B) Zone around the colony indicate chitinolytic property of the strain in LB media; (C) Proteolytic activity of the strain in gelatin agar media; and (D) Orange halo indicates the production of siderophore in CAS agar plate

complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the siderophore production. The isolate produced hydroxamate siderophore as evidenced by positive tetrazolium test and negative in case of Arnow test (Figure 4.2D).

On the basis of these studies it was well understood that the fluorescent *Pseudomonas* BRL-1 isolate was showing significant antagonistic property through combined and/ or individual effect of siderophore, production of proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen (Remeshkumar *et al.*, 2002). These observations might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against *Cymbidium* rot pathogens.

The next chapter deals with (a) development of a cost effective media for large-scale biomass production of the potential antagonistic fluorescent *Pseudomonas* BRL-1, (b) powder formulation of fluorescent *Pseudomonas* BRL-1 and lastly (c) field evaluation of the powdered formulation of the antagonist to check its efficacy.

4.5. Reference

- Alstrom, A. and Burns, R.G. 1989. Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. *Biol. Fertil. Soils.* 7: 232-238.
- Archibald, F. 1983. Lactobacillus plantarum, an organism not requiring iron. *FEMS Microbiol. Lett.* 19: 29-32.
- Arnow, L.E. 1937. Colorimetric determination of the compounds of 3,4-dihydrooxyphenyllalanine tyrosine mixtures, *J. Biol. Chem.* 118: 531-537.
- Bakker, A.W. and Schippers, B. 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.-mediated plant growth stimulation. *Soil Biol. Biochem.* 19: 451-457.
- Basha, S. and Ulagnathan, K. 2002. Antagonism of *Bacillus* species (strain BC121) toward *Curvularia lunata*. *Curr. Sci.* 82: 1457-1463.
- Blumer, C. and Haas, D. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. *Arch. Microbiol.* 173: 170-177.
- Brisbane, P.G. and Rovlra, A.D. 1988. Mechanisms of inhibition of Gaeumannomyces graminis var. tritici by fluorescent pseudomonads.*Plant Pathol.* 37: 104-111.
- Bull, C.T., Weller, D.M. and Thomashow, L.S. 1991. Relationship between root colonization and suppression of Gaeumannomyces graminis var. tritici by Pseudomonas fluorescens strain 2-79. *Phytopathology*. 81: 954-959.
- Buyer, J.S. and Leong. J. 1986. Iron transport-mediated antagonism between plant growth-promoting and plant-deleterious Pseudomonas strains. *J. Biol. Chem.* 261: 791-794.
- Christ, U. and Mosinger, E. 1989. Pathogenesis related protein of tomato: Induction by *Phytophthora infestance* and other biotic and abiotic inducers and correlations with resistance. *Phyisol. Mol. Plant Path.* 35: 53-65.
- Crichton, R.R. and Charloteaux-Wauters, M. 1987. Iron transport and storage. *Eur. J. Biochem.* 164: 485-506.
- Dahiya, J.S., Woods, D.L. and Tewari, J.P. 1988. Control of Rhizoctonia solani, casual agent of brown girdling root rot of rapeseed, by Pseudomonas fluorescens. *Bot. Bull. Acad. Sin.* 29: 135-142.
- de Weger, L.A., van der Vlugt, C.I.M., Wljfjes, A.H.M., Bakker, P.A.H.M., Schlppers, B. and Lugtenberg, B. 1987. Flagella of a plant-growth-stimulating Pseudomonas fluorescens strain are required for colonization of potato roots. *J. Bacteriol.* 169: 2769-2773.

- Dennis, C. and Webster, J. 1971a. Antagonistic properties of species group of *Trichoderma* I. Production of volatile antibiotics. *Trans. Br. Mycol. Soc.* **57:** 25-29.
- Dennis, C. and Webster, J. 1971b. Antagonistic properties of species group of *Trichoderma* II. Production of volatile antibiotics. *Trans. Br. Mycol. Soc.* **57:** 41-48.
- Dunne, C., Crowley, J.J. Moenne-Loccoz, Y., Dowling, D.N., de Brunijn, F.J. and O'Gara, F. 1997. Biological control of Pythium ultimum by stenotrophomonas maltophilia W81 is mediated by an extracellular proteolytic activity *Microbiology*. 143: 3921-3931.
- Elad, Y. and Baker. R. 1985a. Influence of trace amounts of cations and siderophore-producing pseudomonads on chlamydospore germination of Fusarium oxysporum. *Ecol. Epidemiol.* **75:** 1047-1052.
- Elad, Y. and Baker. R.1985b. The role of competition for iron and carbon in suppression of chlamydospore germination of *Fusarium* sp. by *Pseudomonas* spp. *Ecol. Epidemiol.* **75:** 1053-1059.
- Gaffney, T.D., Friedrlch, L., Vernoolj, B., Negmtto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. 1993. Requirement of salicylic acid for lhe induction of systemic acquired resistance. *Science.* 261: 754-756.
- Gallagher, L.A. and Manoil, C. 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J. Bacteriol.* 183: 6207-6214.
- Gaur, R., Shani, N., Johri, B. N., Rossi, P. and Aragno, M. 2004. Diacetylphloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Curr. Sci.* **86:** 453-457.
- Gunasekaaran, P. 1995. In: Laboratory Manual In Microbiology. pp. 125. New Age International (P) Limited, New Delhi.
- Hoffland, E., Pieterse, C.M.J., Blk, L. and van Pelt, J.A. 1995. lnduced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins. *Physiol. Mol. Plant Path.* **46:** 309-320.
- Howell, C.R. and Stipanovic, R.D. 1980. Suppression of Pythium ultimum-induced damping off of cotton seedlings by Pseudomonas fluorescens and its antibiotic pyoluteorin. *Phytopathology.* **70:** 712-715.
- Howell, C.R. 2003. Mechanisms Employed by *Trichoderma* Species in the Biological Control of Plant Diseases: The History and Evolution of Current Concepts. *Plant Dis.* **87:** 4-10.
- Howell, C.R. and Stipanovic, R.D. 1979. Control of *Rhizoctonia solani* in cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology.* **69:** 480-482.

- Janisiewicz, W.J., Tworkoski, T.J. and Sharer, C. 2000. Characterizing the Mechanism of Biological Control of Postharvest Diseases on Fruits with a Simple Method to Study Competition for Nutrients. *Phytopathology.* **90:** 1196-1200.
- Keel, C., Wirthner, P.H., Oberhansli, T.H., Voisard, C., Burger, D., Haas, D. and Defago. G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. *Symbiosis.* **9:** 327-341.
- Knowles, C.J. 1976. Microorganisms and cyanide. *Bacteriol. Rev.* 40: 652-680.
- Kremer, R.J. and Souissi, T. 2001. Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Curr. Microbiol.* 43: 182-186.
- Lindberg, G.D. 1981. An antibiotic lethal to fungi. *Plant Dis.* 65: 680-683.
- Loper, J.E. and Buyer, J.S. 1991. Siderophores in microbial interactions on plant surfaces. *Mol. Plant-Microbe Interact.* 4: 5-13.
- Lorito, M., Peterbauer, C., Hayes, C.K. and Harman, G.E. 1994. Synergistic interaction between fungal cell wall degrading enzymes and different antifungal compounds enhances inhibition of spore germination. *Microbiology.* **140:** 623-629.
- Luckner, M. 1990. *Secondary products derived from amino acids.* In: Secondary Metabolism in Microorganisms, Plants, and Animals. pp. 246-249. Springer-Verlag, Berlin, Germany.
- Matzanke, B.F. 1987. *Mossbauer spectroscopy of microbial iron metabolism,* In: Iron transport in microbes, plants and animals. pp. 251-284. eds. Winkelmann, G., van der Hehn, D. and Neilands, J.B. Verlagsgeselischaft mbH, Weinheim.
- Maurhofer, M., Hase, C., Meuwly, P., Metraux, J.P. and Defago, G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing Pseudomonas fluorescens Strain CHAO: Influence of the gacA gene and pyoverdine production. *Phytopathology.* 84: 139-146.
- Neilands, J.B., Konopla, K.L., Schwyn, B., Coy, M., Frands, R.T., Paw, B.H. and Bagg. A. 1987. *Comparative biochemistry of microbial iron assimilation,* In: Iron transport in microbes, plants and animals. pp. 3-33. eds. Winkelmann, G., van der Helm, D. and Neilands, J.B. Verlagsgeselischaft mbH, Weinheim.
- Nielsen, M.N., Sorensen. J., Fels, J. and Pedersen, H.C. 1998. Secondary metabolite and endochitinase dependent antagonism toward plant pathogenic microfungi of *Pseudomonas fluorescens* isolated from sugar beet rhizosphere. *Appl. Environ. Microbiol.* 64: 3563-3569.
- Nielsen, T.H., Sorensen, D., Tobiasen, C., Andersen, J.B., Christophersen, C., Givskov, M. and Sorensen, J. 2002. Antibiotic and Biosurfactant Properties of Cyclic

Lipopeptides Produced by Fluorescent *Pseudomonas* spp. from the Sugar Beet Rhizosphere. *Appl. Environ. Microbiol.* **68:** 3416–3423.

- Parke, J.L. 1990. Population dynamics of Pseudomonas cepacia in the pea spermosphere in relation to biocontrol of Pythium. *Phytopathology*. **80:** 1307-1311.

- Parke, J.L. 1991. *Root colonization by indigenous and introduced microorganisms*. In: The Rhizosphere and Plant Growth. pp. 33-42. eds. Keister, D.L. and Cregan, P.B. (Boston, MA. Kluwer Academic Publishers).

- Paulitz, T.C. and Baker, R. 1987. Biological control of Pythium damping-off of cucumbers with Pythium *nunn:* Population dynamics and disease suppression. *Phytopathology*. **77**: 335-340.

- Rameshkumar, N., Thirumalai Arasu, V. and Gunasekaran, P. 2002. Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria, *Pseudomonas fluorescens*. *Curr. Sci.* **82**: 1463-1466.

- Ross, A.F. 1961. Systemic acquired resistance induced by localized virus infections in plants. *Virology* 14: 340-358.

- Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Mollna, A., Steiner, H.X. and Hunt, M.D. 1996. Systemic acquired resistance. *Plant Cell.* 8: 1809-1819.

- Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Annal. Bichem.* **160:** 47-56.

- Sharaf, E.F. and Farrag, A.A. 2004. Induced resistance in tomato plants by IAA against Fusarium oxysporum lycopersici *Pol. J. Microbiol.* **53:** 111-116.

- Sneh, B., Dupler, M., Elad, Y. and Baker, R. 1984. Chlamydospore germination of *Fusarium oxysporum* f. sp. *cucumennum* as affected by fluorescent and lytic bacteria from Fusarium suppressive soils. *Phytopathology*. **74:** 1115-1124.

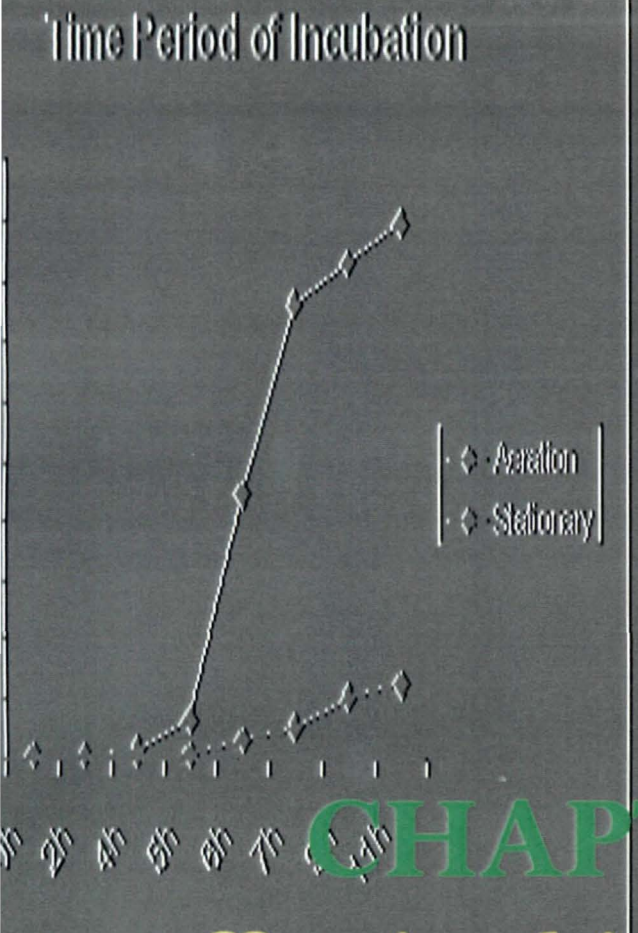
- Snow, G.A. 954. Mycobactin. A growth factor for *Mycobacterium johnei* II-Degradation and identificatin of fragments, *J. Chem. Soc.* 2588-2596.

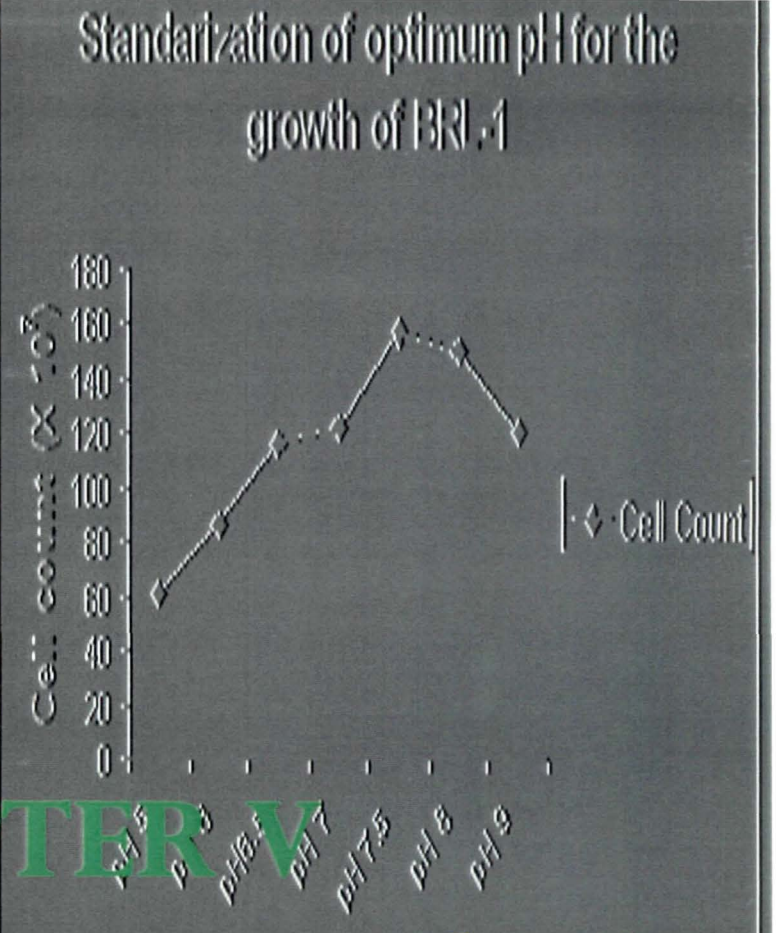
- Thomashow, L.S. and Weller, D.M. 1988. Role of a phenazine antibiotic from Pseudomonas fluorescens in biological control of Gaeumannomyces graminis var. tritici. *J. Bacteriol.* **170**: 3499-3508.

- Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. 1990. Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. *J. Gen. Appl. Microbiol.* **36:** 377.

- Uknes, S., Mauch-Manl, E., Moyer, M., Potter, S., Williams, S., Dincher, S., Chsndler, D., Slusarenko, A., Ward, E. and Ryals, J. 1992. Acquired resistance in Arabidopsis. *Plant Cell.* 4: 645-656.

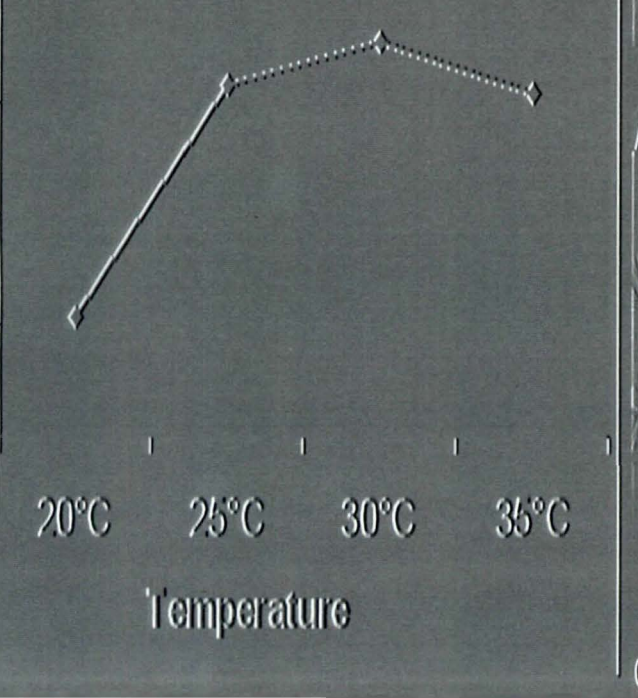
- Wei, G., Kloepper, J.W. and Sadik, T. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth promoting rhizobacteria. *Phytopathology*. **81**: 1508-1512.
- Weller, D.M. 1983. Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. *Phytopathology*. **73**: 1548-1553.
- Weller, D.M. and Cook, R.J. 1983. Suppression of take-ali of wheat by seed treatments with fluorescent pseudomonads. *Phytopathology*. **73**: 463-469.
- Zdor, R.E. and Andemon, A.J. 1992. Influence of root colonizing bacteria on the defense responses of bean. *Plant Soil*. **140**: 99-107.

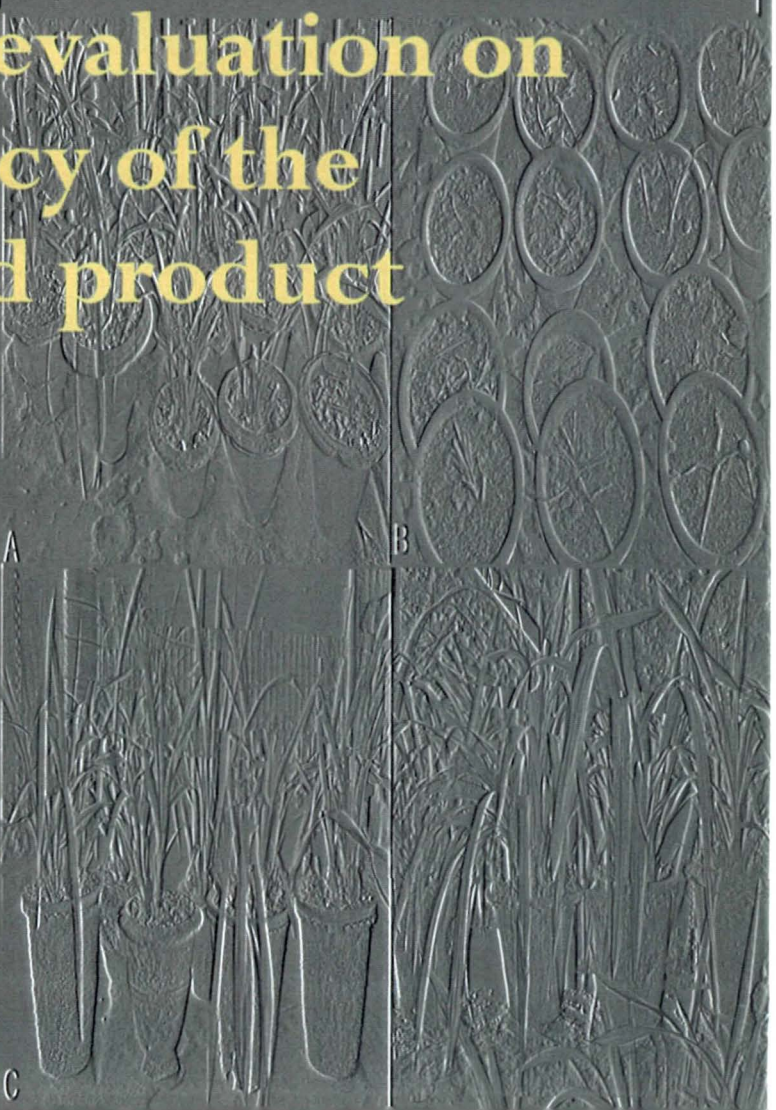




CHAPTER V

Cost effective biomass production and in vivo evaluation on the efficacy of the formulated product





5.1. Introduction

Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). The interactions between microorganisms and plant hosts can be complex. Interactions that lead to biocontrol can include antibiosis, competition, induction of host resistance and predation (Cook and Baker 1983). When testing bacterial and fungal isolates from the environment for biocontrol activities, between 1 and 10% show at least some capacity to inhibit the growth of pathogens *in vitro*. However, fewer isolates can suppress plant diseases under diverse growing conditions and fewer still have broad-spectrum activity against multiple pathogenic taxa. Nonetheless, intensive screens have yielded numerous candidate organisms for commercial development. Some of the microbial taxa that have been successfully commercialized and are currently marketed as Environmental Protection Act or EPA-registered biopesticides in the United States include bacteria belonging to the genera *Agrobacterium, Bacillus, Pseudomonas*, and *Streptomyces* and fungi belonging to the genera *Ampelomyces, Candida, Coniothyrium,* and *Trichoderma* (www.epa.gov/pesticides /biopesticides). In India bacteria like *Bacillus, Pseudomonas* and fungi like *Trichoderma* are marketed as biopesticide. Still, much remains to be learned about the microbial ecology of both plant pathogens and their microbial antagonists in different agricultural systems (Kerry, 2000; Weller *et al.*, 2002). Fundamental work remains to be done on characterizing the different mechanisms by which organic amendments reduce plant disease (Hoitink and Boehm 1999). More studies on the practical aspects of mass-production and formulation need to be undertaken to make new biocontrol products stable, effective, safer and more cost-effective (Fravel *et al.*, 1999).

Industrial interest in the development of microorganism for biological control has fluctuated over the past several decades. Once a biocontrol agent has shown potential for disease control based on laboratory, greenhouse and field tests, production of an effective biomass becomes a major concern. Advances in fermentation technology have

produced bacterial and fungal biomass for use as biocontrol insecticide and herbicides (Bowers, 1982; Churchill, 1982). However, similar technology for the production of biocontrol microorganism effective against plant pathogens is in its infancy. This deficiency is clearly an obstacle to the advancement of biocontrol research. All the biological control systems are based on a single, fundamental concept, the use of a living organism to control a pest. In order to commercialize successfully a biological control agent, this concept must be appreciated at all times. First step in the production of biocontrol agent is development of a suitable medium using inexpensive readily available agricultural by-products with the appropriate nutrient balance. (Latge and Soper, 1977). Acceptable materials include molasses, brewer's yeast, corn steep liquor, sulphite waste liquor, and cotton seed and soy flours (Lisansky, 1985).

For a successful fermentation, not only must appropriate substrates be used, but sufficient biomass containing adequate amounts of effective propagules must be obtained. Isolates of *Trichoderma* and *Gliocladium* performed best for biocontrol if preparations contained the resistant, survival propagules of the fungus i.e. chlamydospore (Lewis and Papavizas, 1984). Of various liquid media tested, small scale fermentation in molasses-brewer's yeast resulted in abundant chlamydospore production (Papavizas *et al.*, 1984). The most common equipment and processes available for the commercial production of microorganism are associated with deep tank fermentation. Deep tank fermentation has been reported by Tabachnik (1988) for the formation of conidia of *Trichoderma*. Liquid cultivation is a standard industrial method of producing cells and their products. Cultivation in batch –stirred tank reactors is the standard method of producing microbial product and such technology has been extensively developed to accommodate the food and pharmaceutical industries. Advantages of this reactor design include flexibility to accommodate multiple processes, good long-term capital investment, reliable processes and quality control, and amenability to automation and minimization of labor costs (Van Brunt, 1986). Liquid fermentation is usually employed for the production of non-obligate

pathogens such as bacteria, some fungi and nematodes (Jenkins and Goettel, 1997). There are several additional factors to consider in liquid fermentation. For example, the rate at which an effective biomass is produced affects cost of production as well as the chance of contamination and viability (Lisansky, 1985). It is desirable to obtain the optimum amount of biomass in the shortest time. With isolates of *Trichoderma, Gliocladium* and *Talaromyces,* satisfactory quantities of biomass were obtained in 6 to 7 days, but this time period is still long compared with that for bacteria. Increased fermentation time also reduces viability of some organisms and increases the risk of contamination.

Since the importance of liquid fermentation has become apparent solid state fermentation facilities have become limited in much of the world. In fact, Solid State Fermentation (SSF) has rarely been used because of insufficient consumer demand for product formed (Cannel and Moo-Young, 1980). Inspite of the rapid advances in the liquid fermentations demonstrated by industrialized nations, most of the researches on mass production of biocontrol fungi continue to emphasis solid (semi-solid) substrate fermentation for the formation of inoculum (Aidoo *et al.,* 1982). SSF are also suitable for the production of fungi which do not sporulate in liquid culture (Lisansky, 1985). New applications of SSF have been suggested for the production of antibiotics (Barrios-Gonzales *et al.,* 1988) secondary metabolites (Trejo-Hernandez *et al.,* 1992, 1993) or enriched foodstuffs (Senez *et al.,* 1980). There are several limitation of SSF such as direct determination of biomass in SSF is very difficult due to problems of separation of the microbial biomass from the substrate, subject to a greater risk of contamination and may require extensive space for processing, incubation and storage.

5.2. Formulation

Once production methods, recovery procedures and storage technology have been worked out, the next step is development of appropriate formulations for field application. A biocontrol formulation with agricultural potential should possess several

desirable characteristics such as ease of preparation and application, stability, adequate shelf life, abundant viable propagules and low cost (Churchill, 1982; Lisansky, 1985). An enormous number of amendments have been utilized in experimental and commercial formulations of different biocontrol agents. In turn, these amendments can be grouped into any number of amendment types. Generally, amendments can be grouped as either carriers (fillers, extenders) or amendments that improve the chemical, physical, or nutritional properties of the formulated biomass. Selections of amendment types along with a limited number of examples of each type are shown in Table 5.1. An extensive treatment of the topic can be found elsewhere (Bernhard *et al.*, 1998). Experimental formulations of *Bacillus* spp. that have effectively reduced plant disease have included clays (Osburn *et al.*, 1995), peat and chitin (Manjula and Podile, 2001; Sid Ahmed *et al.*, 2003), methylcellulose (Racke and Sikora, 1992), Ca-alginate, alginate manucol, or carob (Schmidt *et al.*, 2001), carboxymethyl cellulose, vegetable oil, and polyvinyl pyrrolidone (Kanjanamaneesathian *et al.*, 2000) and peptone (Ferreira *et al.*, 1991; Schmidt *et al.*, 2001) and nutrient medium (Smith *et al.*, 1993). Another various carrier-based formulation was extensively studied by Vidyasekaran and Muthamilan (1995) in case of *Pseudomonas fluorescens* shown that the bacteria can survive well in talc or peat based formulation for more than eight months.

Table 5.1. Types of amendments and example materials for formulating biocontrol biomass

Amendment type	Examples
Liquid carriers	Vegetable oils
Mineral carriers	Kaolinite clay, diatomaceous earth
Organic carriers	Grain flours
Stabilizers	Lactose, sodium benzoate
Nutrients	Molasses, peptone
Binders	Gum arabic, carboxymethylcellulose
Desiccants	Silica gel, anhydrous salts
Thickeners	Xanthan gum
Surfactants	Tween 80
Dispersants	Microcrystalline cellulose
UV protectants	
Sunscreens	Oxybenzone
Optical brighteners	Blankophor BBH
Light blockers	Lignin (PC 1307)
Stickers	Pregelatinized corn flour

To introduce an antagonist in the ecosystem and help it to survive, proliferate, become active and establish itself in a new environment, proper formulations or antagonist preparations have to be evolved. These are intended to make economically inexpensive substrate, which allow production of maximum biomass in the shortest time (Sen, 2000). Among six isolates of fluorescent *Pseudomonas* only fluorescent *Pseudomonas* BRL-1 showed effective antagonistic activity against all the three pathogens of *Cymbidium* pseudobulb rot as described earlier and also produce significant amount of indole acetic acid (IAA). Because of its potentiality the main objectives are (i) formulation of a cost effective media (ii) powder formulation using different inert carriers (iii) survibility of the organism in the inert carriers and finally (iv) *in vivo* application of inert carrier based formulated fluorescent *Pseudomonas* BRL-1.

5.3. Materials and Methods

5.3.1. Standardization of optimum condition: Conditions like method of cultivation, incubation period, pH, temperature, aeration for large scale biomass production of the selected strain BRL-1 of fluorescent *Pseudomonas* was performed utilizing a very common media (PS media) (Peptone-1%; Skim milk-1%; K_2HPO_4, 0.15% and $MgSO_4$ 0.15%) used for liquid cultivation of fluorescent pseudomonads. The biomass production was determined optically and further verified by hemocytometer cell counting method on Leica DSML research microscope. In all cases inoculum concentration was maintained at $1.2 \pm 0.25 \times 10^7$ cells / ml i.e. this would be the cell count at 0 hours in the fermentation media.

5.3.1.1. Effect of method of cultivation on biomass production: Biomass production was carried out with stationary and shake flask method (Mukherjee and Majumdar, 1971) in 100 ml Erlenmeyer flask containing 25 ml of (PS media), inoculated with 12 hours old culture of fluorescent *Pseudomonas* BRL-1. Initial inoculum concentration of the PS was set at $1.42 \pm 0.003 \times 10^7$ cells / ml, incubated at 30^0C. In the stationary method the inoculated broth were kept as such without any shake (static) where as in shake flask method the broth cultures were placed in a rotary shaker at 150 r.p.m. The biomass production was determined after different time intervals.

5.3.1.2. Effect of initial pH of the media on biomass production: Initial pH of the medium was adjusted to 5, 6, 6.5, 7, 7.5, 8 and 9 with 0.1 N HCl or NaOH. The fermentation was carried out in standardized method of cultivation in 100 ml Erlenmeyer flasks each containing 25 ml of PS inoculated with 12 hours old culture of BRL-1. The biomass production was estimated after standardized incubation time.

5.3.1.3. Effect of temperature on biomass production: To determine the optimum temperature for the large scale biomass production fermentation was carried out in standardized method of cultivation and pH at different temperatures such as 20^0C, 25^0C, 30^0C and 35^0C. Culture was taken after standardized incubation period for the assay of highest biomass yield (Mukherjee and Majumdar, 1971).

5.3.1.4. Effect of the initial number of cell in the PS Media on the biomass production: Start point cell number plays an important role for shortening of lag phase and there by increasing the biomass yield in short period. To obtain the optimum initial cell number, fermentation was carried out in 100 ml Erlenmeyer flask containing 25 ml of PS where start point cell number was varied from 0.5×10^7 to 5×10^7 cells / ml utilizing all earlier standardized parameters. Biomass yield was measured after proper incubation period.

5.3.1.5. Effect of aeration of biomass production: Effect of aeration was studied by placing different volume of medium; 15 ml 20 ml, 25 ml, 30 ml, 35 ml, 40 ml and 45 ml in 100 ml of Erlenmeyer flasks utilizing the standardized parameters. The cellular mass yield was measured after standardized incubation period.

5.3.2. Identification of a cost effective carbon and nitrogen source: After standardization of various growth factors, cost effective carbon and nitrogen sources were investigated. For identification of cost effective carbon source various sources of carbon were taken such as glucose, sucrose, fructose, molasses, skim milk at 4% and nitrogen source as peptone at 1% (w/v). For investigation of cost effective nitrogen source various organic and inorganic nitrogen sources were taken like peptone, yeast flour, mustard cake extract, fishmeal, urea, soybean flour, ammonium nitrate, ammonium chloride, ammonium sulphate, sodium nitrate and gelatin amended with selected carbon source (4% w/v).

5.3.3. Formulation of media: Finally C/N ratio was established for designing of a cost effective suitable, commercial media. As the carbon to nitrogen ratio of the media plays a very important role in biomass production investigation was next made to determine optimum C/N ratio of media using standardize carbon and nitrogen source. For this study first the cost effective standardized carbon source at fixed concentration was taken but amount of standardized nitrogen source was varied to obtain to desired C/N ratios. Further the nitrogen source at optimum ratio was fixed but amount of carbon source was varied to get the desired C/N ratio.

5.3.4. Survivility test: To test the survivility of fluorescent *Pseudomonas* BRL-1, the strain was cultured on formulated liquid broth utilizing standardized growth condition. The survival was tested in different carriers like peat, talc, farmyard manure and vermiculite. Ten grams of carboxymethyl cellulose was added to 1 kg of the carrier and mixed well. The pH was adjusted to 7.0 by adding calcium carbonate. The carriers were autoclaved twice for 30 minutes on two consecutive days. One kg of the carrier was inoculated with 400 ml of the bacterial suspension containing 8 x 10^9 cfu / ml was mixed well under sterile conditions and kept in sealed polythene bags at room temperature (22 ± 2^0C). Samples were drawn at intervals up to six months and the bacterial population was assessed by serial dilution method. Treatments were replicated three times and Petri dishes were incubated at 30^0C. After 48 hours, colony-forming units of the bacterium was recorded.

5.3.5. *In vivo* application of selected inert carrier formulation of fluorescent *Pseudomonas* BRL-1: *In vivo* evaluation of inert carrier based formulation of fluorescent *Pseudomonas* BRL-1 was performed in a polyhouse at Darjeeling. *In vivo* experiment was performed by slightly modified method of Vidhyasekaran and Muthamilan (1995). Healthy young plantlets of same age

were collected from different nurseries of several localities in Darjeeling and its adjoining areas. Collected samples were washed thoroughly with tap water and potted into earthen pots. Each pot was filled with 3 kg unsterilized soil (3 part loam and 1 part well composed farmyard manure). Experimental sets having five replicas were exposed in the polyhouse for *in vivo* experiment (Table 5.2).

Table 5.2. In vivo arrangement of pots for various treatments

Set No	Treatment
Set I	Bulbs along with roots were dipped in mixture of spore and cell suspension of all three pathogens responsible for *Cymbidium* rot.
Set II	Bulbs along with roots were dipped in mixture of spore and cell suspension of all three pathogens and uniformly coated with powdered formulation of fluorescent *Pseudomonas* BRL-1.
Set III (Control)	Bulbs were coated with powdered formulation of fluorescent *Pseudomonas* BRL-1 only.
Set IV (Blank)	Bulbs were treated with sterile distilled water only.

- *Set I*: In this set, the healthy pseudobulbs along with roots were dipped in mixture of spore / cell suspension (10^6 spores / cell /ml) of all three pathogens for two minutes and then the bulbs were potted in earthen pots.
- *Set II*: The pseudobulbs were dipped in mixture of all three pathogens (10^6 spores / cell / ml) and these dipped bulbs were then uniformly coated with powered formulation of fluorescent *Pseudomonas* BRL-1 (about 10^7 cfu/gm of powder) supplemented with 1% Carboxy Methyl Cellulose (CMC) as adhesive and planted in earthen pots.

- *Set III*: This was a control set, where bulbs were coated with powdered formulation of fluorescent *pseudomonas* BRL-1 only and transferred to earthen pot.
- *Set IV*: This was a blank set were bulbs were treated with sterile distilled water and planted in the pots.

Pots were arranged in rows, well sanitation system was maintained in the polyhouse, plants were watered regularly and disease incidence was monitored periodically.

5.4. Results and Discussion

5.4.1. Effect of method of cultivation on biomass production

Initial efforts were made to carry out the effect of method of cultivation like stationary and shake flask culture method on the growth of the organism in PS media. In both the cultivation method initial count at 0 hours was 1.42 ± 0.003 × 10^7 cells / ml. Growth was measured at different time period. Results were presented in figure 5.1.

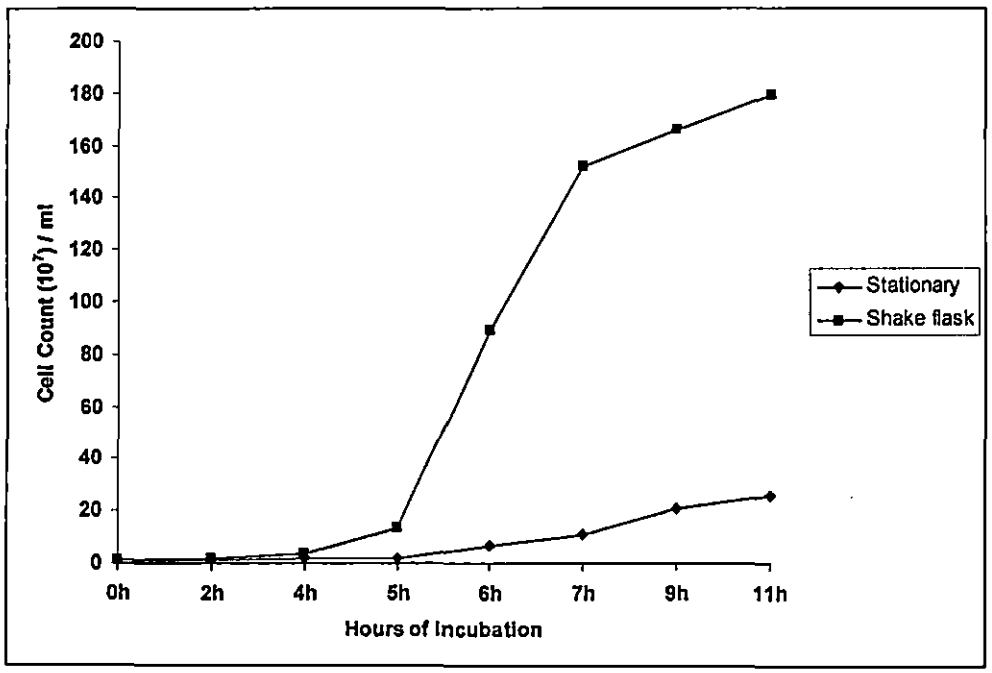


***Figure 5.1:* Effect of method of cultivation on biomass production. Results were presented mean of three separate experiments each in triplicate.**

It is evident from the figure that the shake-flaks method is superior to the stationary process. From the figure it is also evident that within 4-5th hour of incubation a sharp change in growth in shake-flask system but extended lag period was observed in stationary culture method. Furthermore, in shake-flask method cells reached to the stationary phase at short time period i.e. highest cell yield in shorter period of incubation.

5.4.2. *Effect of initial pH of the media on the biomass production*

Initial pH of the medium was adjusted to 5, 6, 6.5, 7, 7.5, 8 and 9 with 0.1 N HCl or NaOH. The fermentation was carried out in 100 ml Erlenmeyer flasks, each containing 25 ml of media. Initial count at 0 hours was 0.93×10^7 cells / ml. The broths were incubated for 7 hours at rotary shaker (150 rpm). The results were shown in Figure 5.2.

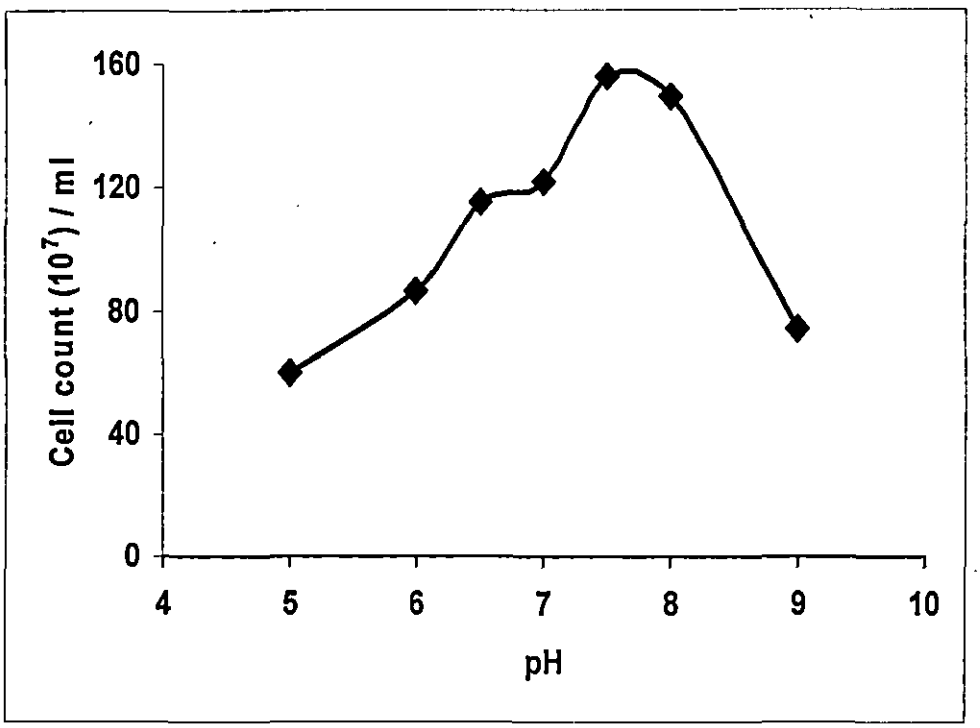


Figure 5.2: **Effect of pH on biomass production. Results were presented mean of three separate experiments each in triplicate.**

From the figure it is clear that when the initial pH of the medium was set at 7.5 the biomass yield was maximum.

5.4.3. *Effect of temperature on biomass production*

Fermentation was carried out in shake culture method at different temperatures (20^0C, 25^0C, 30^0C and 35^0C), keeping other parameters constant (incubation

period- 7 hours; method of cultivation- shake-flask; pH- 7.5) results were depicted in Figure 5.3.

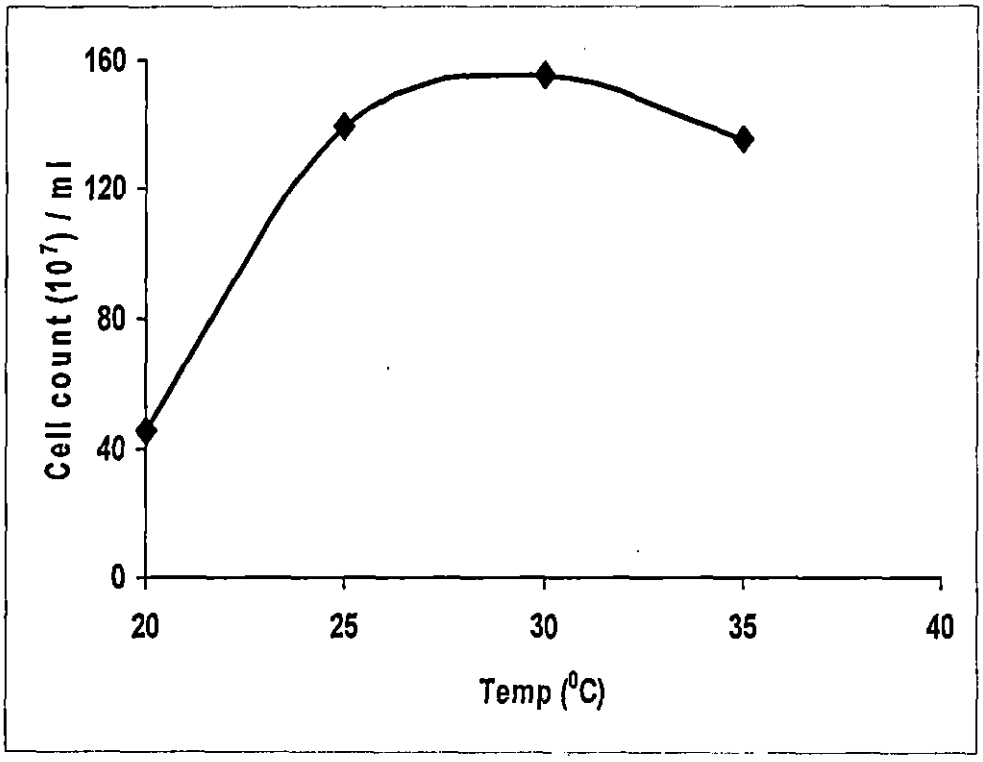


Figure 5.3: **Effect of temperature on biomass production. Results were presented mean of three separate experiments each in triplicate.**

Of the various temperatures tested for biomass yield, it was found that 30°C favoured the optimum biomass production.

5.4.4. Effect of the initial number of cell in the PS Media on the biomass production: As the start point cell number plays an important role for shortening of lag phase and there by increasing the biomass yield in short period, an attempt have been made to evaluate the role of initial cell number in the media on biomass production utilizing the earlier standardize parameters (incubation period- 7 hours; pH- 7.5; temperature- 30°C; method of cultivation- shake-flask method). Results are presented in Figure 5.4.

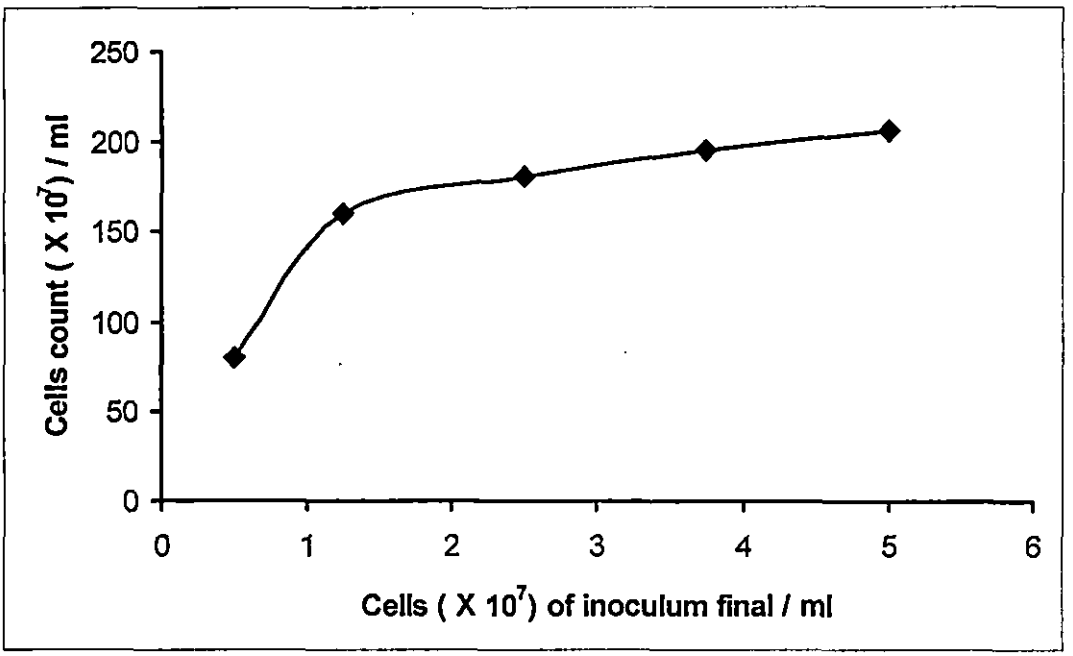


Figure 5.4: **Effect of inoculum volume of growth. Results were presented mean of three separate experiments each in triplicate.**

From the figure it is evident that the start point cell number in the PS was manipulated from 0.5 × 10^7 to 5 × 10^7 cells / ml. After the incubation period (7 hours), optimum initial cell number was found 1.25 × 10^7 cells / ml.

5.4.5. *Effect of aeration on biomass production*

Effect of aeration was studied by placing different volume of medium; 15 ml, 20 ml, 25 ml, 30 ml, 35 ml, 40 ml and 45 ml in 100 ml of Erlenmeyer flasks which were incubated for 7 hours at 30^{0}C at pH 7.5 on a rotary shaker (150 rpm). Initial number of cells in the medium was set at 1.25 × 10^7 cells /ml Degree of aeration of the fermentation broth in the shaker flask was inversely proportional to volume of liquid. The results are shown in Table 5.3.

Table 5.3. Effect of aeration on the production of biomass.

Volume of media	Cell count
15 ml	29.19×10^7
20 ml	97.6×10^7
25 ml	174.4×10^7
30 ml	817.6×10^7
35 ml	203.2×10^7
40 ml	102.4×10^7
45 ml	86.4×10^7

From the Table it is quite interesting that 30 ml of media volume in a 100 ml flask gives sudden shifting in biomass production which tempts us to speculate that adequate but optimum aeration is essential for large scale production of biomass.

After standardization of different physical factors like incubation period (7 hours), method of cultivation (shake-flaks), pH (7.5), temperature (30°C), inoculum volume (30ml) and aeration, further experiments were designed to identify a suitable cost effective carbon and nitrogen source.

5.4.6. Effect of different Carbon source on biomass production

Effect of different carbon source (4%) like glucose, sucrose, fructose, molasses, and skim milk on biomass production was determined after 7 hours of fermentation on a rotary shaker at pH 7.5 and incubated at 30°C in a total volume of 30 ml in 100 ml Erlenmeyer flask where 1% peptone used as nitrogen source in each cases. The results are shown in Figure 5.5.

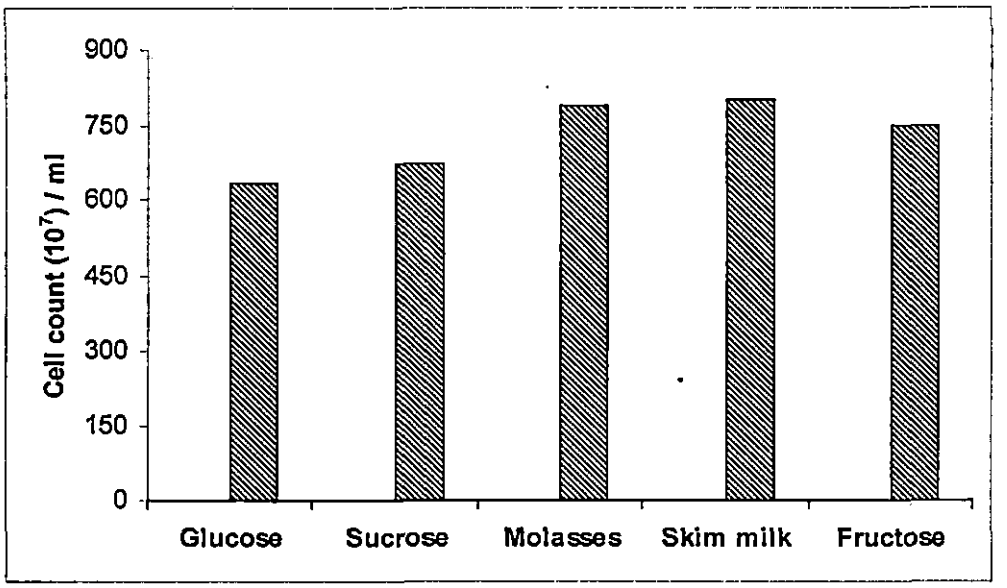


Figure 5.5: **Effect of different carbon source on biomass production. Results were presented mean of three separate experiments each in triplicate.**

Different carbon sources gave different degree of biomass yield in the fermentation broth i.e. skim milk ≅ molasses > fructose > sucrose > glucose. However, considering the cost effectiveness and the biomass production, molasses has been chosen as best carbon sources for commercial production of the strain. Now the optimum concentration of standardized carbon source i.e. molasses was determined in media ranging from 2 to 6% (Table 5.4)

Table 5.4. Optimization of concentration of Carbon source (MOLASSES) on highest biomass yield.

Carbon Source (Molasses) in %	Initial cell count /ml	Final cell Count /ml
2%	1.03×10^7	364.38×10^7
3%	1.03×10^7	556.96×10^7
4%	1.03×10^7	902×10^7
5%	1.03×10^7	668.35×10^7
6%	1.03×10^7	570.18×10^7

From this experimental result it is quite clear that at 4% concentration of molasses gave the highest yield of cellular mass.

5.4.7. Effect of different Nitrogen source on biomass production

Different nitrogen sources, including both inorganic and organic compounds were examined. To the basal medium containing 4% molasses as C source, $MgSO_4$ – 0.15%, K_2HPO_4- 0.15% and different nitrogen sources were added. Fermentation condition and the cell count method were the same as described previously. The results are shown in Figure.5.6.

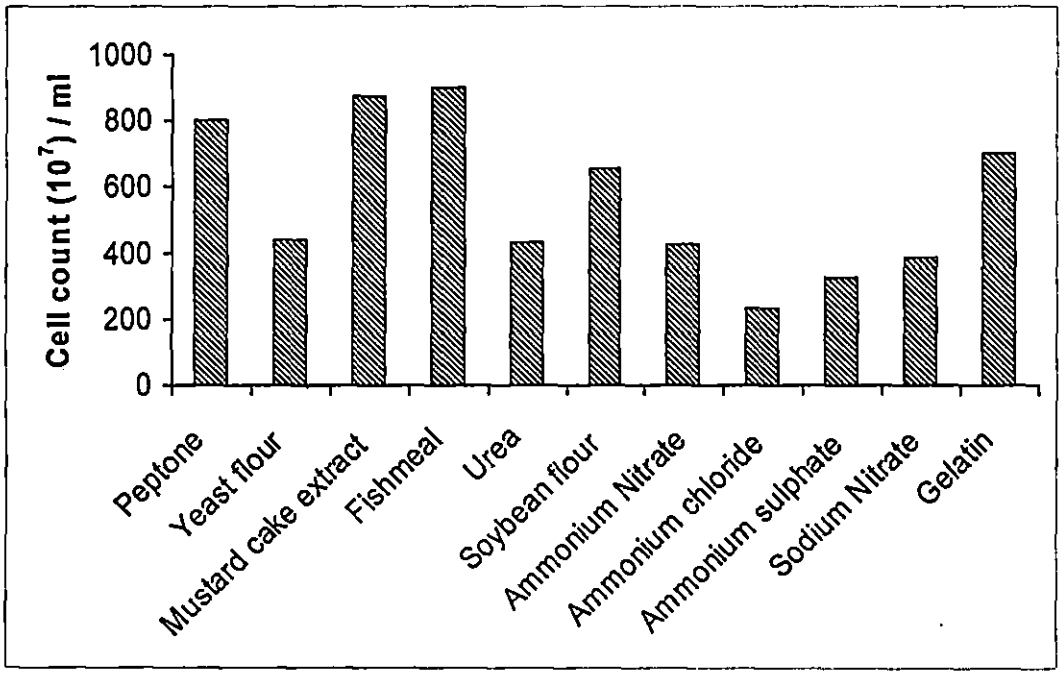


Figure 5.6: **Effect of different Nitrogen source on biomass production. Results were presented mean of three separate experiments each in triplicate.**

The figure shows that organic N_2 sources like peptone, and fishmeal were superior to inorganic N_2 sources and other organic nitrogen sources in giving higher cellular yield in said time period (7 hours). Of the various organic N_2 sources tested, fishmeal gave maximum yield and from the commercial production point of view fishmeal was the best option so it has been selected as the cheapest nitrogen source for further experiment.

5.4.8. Effect of C/N ratio on biomass production

As the carbon to nitrogen ratio of the media plays a very important role in biomass production, investigation was next made to determine optimum C/N ratio of media using molasses as carbon source and fishmeal as N_2 source. In this study molasses was used in a 4% concentration. Amount of N_2 source i.e. fishmeal was varied to obtain to desired C/N ratios. The results are indicated in Table 5.5.

Table 5.5: Optimization of C/N ratio on biomass production

C/N ratio	Cell count / ml
40	191.8×10^7
20	326.78×10^7
8	618.05×10^7
4	898×10^7
2	902×10^7

Maximum production of cells was obtained in medium with a C/N ratio of 2 but the biomass yield difference between C/N ratio 2 and 4 was very negligible and to minimize the cost of production C/N ratio at 4 has been selected as the optimum condition.

Finally, from the above experimental result a new, cost effective media was formulated with the composition of 4% molasses, 2% fishmeal, $MgSO_4$ – 0.15% and K_2HPO_4-0.15% with pH 7.5. Furthermore cost effective fermentation media formulation is not end point of biopesticide production. The next step was the development of an appropriate formulation for field application that should be inert, low cost, easy to preparation and application, should provide stability and adequate self life. Fluorescent *Pseudomonas* BRL-1 was grown in the newly formulated media, maintaining all the optimum physical condition for the strain and mixed with different types of inert, low

cost carrier like peat, talc, farmyard manure and vermiculite to check the survivility of the strain in the formulated carrier. Results were presented in the figure 5.7.

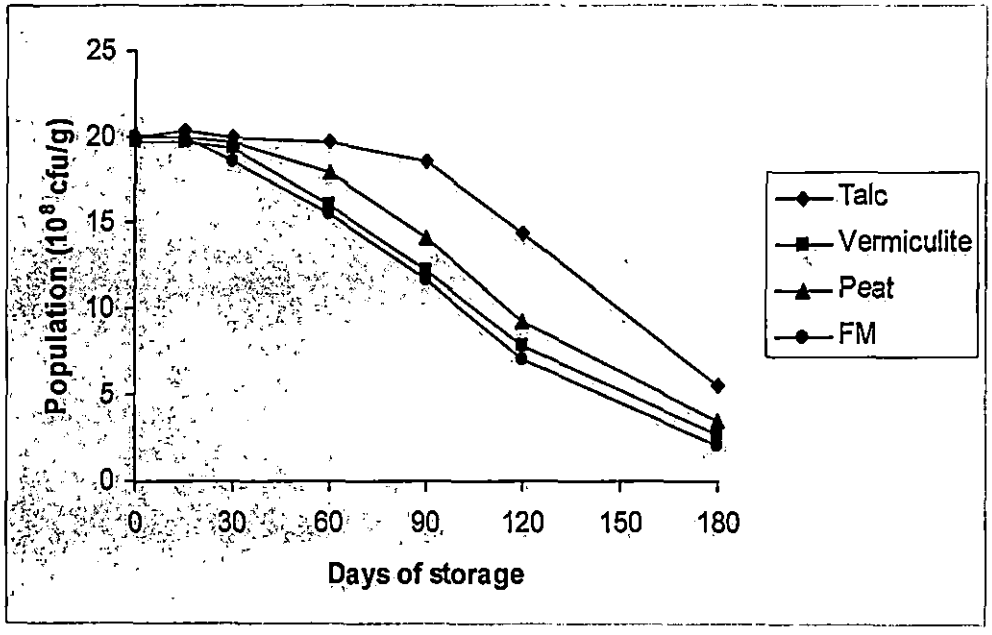


Figure 5.7: **Survivility of fluorescent *Pseudomonas* BRL-1. Results were presented mean of three separate experiments each in triplicate.**

Among the different formulated carrier for fluorescent *Pseudomonas* BRL-1, talc based formulation was the superior one. Upto 90 days the organism survived without any drastic decline from the initial population. After 6 months of storage only 4% reduction in total population was detected in talc based formulation.

5.4.9. In vivo application of inert carrier based formulation of fluorescent Pseudomonas BRL-1

The experimental sets were monitored for disease incidence upto one and half years of growth in a polyhouse at Darjeeling. All the plants of *Set I* where the bulbs were treated with the mixture of all the pathogens showed dry, fibrous, hollow pseudobulbs without any leaf and ultimately causing death of the plants (Figure 5.8 B).

Figure 5.8. In vivo experiments: (A) Blank Set: pseudobulbs were treated with sterile distilled water; (B) Bulbs were dipped in mixture of all three pathogens; (C) Treated Set: Bulbs were dipped in mixture of three pathogens and uniformly coated with talc based formulation of fluorescent Pseudomonas BRL-1; and (D) Control Set: Bulbs were coated with powdered formulation of the antagonist.

From the survivility test it was evident that talc based formulation was superior to other inert carrier so talc based formulation of fluorescent *Pseudomonas* BRL-1 was used for *in vivo* application.

In comparison to *Set I* (when bulb were dipped in the mixture of three pathogens the disease severity was maximum causing death of the plant) in *Set II* where bulbs were dipped in the mixture of three pathogens and uniformly coated with the talc based formulation of the antagonist, the plants were not only protected from the disease significantly but also showed healthy and vigorous growth (Figure 5.8 C). Uprooted plantlets of this set showed normal and healthy condition of the pseudobulbs, roots were well developed and leaves were green in colour.

In *Set III* which was the control set where the bulbs were treated with talc based formulation of fluorescent *Pseudomonas* BRL-1 only, the results showed enhanced growth both in shoot and root system (Figure 5.8 D). Plants of these set and of *Set II* showed more vigourous growth than that of the blank set (*Set IV)* (Figure 5.8 A). It has been seen that plant growth promoting rhizobacteria (PGPR) increase plant growth in various ways one of them is by releasing phytohomones (Arshad and Frankenberger, 1991; Beyeler *et al.*, 1999). The luxuriant growth of the plants of *Set II* and *Set III* could be explained by the fact that production of significant amount of phytohormone (Indole Acetic Acid) by the strain fluorescent *Pseudomonas* BRL-1.

5.5. References

- Aidoo, K.E., Hendry, R. and Wood, B.J.B. 1982. Solid substrate fermentations. *Adv. Appl. Microbiol.* 28: 201-235.
- Arshad, M. and Frankenberger, Jr W.T. 1991. *Microbial production of plant hormones.* In: The rhizosphere and plant growth. pp. 327-334. eds. Keister, D.L. and Cregan, P.B. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Barrios-Gonzales, J., Tomasini, A., Viniegra-Gonzalez, G. and Lopez, L. 1988. *Penicillin production by solid state fermentation.* In: Solid State Fermentation in Bioconversion of Agro-industrial Raw Materials. pp. 39-51. ed. Raimbault, M. ORSTOM, Montpellier Fr.
- Bernhard, K., Holloway, P.J. and Burges, H.D. 1998. *Appendix I: A catalogue of formulation additives: Function, nomenclature, properties and suppliers.* In: Formulation of Microbial Biopesticides: Beneficial Microorganisms, Nematodes and Seed Treatments. pp. 333-365. ed. Burges, H.D. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Beyeler, M., Keel, C., Michaux, P. and Haas, D. 1999. Enhanced production of indole-3-acetic acid by a genetically modified strain of *Pseudomonas fluorescens* CHA0 affects root growth of cucumber, but does not improve protection of the plant against *Pythium* root rot. *FEMS Microbiol. Ecol.* 28: 225–233.
- Bowers, R.C. 1982. *Commercialization of microbial biological control agents.* In: Biological Control of Weeds with Plant Pathogens. pp. 157-173. eds. Charudattan, R. and Walker, H.L. John Wiley and Sons: New York.
- Cannel, E. and Moo-Young, M. 1980. Solid-state fermentation system. *Pro. Biochem.* 9: 24-28.
- Churchill, B.W. 1982. *Mass production of microorganisms for biological control.* In: Biological Control of Weeds with Plant Pathogens. pp. 139-156. eds. Charudattan, R. and Walker, H.L. John Wiley and Sons: New York.
- Cook, R.J. 2000. Advances in plant health management in the 20th century. *Annu. Rev. Phytopathol.* 38: 95-116.
- Cook, R.J. and Baker, K.F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. American Phytopathological Society, St. Paul, MN.
- Ferreira, J.H.S., Matthee, F.N. and Thomas, A.C. 1991. Biological control of *Eutypa lata* in grapevine by an antagonistic strain of *Bacillus subtilis*. *Phytopathology*. 81: 283-287.
- Fravel, D.R., Rhodes, D.J. and Larkin, R.P. 1999. *Production and commercialization of biocontrol products.* In: Integrated Pest and Disease Management in Greenhouse Crops.

pp. 365-376. eds. Albajes, R., Gullino, M.L. van Lenteren, J.C. and Elad, Y. Kluwer Academic Publishers, Dordrecht.

- Hoitink, H.A.J. and Boehm, M.J. 1999. Biocontrol within the context of soil microbial communities: A substrate dependent phenomenon. *Annu. Rev. Phytopathol.* 37: 427-446.
- Jenkins, N.E. and Goettel, M.S. 1997. Methods for mass-production of microbial control agents of grasshoppers and locusts. *Mem. Entomol. Soc. Can.* **171:** 37-48.
- Kanjanamaneesathian, M., Pengnoo, A., Jantharangsri, A., Niratana, L. and Kusonwiriyawong, C. 2000. Scanning electron microscopic examination of a pellet formulation of *Bacillus megaterium* and *B. pumilus*, antagonists of *Rhizoctonia solani*, and survival during storage. *World J. Microbiol. Biotechnol.* 16: 523-527.
- Kerry, B.R. 2000. Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-pathogenic fungi. *Annu. Rev. Phytopathol.* 38: 423-441.
- Latge, J.P. and Soper, R.S. 1977. Media sutiable for industrial production of *Entomophthora virulenta* zygospores. *Biotechnol. Bioenginer.* 19: 1269-1284.
- Lewis, J.A. and Papavizas, G.C. 1984. A new approach to stimulate population proliferation of *Trichoderma* spp. and other potential biocontrol fungi introduced into natural soil. *Phytopathology.* 74: 1240-1244.
- Lisansky, S.G. 1985. *Production and commercialization of pathogens.* In: Biological Pest Control. pp. 210-218. eds. Hussey, N.W. and Scopes N. Blandford. Press: Poole, England.
- Manjula, K., and Podile, A.R. 2001. Chitin-supplemented formulations improve biocontrol and plant growth promoting efficiency of *Bacillus subtilis* AF 1. *Can. J. Microbiol.* 47: 618-625.
- Mukherjee, S.K. and Majumdar, S.K. 1971. Pectinase production by *Aspergillus niger*. *J. Ferment. Technol.* 49: 760-769.
- Osburn, R.M., Milner, J.L., Oplinger, E.S., Smith, R.S., and Handelsman, J. 1995. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. *Plant Dis.* 79: 551-556.
- Papavizas, G.C., Dunn, M.T., Lewis, J.A. and Beagle-Ristaino, J. 1984. Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopathology.* 74: 1171-1175.
- Racke, J. and Sikora, R.A. 1992. Isolation, formulation and antagonistic activity of rhizobacteria toward the potato cyst nematode *Globodera pallida*. *Soil Biol. Biochem.* 24: 521-526.

- Schmidt, C.S., Lorenz, D., Wolf, G.A. and Jager, J. 2001. Biological control of the grapevine dieback fungus *Eutypa lata* II: Influence of formulation additives and transposon mutagenesis on the antagonistic activity of *Bacillus subtilis* and *Erwinia herbicola*. *J. Phytopathol.* 149: 437-445.

- Senez, J.C., Raimbault, M. and Deschamps, F. 1980. Protein enrichment of starchy substrates for animal feeds by solid state fermentation. *World Ani. Rev.* 35: 36-40.

- Sid Ahmed, A., Ezziyyani, M., Perez Sanchez, C. and Candela, M. E. 2003. Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *Eur. J. Plant Pathol.* 109:633-637.

- Smith, K.P., Havey, M.J. and Handelsman, J. 1993. Suppression of cottony leak of cucumber with *Bacillus cereus* strain UW85. *Plant Dis.* 77: 139-142.

- Tabachnik, M. 1988. *Trichoderma*: Production and formulation. *Trichoderma Newsletter.* 4: 7.

- Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029c in solid state fermentation system: Influence of the composition of liquid medium used for impregnating sugar cane pith bagasse. *Proc. Biochem.* 28: 23-27.

- Trejo-Hernandez, M.R., Raimbault, M., Roussos, S. and Lonsane, B.K. 1992. Potential of solid state fermentation for production of ergot alkaloids. *Lett. Appl. Microbiol.* 15: 156-159.

- Van Brunt, J. 1986. Fermentation economics. *Biotechnol.* 4: 395-401.

- Vidyasekaran, P. and Muthamilan, M. 1995. Development of Formulation of *Pseudomonas fluorescens* for Control of Chickpea Wilt. *Plant Dis.*79: 782–786.

- Weller, D.M., Raaijmakers, J.M., McSpadden Gardener, B. and Thomashow, L. S. 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40: 309-348.

CHAPTER VI

Discussion

6. Discussion

Orchid is the most beautiful flower in God's creation. Taxonomically they represent the most highly evolved family among monocotyledons. Orchid exhibit an incredible range of diversity in size, shape and colours of the flower. They are the most pampered of the plants and occupy top position among all the flowering plants valued for cut flower production and as potted plants. Orchid is an important plant for earning foreign exchange and also plays a significant role in agricultural economy. It is an important plant especially in developing economy of the developing country. Many orchid specialists have developed tissue culture laboratories and green houses for the mass propagation of commercially valuable orchids like *Cymbidium, Cattleya, Vanda* hybrids etc. Small growers buy these clonally propagated elite plantlets and grow them at a small scale for the commercial purpose. Cut flowers and the whole plant are sold in domestic and international markets. *Cymbidium,* the only cut flower among the orchid occupies proud position in global cut flower trade. The North-East Indian hills are richest phytogeographycal habitat for this genus because of the prevailing supporting climate.

In Eastern Himalaya especially in the hills of Darjeeling and its adjoining area, *Cymbidium* have great horticultural value and have been cultivated extensively and plays a vital role, because it is an only source of income of many orchid growers. Major part of orchid growers in this region suffered from huge crop loss due to an epidemic pseudobulb rot since 1995 during every monsoon months. Infected pseudobulb initially turns soft and pulpy followed by oozing out of foul odor liquid. With severity the bulbs and roots lose weights as the internal tissue gradually disintegrates. Finally the bulbs become hollow, fibrous and dry causing death of the plant. The recorded incidence ranged from 60 to100% during the survey period between 2002 to 2005. The severity of the disease is generally associated with high humidity, soil moisture level and often related to poor drainage system and irrigation water. Three organisms were consistently isolated from infected samples collected from various localities. They were

identified as *Erwinia carotovora, Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis,* predominant at early, middle and later stages of infection. The presences of the organisms were confirmed histopathologically and conventional pathogenecity test. Pathogenicty tests were performed by dipping method in two ways. In the first case the bulbs were dipped separately into fungal as well as bacterial spore /cell suspension (10^6 spores / cells /ml) for 1 min. In another set the sterilized bulbs were dipped first into *E. carotovora,* than into *F. oxysporum* 12 days later and then into *M. hiemalis* f. *hiemalis* 15 days after the second dip. For control set the pseudobulbs were dipped into sterilized water. The samples were incubated aseptically at 20^0C with the relative humidity of 80% and all inoculated bulbs were evaluated for disease 47 days after the first inoculation. When the pathogenicity tests were performed separately *E. carotovora* exhibit maximum (70%) tissue disintegration followed by *F. oxysporum* (30%) and *M. hiemalis* f. *hiemalis* (10%) but none of the individual pathogen caused 100% tissue disintegration. Complete destruction was observed after 47 days of the first inoculation when these three pathogens were inoculated consecutively according to their serial occurrence. Even it was found that when the pathogenicity test was performed in consecutive dipping method symptoms mimicked the natural symptoms found in the field condition. It is an interesting finding on host-pathogen combination as three pathogens act in sequence towards ultimate demolition of the host. This *Cymbidium* pseudobulb rot is a synergistic activity of three pathogens to cause an uncontrolled epidemic disease of *Cymbidium.*

Several chemical control measures have been practiced by the orchid cultivars to control this epidemic disease but till date no significant achievements have been made. So, it was very essential to isolate a biocontrol agent that effectively controls these three pathogens. Literature surveys suggest that bacterial pathogen is unable to control by fungal antagonist. So the objective was to isolate a putative bacterial antagonist that can show the strong inhibitory effect against all three pathogens. Though a range of different bacterial genera and species have been studied but the genus *Pseudomonas* is the

choice of many workers since pseudomonads are not only the dominant bacterial group in the rhizosphere ecosystem but are functionally more versatile, characteristically fast growing, easy to culture and manipulate genetically in laboratory and are able to utilize a range of easily metabolizable organic compounds making them amenable to experimentation.

Fifty-four fluorescent pseudomonad strains were isolated from rhizosphere of potato and *Cymbidium*. Morphological, physiological, cultural and biochemical parameters were evaluated for characterization of these native isolates. All the rhizospheric fluorescent pseudomonads were preliminary screened for antagonistic activities against both fungal as well as bacterial pathogens. During preliminary screening of potential antagonist it has been found that six isolates showed antagonism against all three pathogens. *In vitro* assay of these six screened antagonists were evaluated against *Cymbidium* rot pathogens by dual plate and dual liquid culture technique. In dual culture, growth inhibitions of the fungal pathogens were variable inhibited by all the six strains which were evident with clear inhibition of colony diameter. Among these six isolates and when comparing their effect of antagonism it was evident that the strain fluorescent *Pseudomonas* BRL-1 was found to be the most potent one. Maximum reduction of mycelial dry weight was furnished by strain BRL-1 which was 68% and 61% in case of *Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis* respectively. Growth inhibition of *E. carotovora* was seen after 48 hours of incubation. All the six isolates showed positive result to check the growth the pathogen, but it was more prominent in case of fluorescent *Pseudomonas* BRL-1 which showed no growth in the interacting zone and abundant growth away from the interaction.

Microscopic observation of the mycelium from the interacting zone showed hyphal shriveling, mycelial deformities, swelling, fragmentation, short branching and granulation of cytoplasm ultimately resulting into lysis. Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the

antagonist. Further study was made to screen for the production of different secondary metabolites including siderophore, hydrogencyanide, non-volatile and volatile compound, hydrolytic enzymes, IAA investigated for their effect on fungal as well as bacterial pathogens. It was well understood that the fluorescent *Pseudomonas* BRL-1 isolate was showing significant antagonistic property through combined and / or individual effect of siderophore, production of proteolytic enzyme, IAA and chitinolytic activity.

To introduce a potential antagonist form laboratory to the trial field and its survival, proliferation, growth activity and establishment to a new environment needs proper formulation after cost effective biomass production, if it is considered from the commercial view point. Fluorescent *Pseudomonas* BRL-1 has been shown to act as an effective biocontrol agent as well as having the ability to produce significant amount of IAA. Considering these phenomenons, it was aimed to develop a cost effective media, its powder formulation, survivility of the organism in the inert carrier and finally *in vivo* application of inert carrier based formulated organism. After standardization of various growth factors, cost effective carbon and nitrogen sources were investigated. Different carbon sources gave various degree of biomass yield in the fermentation broth. However, considering the cost effectiveness and the biomass production, molasses has been chosen as best carbon sources for commercial production of the strain. Different nitrogen sources, including both inorganic and organic compounds were examined. It has been found that organic N_2 sources like peptone, and fishmeal were superior to inorganic N_2 sources and other organic nitrogen sources in giving higher cellular yield. Of the various organic N_2 sources tested, fishmeal gave maximum yield and from the commercial production point of view fishmeal was the best option so it has been selected as the cheapest nitrogen source. Finally, from the experimental result a new, cost effective media was formulated with the composition of 4% molasses, 2% fishmeal, $MgSO_4$ – 0.15% and K_2HPO_4- 0.15% with pH 7.5. The very next step was the development of low cost inert carrier which should be easy to preparation and

application as well as provide stability and adequate self life. Among the different formulated carrier for fluorescent *Pseudomonas* BRL-1, talc based formulation was the superior one. Upto 90 days, the organism survived without any drastic decline from the initial population. After 6 months of storage only 4% reduction in total population was detected in talc based formulation. This inert carrier based formulation was used for *in vivo* application in a polyhouse at Darjeeling. The *Sets* of different treatments were monitored for disease incidence after 1½ years growth. It was very evident that application of the powdered formulation of the antagonist not only protects the plant from the disease but also showed healthy and vigorous growth of the plant. If this result is applied to the grower's field then it will help the horticulturalists to protect this economically important plant and may ultimately boost up the economy of this area.

Publications

Lists of Publications

1. K. Acharya, M. Rai, N. P. Rai and **S. Sen,** Three new species of *Russula*: Addition to the macrofungi of Sikkim Himalaya. ***Indian Forester,*** 130: 953 – 955, 2004.
2. R. Acharya, M. Mukhia, **S. Sen,** and K. Acharya, Nitric oxide: A common antipathogenic factor of plants. ***Indian Journal of Experimental Biology,*** 43: 100 –103, 2005.
3. **S. Sen,** A. Saha and K. Acharya, *In vitro* inhibition of *Fusarium oxysporum* by florescent *Pseudomonas* BRL-1. ***Journal of Botanical Society of Bengal,*** 59: 31-36, 2005.
4. **S. Sen,** R. Acharya and K. Acharya, *In vitro* antagonistic effect of Fluorescent *Pseudomonas* BRL-1 against *Aspergillus niger*. ***Journal of Mycopathological Research,*** 44: 97-100. 2006.
5. **S. Sen,** A. Saha and K. Acharya, Management of an epidemic disease of *Cymbidium* rot by fluorescent *Pseudomonas*. ***Journal of Mycopathological Research,*** 44: pp. 160, 2006 (Abstract).
6. **S. Sen,** R. Pal, A. Saha and K. Acharya, Optimization of different physiological factors on growth for the development of a cost effective media for large scale production of an antagonistic PGPR fluorescent Pseudomonas BRL-1. ***Journal of Mycopathological Research,*** 44: pp. 184, 2006 (Abstract).
7. **S. Sen,** M. Bhattacharyya, S. Tahsin Khandakar, S. Giri and K. Acharya, Evaluation of biocontrol potentiality and plant growth promoting capability of a fluorescent Pseudomonas BRL-1 against several phytopathogen. ***Journal of Mycopathological Research,*** 44: p. 184-185, 2006 (Abstract).
8. C. K. Maiti, **S. Sen,** A. Karan, S. Ghosh and K. Acharya, Isolation, characterization and screening of fluorescent Pseudomonads population of medicinal plant's rhizosphere against alternaria alternata. ***Journal of Mycopathological Research,*** 44: pp. 185, 2006 (Abstract).
9. **S. Sen,** T. Bose, K. Basu, R. Acharya, N. Samajpati and K. Acharya, *In vitro* antagonistic effect of Fluorescent *Pseudomonas* BRL-1 against *Sclerotium rolfsii*. ***Indian Phytopathology,*** 59: 227-230, 2006.
10. **S. Sen,** A. Saha and K. Acharya, *In vitro* inhibition of *Mucor hiemalis* f. *hiemalis* by florescent *Pseudomonas* BRL-1, ***Ecology and Environment, (In press).***
11. **S. Sen,** R. Acharya, A. Saha, and K. Acharya, A new report of *Cymbidium* pseudobulb rot orchestrated by *Erwinia carotovora, Fusarium oxysporum* and *Mucor hiemalis* f. *hiemalis*, ***Plant Disease (In press).***

12. **S. Sen,** S. Ghosh, R. Acharya, A. Saha and K. Acharya, Isolation, characterization and screening of fluorescent pseudomonads against the pathogens of *Cymbidium* pseudobulbs rot, ***Ecology and Environment.*** **(Communicated).**

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(III)

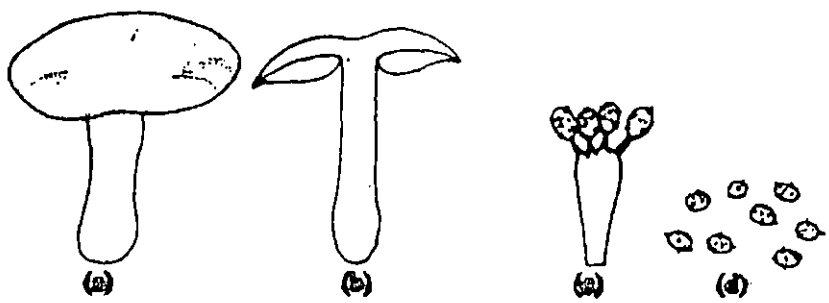
THREE NEW SPECIES OF *RUSSULA* : ADDITION TO THE MACROFUNGI OF SIKKIM HIMALAYA

The Sikkim Himalayas are spread over an area of 7,096 km^2. The general physiography of the region is hilly and the sloping terrain is the major feature over which most habitations, agricultural activity and forest cover may be found. The vertical range is from 1,000 m amsl (foothills), through 4,000 m amsl (timber line) upto 8,500 m amsl (the Kanchanjunga peak) (Rai and Sharma, 1994). The Himalayan ecosystem is known for its richness in biodiversity (Chakraborty, 1997). After Berkeley (1956) very few work have been undertaken regarding the biodiversity of macrofungi of this area (Sarbhoy *et al.*, 1975; Bilgrami *et al.*, 1979, 1991). Three new species of *Russula* have been reported for the first time from this area.

The study materials were collected during field trips in the year 2000 and 2002. The morphological and ecological features were noted and colour photographs were taken. The chemical colour reaction were noted on the fresh collection. After the specimens were brought to the laboratory, their microscopic properties were determined by using an Olympus research microscope. All the microscopic structure were drawn with the help of camera lucida. Then, they are identified according to Ramsbottom (1965), Bessey (1978) and Singer (1986). The voucher specimen has been deposited in Mycological Herbarium of Darjeeling Government College.

Russula ochroleuca Pers. ex. Fr. : Pileus 10 cm, yellow but variously tinged pale lemon yellow colour with orange or brownish at center, convex-umbilicate then flat, slightly depressed, margin thick, curved, sometimes lobed (Fig. 1). Pileus context thick, soft, odour indistinct. Lamellae upto 2 cm broad, thin, adnate,

Fig. 1



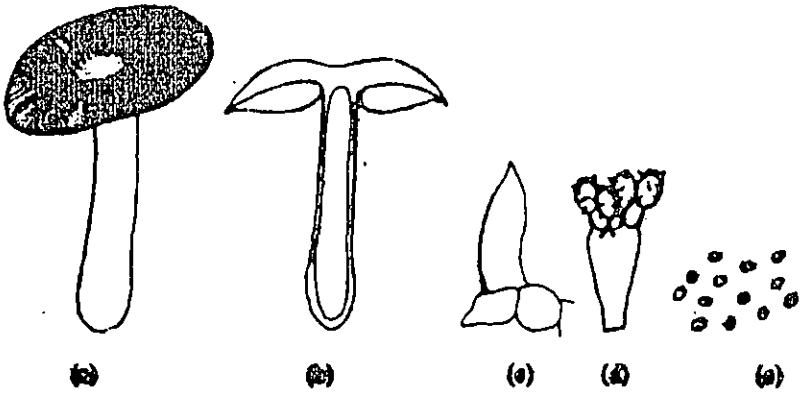
Russula ochroleuca
(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Basidia, (d) Basidiospores.

lamellae of only one length. Stipe 3-7x1.5-2.5 cm, long, cylindrical, central, fistular, white, surface smooth, moist. Annular veil and volva absent. Spore print white. Spore white, ovoid, apiculate 7-9x6.3-9 µ, amyloid. Basidia 20-30x6-8 µ, clavate, thin walled bearing 4 sterigmata along with four basidioles. Subhymenium distinct, psedoparenchymatous, nearly fifty per cent cells are sphaerocysts. Hymenophoral trama regular. Cystidia absent. Pileus cuticle and context made up of septate, branched, interwoven hyphae, clamp connection absent. Collected from the humus forest floor of Darjeeling. The voucher specimen no. DGC/MP/MF-085.

***Russula sororia* (Fr.) Romell.** : Pileus 5.4 cm, dark brown or cigar brown at center, yellowish brown at edge, sometimes with olive markings, subglobose to convex, depressed (Fig. 2). Pileus context thin, soft, fruity smell. Lamellae 2.7 cm long, finely adnexed, lamellae of one length. Stipe fistular, central, 5x1.5 cm, cylindrical, white, surface smooth. Annular veil and volva absent. Spore print creamy white. Spores 6.5-8.5x5.7 µ, amyloid (positive test with Melger regent), pale cream-colored, ovoid with conical obtuse or truncated warts. Basidia with four sterigmata with four basidioles. Subhymenium distinct with fifty percent of cells spherocysts. Hymenophoral trama is intermixed with cystidia. Collected from the forest floor of broadleaf trees in Lebong area, Darjeeling in mid-June. The voucher specimen no. is DGC/MP/MF-098.

***Russula xerampelina* Fr.**: Pileus 10 cm, purple to crimson pale lilac red with central blackish often brownish wine colored, convex then flat, slightly viscous when damp but shiny in nature, velvety, margin thin (Fig. 3). Pileus context thin soft; lamellae upto 2 cm broad, thin, adnate, lamellae of one length only. Stipe cylindrical, central, smooth, fistular 7x2.2 cm whitish tinged with glowing red-pink. Annular veil and volva absent. Spore print white, spore ovoid with obtuse on conical warts, 8-9x5-8 µ, amyloid. Basidia thin walled with four sterigmata along four

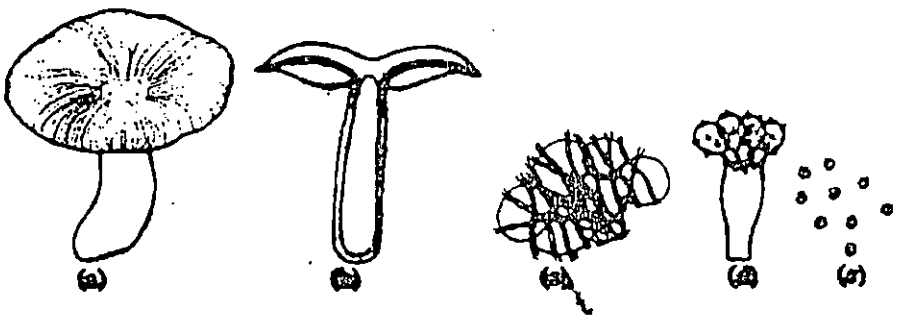
Fig. 2



Russula sororia

(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Cystidia, (d) Basidia, (e) Basidiospores

Fig. 3



Russula xerampelina
(a) Basidiocarp, (b) Basidiocarp with longitudinal section, (c) Trammel cells, (d) Basidia, (e) Basidiospores.

basidioles. Subhymenium distinct with nearly fifty per cent of sphaerocysts cell. Hymenophoral trama regular, cystidia absent, grayish green reaction with ferrous sulphate. Pileus cuticale and context made up of thin walled cells. Spore amyloid (positive test with Melger regent). Collected from the Lebong forest area on June. The voucher specimens no. DGC/ MP/MF - 080.

References

Berkeley, M.J. (1956). Decades of Fungi, Decas 1-62, Nos. 1-620. *Hooker's London, J. Bot.* 3-8: 1844-1856.

Bessey, E.A. (1978). *Morphology and Taxonomy of Fungi*. Vikas Publising House Pvt. Ltd., New Delhi.

Bilgrami, K.S., S. Jamaluddin and M.A. Rizwi (1979). *Fungi of India (Part I) :- List and References*. Today and Tomorrow's Printers and Publishers, New Delhi.

Bilgrami, K.S., S. Jamaluddin and M.A. Rizwi (1991). *Fungi of India*. Today and Tomorrow's Printers and Publishers, New Delhi.

Chakraborty, T. (1997). *Himalayan Paryavaran*, 5: 67-69.

Sarbhoy, A.K., Girdharilal and J.L. Varshney (1975). *Fungi of India*. Navajug Traders, New Delhi.

Singer, R. (1986). *The Agaricales in Modern Taxonomy*. Bishen Singh Mahendra Pal Singh, Dehra Dun. VIII, 981 pp.

Ramsbottom, J. (1965). *A Handbook of the Larger British Fungi*. Alden & Mowbray Ltd. Great Britain.

Rai, L.K. and E. Sharma (1994). *Medicinal Plants of the Sikkim Himalaya : Status, Uses and Potential*. Bishen Singh Mahendra Pal Singh, Dehra Dun. pp. 15-17.

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Nitric oxide: A common antipathogenic factor of plants

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In the present study, nitric oxide synthase/nitric oxide (NOS/NO) status was tested in the host plants infected with fungi, bacteria and virus. In each case cytosolic nitric oxide synthase (Cyt-NOS) of diseased plants was inhibited and inhibition was competitive in nature in respect to l-arginine, the substrate for the enzymic activity. Elevation of host nitric oxide (NO) level before infection using nitric oxide (NO) donor protected disease initiation significantly. The nature of enzyme kinetics and the manner of disease protection by nitric oxide donor (NO-donor) was similar in all the three cases of infection. It was concluded that nitric oxide was a common antipathogenic factor of plants.

Keywords: *Nitric oxide/nitric oxide synthase, Plant defense*

Disease resistance in plants is usually associated with different kinds of passive and active responses. Current concept of active defense mechanism includes programmed cell death (PCD)[1], hypersensitive reaction (HR)[2,3] production of different diffusible signaling molecules like salicylic acid (SA), jasmonic acid (JA), ethylene etc[4] and ultimately the establishment of systemic acquired resistance (SAR)[3,5]. Recently a new concept ie, the concept of nitric oxide (NO) has been introduced in this field[6,7]. It is a potent signaling molecule[8]. NO is well studied in animal system and was first identified as endothelial derived relaxing factor (EDRF)[9]. It is a versatile and powerful effector of animal redox regulated signaling system and immune responses[10] and it also functions as a second messenger of insulin[11]. Nitric oxide synthase (NOS) is the key enzyme for the production of NO from L-arginine in a system[10,11]. In contrast to extensive studies throughout the last two decades or more on role of NO in the mammalian immunity, recently its involvement in the plant defense system has been addressed[6,7]. Some basic analogies at the molecular level regarding the signal transduction of NO in animals to that of plants focus interest to study the involvement of this molecule in plant defense mechanism[12]. It has been reported that NO is produced by plants[6,7]. It mediates leaf expansion[13], root growth[14], phytoalexin production[15] and defense responses against pathogens[12]. In our earlier papers, we have reported the status of NOS in different healthy and diseased plants[16], impact of disease initiation on host NOS[17] and involvement of NOS in host pathogen interaction[18]. In this report we have demonstrated NO as universal effector molecule of plant defense responses. By inhibiting the host NOS activity and thereby restraining NO production, a pathogen makes its way more effective to establish a disease. Furthermore, this finding has been supported by opposite experiments where elevated level of host NO provide significant disease protection after challenging with efficient pathogen (s).

Chemicals – N^G methyl-l-arginine acetate ester (NAME) was purchased from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade.

Collection of healthy and diseased plants – Three different host plants of *Brassica campestris* L. var. sarson Prain, *Citrus aurantifolia* Swingle and *Ammomum subulatum* Roxburg were collected from field in their healthy as well as pathologically disordered condition caused by *Alternaria brassicae* (Bark.) Sacc., *Xanthomonas citri* Hasse. and chirke (mosaic streak) virus, a fungal, bacterial and viral pathogen, respectively.

Assay of NOS activity of intact tissue and homogenates – The reaction mixture containing intact leaf tissue (100 mg) or tissue homogenate (200 – 300 μg of protein) or cytosolic fraction (150 – 200 μg of protein) or soluble particulate fraction (250 – 400 μg of protein) of both healthy and diseased sample was incubated with 10 μM of l-arginine, 64 mM of haemoglobin, in a total volume of 2.5 ml Tris – HCl buffer (pH 7.4) for different periods at 25° ± 1°C. At different time periods, portions of reaction mixture was centrifuged at 10,000 × g for 5 min and NO content of the supernatant was determined and compared with an appropriate control set.

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Determination of NO – Nitric oxide was determined according to Jia *et al.*[19] using scanning Beckman spectrophotometer (Model DU6). Typically, NO content was determined by adding 64 m*M* of oxyhemoglobin to the isolated supernatant and the formation of NO was quantified by determining the conversion of oxyhemoglobin to methemoglobin[19].

Preparation of tissue homogenate – Leaf tissue (100 mg) of diseased and healthy plants was taken separately in a morter with 1 ml of chilled 50 m*M* ofTris-HCl buffer (*p*H 7.4) and homogenized by pestle. The homogenate was centrifuged at 10,000 × *g* for 30 min at 4°C and then the supernatant was further centrifuged at 20,000 × *g* for 30 min at 4°C for the separation of cytosolic and particulate fractions. Both the cytosolic and particulate fractions were collected separately and stored at 0°C. Before further use, particulate fraction was resuspended in equal volume of the same buffer. All the experiments were done at 4°C. Protein was estimated using Folin-ciocalteu reagent, according to the method of Lowry *et al*[20].

Determination of enzyme kinetics – K_m and V_{max} values of the cytosolic NOS were determined by Lineweaver-Burk Plot.

Statistical analyses – Results presented as mean ± SD (Standard deviation) of at least six individual experiments. Data were analyzed by Student's *t* test and values of $P< 0.01$ were considered significant.

In each cases of pathological conditions irrespective of fungi, bacteria and virus attack, the host NOS activity of the intact tissue declined, as determined by NO production from 0.594 ± 0.027 to 0.333 ± 0.012; 0.462 ± 0.024 to 0.379 ± 0.035 and 0.810 ± 0.051 to 0.525 ± 0.033 nmole/100 g of tissue/hr, respectively when compared with healthy subjects. To determine whether the broken cell preparation could possess NOS activity, tissue homogenate and broken cells was prepared from diseased plants as described earlier and observed enzyme inhibition as found in the intact or whole tissue of disease plants. Addition of 10 μM of NAME to the reaction mixture in all cases completely inhibited NO production. Furthermore, both the particulate and soluble cytosolic fractions of healthy tissue homogenate prepared by ultracentrifugation contained detectable amount of NOS activity. But in case of diseased host only the cytosolic NOS activity was inhibited. The basal NOS activity of the cytosolic fractions of healthy plants were 1.107 ± 0.063; 1.95 ± 0.02; 2.08 ± 0.033 nmole NO produced/mg protein/hr (n=3), respectively, decreased to 0.888 ± 0.022; 1.70 ± 0.04 and 1.69 ± 0.049 nmole NO produced/mg protein/hr, respectively in case of diseased plants. In contrast, NOS activity of the particulate fraction of the host tissue was 0.490 ± 0.016; 0.908 ± 0.029 and 0.751 ± 0.087 nmole NO produced/mg protein/hr respectively at their healthy condition was likely to be changed to 0.475 ± 0.027; 0.900 ± 0.046 and 0.760 ± 0.021 nmole NO produced/mg protein/hr, respectively at their diseased condition.

Lineweaver-Bark plot of the cytosolic NOS activity of the diseased and healthy plants demonstrated that inhibition of NOS activity during diseased phase was related to the increase of K_m of l-arginine from 0.480, 0.344, 0.442 μM (n=6) to 1.0, 0.485, 0.757 μM, respectively when compared to diseased phase with simultaneous decrease of V_{max} from 1.05, 1.98 and 2.17 nmole NO produced/mg protein/hr to 0.869, 1.84 and 1.96 nmole NO produced/mg protein/hr (Table 1). Addition of 10 μM N^G nitro-l-arginine-methyl ester

Table 1 — Effect of diseased condition of the plants on NOS activity of cytosolic fraction. Cytosolic fraction of healthy and diseased plant tissue homogenate (n = 6) was incubated with different concentration of l-arginine for 1 hr. The NOS activity of the cytosolic fractions were analyzed by Lineweaver-Burk plot as described in materials and methods

Host name	Cytosolic fraction of	K_m (μM)	V_{max} (nmole NO produced/mg protein/hr)
Brassica campestris	A	0.480 ± 0.037	1.05 ± 0.05
	B	1.00 ± 0.06*	0.869 ± 0.056*
Citrus aurantifolia	A	0.344 ± 0.042	1.98 ± 0.017
	B	0.485 ± 0.036*	1.84 ± 0.016*
Ammomum subulatum	A	0.442 ± 0.047	2.17 ± 0.039
	B	0.656 ± 0.023*	2.04 ± 0.018*

A – Healthy plant; and B – Diseased plant.
*Significant at $P<0.01$

(NAME) to reaction mixture containing various concentrations of l-arginine (added for the demonstration of K_m and V_{max}) completely inhibited NO production. Durner *et al*[12] have reported that NO induces PR-1 gene expression through salicylic acid. Our study complements their findings by demonstrating the present work that pathogen might somehow be blocking the NOS activity mediating a competitive inhibition and thus, inhibiting the defense gene expression to make the plant more vulnerable for susceptibility. Interestingly, the NOS activity was found to be present in both particulate and cytosolic fraction, but results suggested that only cytosolic NOS participates in this mechanism. Further work on pathogen blocking the NOS activity is going on.

Since NOS activity was inhibited in a similar manner in each cases of infection, we tested the possibility of elevated level of NO to protect plants from disease initiation. Sodium nitroprusside (SNP) is well known for its NO generating character. Other workers[2] have also demonstrated the production of NO (~2 μM) from SNP (0.5 mM). To determine whether SNP could be substantiated for NO production of plants against infection, we experimented the effect of SNP on the progression of pathogenic activity. Administration of SNP (0.1 mg/ml) by foliar spray before 24 hr of infection protected fungal diseases up to 72% and bacterial diseases up to 65% as resulted from cell count of micro-HR detected by Evans Blue staining[21]. Since SNP did not generate NO spontaneously in aqueous medium[22], its effect was quite less. However, there are many other NO donors available which are known to generate NO spontaneously in aqueous medium[21] and it is possible that some of these NO donors may be useful for protection of the disease. Mechanism of this NO protection might be related to self amplifying character of NO as described by Klessig[4]. Therefore, NO donor can possibly be used as an antipathogenic agent and feasibility of using NO donors as a routine adjunct in the crop field can be considered. Thus, a new concept of internal scenario of diseased plants has been exposed in this paper denoting: (i) insufficient production of NO through suppression of cytosolic NOS activity: (ii) exhibition of a competitive inhibition of a cytosolic NOS; and (iii) partial protection from disease by elevating host NO level using NO donor which furnish strength to conclude that NO may play a direct protective role as a general antipathogenic molecule to resist disease occurrence in plants. Further work is in progress to evaluate the potentiality of other NO donors on disease protection in the field.

References

1 Greenberg J T, Guo A, Klessig D F & Ausubel F M, Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense function, *Cell*, 77 (1994) 551.

2 Delledonne M, Xia Y, Dixon R A & Lamb C, Nitric oxide functions as a signal in plant defense resistance, *Nature*, 394 (1998) 585.

3 Hausladen A & Stamler J S, Nitric oxide in plant immunity, *Proc Natl Acad Sci USA*, 95 (1998) 10345.

4 Klessig D F, Durner J, Noad R, Navarre D A, Wendehenne D, Kumar D, Zhou J M, Shah J, Zhang S, Kachroo P, Trifa Y, Pontier D, Lam E & Silva H, Nitric oxide and salicylic acid in plant defense, *Proc Natl Acad Sci USA*, 97 (2000) 8849.

5 Shirasu K, Dixon R A & Lamb C, Signal transduction in plant immunity, *Curr Opinion Immunol*, 8 (1996) 3.

6 Ninnemann H & Maier J, Indications for the occurrence of nitric oxide synthase in fungi and plants and the involvement in photoconidiation of *Neurospora crassa*, *Phytochem Phytobiol*, 64 (1996) 393.

7 Wildt J, Kley D, Rockel A. Rockel P & Segschneider H, Emission of NO from higher plant species, *J Geophys Res*, 107 (1997) 5919.

8 Ignaroo L J, Nitric oxide: A novel signal transduction mechanism for transcelluler communication, *Hypertension*, 16 (1990) 477.

9 Ignaroo L J, Buga M, Wood K S, Byrns R E & Chaudhuri G, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide, *Proc Natl Acad Sci USA*, 84 (1987) 9265.

10 Klessig D F & Durner J, Nitric oxide as a signal in plants, *Curr Opinion Plant Biol*, 2 (1999) 369.

11 Kahn N N, Acharya K, Bhattacharya S, Acharya R, Mazumder S, Bawmann A W & Sinha A K, Nitric oxide: The "Second messenger" of insulin. *IUBMB Life*, 49 (2000) 441.

12 Durner J, Wendehenne D & Klessig D F, Defense gene induction in tobacco by nitric oxide, cGMP and cADP ribose, *Proc Natl Acad Sci USA*, 95 (1998) 10328.

13 Klepper L, NOx evolution by soybean leave treated with salicylic acid and selected derivatives, *Pest Biochem Physiol*, 39 (1991) 43.

14 Cueto M, Hernandez-Perera O, Martin R, Benture M L, Rodrigo J, Lamas S & Galvano M P, Presence of nitric oxide synthase in roots and nodules of *Lupinus albus*, *FEBS Lett*, 398 (1996) 159.

15 Noritake T, Kawakita K & Doke N, Nitric oxide induces phytoalexin accumulation in potato tuber tissue, *Plant Cell Physiol*, 37 (1996) 113.

16 Acharya K, Pokhrel B, Sherpa A R, Katel O N & Acharya R, Inhibition of nitric oxide synthesis an important step in plant pathogenesis, *Bull Pure Appl Sci*, 20 (2001) 7.

17 Acharya K & Acharya R, Nitric oxide: The signal molecule of plant defense blocked during pathogenesis, *Indian J Pure Appl Biol*, 17 (2002) 128.

18 Acharya K & Acharya R, Involvement of nitric oxide synthase in *Solanum tuberosum – Phytopthora infestans* interaction, *J Mycopathol Res*, 40 (2002) 29.

19 Jia L, Bonaventua C, Bonaventura J & Stamler S J, S-nitrosohemoglobin: A dynamic activity of blood involved in vascular control. *Nature*, 380 (1996) 221.

20 Lowry O H, Rosebrough N J, Farr A L & Randall R J, Protein measurement with folin-phenol reagent, *J Biol Chem*, 193 (1951) 265.
21 Dietrich R A, Delaney T P, Uknes S J, Ward E R, Ryals J A & Dangl J, *Arabidopsis* mutants stimulating disease resistance response, *Cell*, 77 (1994) 565.
22 Hogg N, Struck A, Goss S P A, Santanam N, Joseph J, Parthasarathy S & Kalyanaraman B, Inhibition macrophase dependent low density lipoprotein oxidation by nitric oxide donors, *J Lipid Res*, 36 (1995) 1756.

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In vitro inhibition of *Fusarium oxysporum* by fluorescent *Pseudomonas* BRL-1

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Fluorescent *Pseudomonas* BRL-1, a rhizosphere isolate, showed *in vitro* antagonistic activity against *Fusarium oxysporum*. Microscopic examination after antagonism showed hyphal shriveling, swelling, vaculation, short branching and granulation of cytoplasm resulting in lysis of hyphae of *F. oxysporum*. Correlation of antifungal activity of this isolate has been found to be linked with the production of siderophore, proteases, chitinases and IAA.

Key words: Fluorescent *Pseudomonas*, *Fusarium oxysporum*.

Introduction

Fusarium oxysporum Schlecht. is an economically important soilborn plant pathogen which mediate many diseases like pod rot, wilt, root rot of *Archis hypogaea* (Mustafee, 1971), fruit rot of *Citrullus vulgaris* (Mathur and Mathur, 1958), wilt of *Dolichos biflorus* (Chakravarti and Kumar, 1972), wilt of *Helianthus annuus* (Ghodajkal *et al.*, 1976), wilt of *Musa paradisica* (Malik and Mehta, 1963), wilt of *Piper bitle* (Dastur, 1935), dry rot of rhizome of *Zingiber officinali* (Gupta and Sohi, 1967) etc. causes persistent and significant losses in crop yields. In recent years, fluorescent Pseudomonads have drawn attention world wide because of production of secondary metabolites such as siderophore (Neilands, *et al.*, 1991), antibiotics (Keel *et al.*, 1992; O'Sullivan 1981) volatile compound, HCN (Wei et al., 1991) enzyme and phytohormones (Keel *et al.*, 1992; O'Sullivan *et al.*, 1991). These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneously induced plant growth. Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, because chemical control is expensive and hazardous to soil ecosystem.

Here attempt has been made to evaluate *in vitro* antagonistic activity of an isolate of florescent *Pseudomonas* BRL-1 against this lethal phytopathogenic fungi *Fusarium oxysporum*.

Materials and Methods

Isolation of fluorescent pseudomonads

A strain of fluorescent *Pseudomonas* BRL-1 was isolated from the rhizosphere of potato and was characterized according to Bergy's Manual of Determinative Bacteriology (Holt, *et al.*, 1994). It was maintained on Tryptic Soy Agar (TSA) medium.

Interaction of fluorescent Pseudomonas BRL-1 against F. oxysporum in dual plate culture

The antagonistic effect of fluorescent *Pseudomonas* BRL-1 were tested against *F. oxysporum* supplied

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from the laboratory of Molecular and Applied Mycology and Plant Pathology, University of Calcutta on Peptone Glucose Agar (PGA) plates using dual culture technique (Randhawa, *et al.*,2002). This was done by streaking the fluorescent *Pseudomonas* BRL-1 in a circle / O shaped and semicircle / U shaped pattern, where as spore suspension of *F. oxysporum* was point inoculated in the centre on the plates. The fungal pathogen inoculated centrally on PGA plate, but uninoculated by fluorescent *Pseudomonas* BRL-1 served as control. The inoculated plates were incubated at 30°C for 6 days and inhibition of colony growth was measured.

Interaction of fluorescent Pseudomonas BRL-1 against F. oxysporum in dual liquid culture

To test the antifungal activity of the fluorescent *Pseudomonas* BRL-1, dual liquid culture method was employed (Basha and Ulagnathan, 2002). One ml of 24 hours grown fluorescent *Pseudomonas* BRL-1 culture (containing 10^7 cfu / ml) and an agar block (5 mm diameter) of 5 days old culture of *F. oxysporum* was inoculated onto 50 ml of Peptone Glucose Broth medium in 250 ml of conical flask. The culture was incubated on a rotary shaker at 30°C for 72 hours. For the control experiments the fungal pathogen was grown alone. Experiments were performed thrice taking triplicate for every set. A drop of fungal culture from each flask was taken out and placed on a clean glass slide and stained with lactophenol and cotton blue and observed under Leica DMLS Research Microscope.

The differences in dry weights (72 hours old culture was dried for 24 hours at 70°C) between the fungal cultures grown with fluorescent *Pseudomonas* BRL-1 strain or the control culture grown without any bacterium were recorded (Basha and Ulagnathan, 2002).

Inhibitory effect of fluorescent Pseudomonas BRL-1

To investigate the inhibitory effect on fluorescent *Pseudomonas* BRL-1 for the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971). An agar block (5 mm diameter) of 4 days old culture of *F. oxysporum* placed on the centre of the Petri plate containing 20 ml of PGA. A loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture was strike on Petri plate having 20 ml of PGA then inverted over the plates inoculated with the fungal pathogen *F. oxysporum*. Two plates were sealed together (mouth to mouth) with parafilm, control plate consist of *F. oxysporum* inverted over uninoculated PGA plate. The plates were incubated at 30°C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the test pathogen was measured and compared with the control.

Production of hydrogen cyanide was tested qualitatively according the method of Wei *et al* (1991). The fluorescent *Pseudomonas* BRL-1 was inoculated in TSA medium supplemented with amino acid glycin (4.4 g l^{-1} of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30°C for 4 days. A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded.

To test the production of IAA, active culture of fluorescent *Pseudomonas* BRL-1 was inoculated in 10 ml of minimal salt media supplemented with 100 µg / ml of tryptophan, and incubated at 30°C under shaking for 48 hours. Culture broth was centrifuged at 7500 rpm for 10 minuets. To 1 ml of culture supernatant, 2 ml of Salkovsky reagent was added and contents incubated at 30°C for 25 minutes. Absorption was read at 530 nm and levels quantified from standard curve of IAA.

To test the chitololytic property of fluorescent *Pseudomonas* BRL-1 it was inoculated on LB medium (Gunasekaaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicates

chitinase activity of the strain (Frandberg and Schnurer, 1994).

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (Schwyn and Neilands, 1987) to detect the siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24 hours old fluorescent fluorescent *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72 hours. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxymethyl cellulose (CMC) for protease, amylase, pectinase and cellulase respectively. Plates were incubated for 48 hours at 30°C and formation of clear zone around bacterial colonies was read as positive (Gaur, *et al.*, 2004).

Results and Discussion

In dual culture, significant growth inhibition of *F. oxysporum* occurred due to antagonistic effect of fluorescent *Pseudomonas* BRL-1, which was evident with clear inhibition of colony diameter. The results are presented in Table1 and depicted in figure 1. At 48 hours of incubation, *F. oxysporum* was strongly inhibited by fluorescent *Pseudomonas* BRL-1 and simultaneous increase in incubation time corresponded escalation in percent growth inhibition. After 96h of incubation the mycelia growing toward the interaction zone, which was prominently evident in semicircular/ U-shaped streak stopped, and the mycelia gradually lost vigor. Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching and finally resulting into lysis (figure 2). The difference in dry weights between the fungal culture grown with and without fluorescent *Pseudomonas* BRL-1 strain was recorded. There was more than 68% reduction in dry weight of the culture grown with fluorescent *Pseudomonas* BRL-1 strain when compared to the control.

Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the bacterium (Arora *et al.*, 2001). Further study was made to screened for the production of different secondary metabolites including siderophore, hydrogencyanide, antimicrobial compound, volatiles, hydrolytic enzymes, IAA investigated for their effect on *F. oxysporum in vitro* (Table 2).

The results (Table 2) showed fluorescent *Pseudomonas* BRL-1 produced mainly siderophore, protease,

Table 1. *In vitro* antagonistic activity of fluorescent *Pseudomonas* BRL-1, cultured as semi-circular and circular streaks, on *Fusarium oxysporum* point-inoculated in the center PGA plates incubated at 30°C.

Colony diameter (in cm) of F. oxysporum after incubation period of						
	0 h	24 h	48 h	72 h	96 h	120 h
Control	0.00	0.80 ± 0.10	2.28 ± 0.02	3.75 ± 0.07	5.00 ± 0.21	7.47 ± 0.53
Semi-circular	0.00	0.27 ± 0.10	0.74 ± 0.15	1.09 ± 0.21	1.41 ± 0.12	1.51 ± 0.02
Circular	0.00	0.00	0.52 ± 0.03	0.78 ± 0.12	1.01 ± 0.01	1.02 ± 0.02

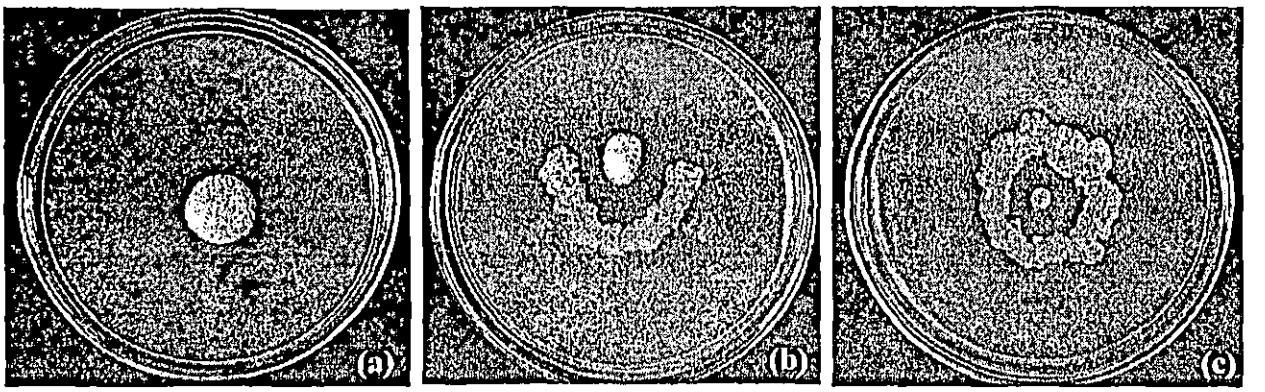


Fig.1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *F. oxysporum.* (a) Control; *F. oxysporum* colony in pure culture showing more rapid growth at 30°C; (b) Colony of *F. oxysporum* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist fluorescent *Pseudomonas* BRL-1 and (c) Colony of *F. oxysporum* showing restricted growth due to fluorescent *Pseudomonas* BRL-1 inoculated as a circular streak .

Table 2. Showing different secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Antibiotic Substances	-
HCN production	-
Volatile substances production	-
Chitinase activity	+
Protease activity	++
IAA production	+++

'+++' Stronger production, '++' Moderate production, '+' Low production,
'-' No production

chitinase and IAA.

The isolate was further studied for role of chitinase and protease activity, characterization of siderophore for their nature and binding properties and IAA production.

Mycolytic enzymes consist of chitinase, protease etc. based formulations have been used to control fungal plant pathogens (Despande, 1999). Fluorescent

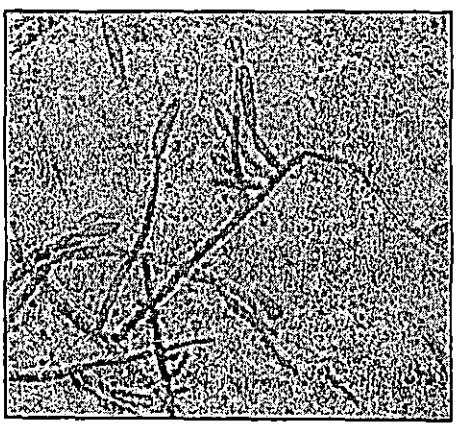
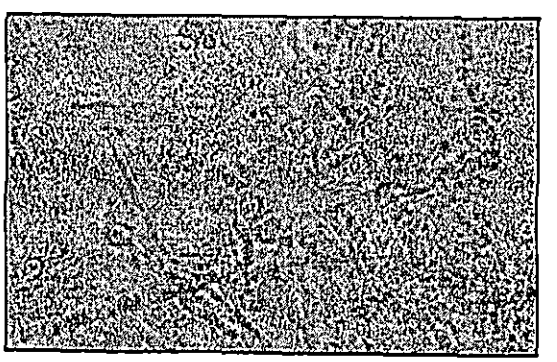


Fig. 2. Microscopic observations of mycelium inhibited by fluorescent *Pseudomonas* BRL-1 strain. Mycelium of *F. oxysporum* (a) grown on Peptone Glucose Agar (PGA) media (Control),



(b) present in the inhibition zone, when grown with fluorescent *Pseudomonas* BRL-1 on PGA.

Pseudomonas BRL-1 has been found to produce chitinase and protease, when they were grown in

chitin and gelatin media respectively, as a sole carbon source. As the fungal cell wall contains chitin as the major component and chitinase are well known to lyse the cell walls of both live and dead fungi (Ueno *et al.*, 1990). The reduction in dry weight of the fungal pathogen may be due to the proteolytic and chitinolytic activity of fluorescent *Pseudomonas* BRL-1.

CAS agar (blue agar) plate assay indicate siderophore production by fluorescent *Pseudomonas* BRL-1. The strain forms colonies with an orange halo. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the siderophore production.

The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Despande , 1999).

In general Indole acetic acid is universally accepted as a plant growth promoter. Other than that, it has been reported by Chirst and Mosinger (1989) that this phytohormone could also induce resistance through PR protein production. Again in the year 2004, Sharaf and Farrag reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by soil pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1.

On the basis of these studies it can be concluded that the fluorescent *Pseudomonas* BRL-1 isolate is showing significant antagonistic property through combined and/ or individual effect of siderophore, production of proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen (Rameshkumar *et al.*, 2002). These observations and further studies might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against *F. oxysporum.*

References

Arora, N.K., Kang, S.C. and Maheshwari, D.K. 2001. Isolation of siderophore producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of ground nut. *Curr. Sci.* 81: 673 –677.

Basha, S. and Ulagnathan, K. 2002. Antagonism of *Bacillus* species (strain BC121) toward *Curvularia lunata. Curr. Sci.* 82: 1457-1463.

Chakravarti, B. P. and Anil Kumar T. B. 1972. Wilt of *Dolichos biforus* caused by *Fusarium oxysporum. Ind. Phytopathol.* 25: 583.

Christ, U. and Mosinger, E. 1989. Pathogenesis related protein of tomato: Induction by *Phytophthora infestance* and other biotic and abiotic inducers and correlations with resistance. *Phyisolo.Mol. Plant Pathol* 35:53-65.

Dastur J F, Diseases of Pan (*Piper bitle*) in the central provinces 1935. *Proc. Ind. Acac. Sci.* 1: 778-815.

Dennis, C. and Webster, J. 1971. Antagonistic properties of species group of *Trichoderma* II. Production of volatile antibiotics. *Trans. Bri. Mycolo. Soc.* 82: 41-48.

Despande, M.V. 1999. Mycopesticide production by fermentation: Potential and challenges. *Crit. Rev. Microbiol.* 25: 229.

Frandberg, E. and Schnurer, J. 1994. Chitinolytic properties of Bacillus Pabuli K1[J] *.J Appl Bacteriol.*76: 361-367.

Gaur, R., Shani, N., Kawaljeet, Johri, B.N., Rossi, P. and Aragno, M. 2004. Diacetylphloroglucinol producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Curr. Sci.* 86: 453-457.

Ghodajkal, B. N., Ganacharya, N. M., Bindu, K. J. and Jahav, V. T. 1976. Sunflower wilt, anew disease from India. *Curr. Sci.* 45: 844.

Gunasekaaran, P. 1995. *Laboratory Manual In Microbiology.* New Age International (P) Limited, New Delhi. pp. 125.

Gupta, G. K. and Sohi, H. S. 1967. Fungal foes that menace ginger crop. *Ind. Fmg.* 16: 15.

Holt, G.J., Krieg, R.N., Sneath, P.H.A., Staley, J.T. and Williams, S.T. 1994. *Bergy's Manual of Determinative Bacteriology.* Willims and Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp.151-168.

Keel C. U., Schnider, M., Maurhofer, C., Voisard, J., Laville. U., Burger, P., Wirthner, D., Haas and Défago,

G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol, *Mol Plant-Microbe Interact.* 5: 4-13.

Malik, P. C. and Mehta, P. P. 1963. Banana wilt in Bihar. *Sci and Cult,* 29: 245.

Mathur, R. L. and Mathur, B. L. 1958. Fruit rot of *Citrullus vulgaris* var. *fistulosus* L.,*Ind Phytopathol,* 11: 66-69.

Mustafee, T. P. 1971. Fiber deteriorating microflora of Ramie, *Sci and Cul.* 37: 386-387.

Neilands J B. 1981. Microbial iron compounds. *Annl. Rev. Biochem.* 50: 715-731.

O'Sullivan, M., Stephens, P. M. and O'Gara, F. 1991. Extracellular protease production by fluorescent *Pseudomonas* spp. and the colonization of sugarbeetroots and soil, *Soil Biol Biochem.* 23: 623-627.

Rameshkumar, N., Thirumalai Arasu, V. and Gunasekaran, P. 2002. Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria, *Pseudomonas fluorescens. Curr. Sci.* 82: 1463-1466.

Randhawa, H.S., Sandhu, R.S. and Kowshik, T. 2002. *In vitro* inhibition of *Aspergillus fumigatus* by *Candida* species, especially *C. albicans* and *C. glabrata. Curr. Sci.* 87: 860-865.

Schwyn, B. and Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Annal. Bichem.* 160: 47-56.

Sharaf, E.F. and Farrag, A.A. 2004. Induced resistance in tomato plants by IAA against Fusarium oxysporum lycopersici *Pol J Microbiol.* 53: 111-116.

Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. 1990. Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. *J. Gen. Appl. Microbiol.* 36: 377.

Wei, G., Kloepper, J.W. and Sadik, T. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth promoting rhizobacteria. *Phytopathology.* 81: 1508-1512.

the centre on the plates. The fungal pathogen inoculated centrally on PGA plate, but uninoculated by fluorescent *Pseudomonas* BRL-1 served as control. The inoculated plates were incubated at 30°C for 6 days and inhibition of colony growth was measured.

Interaction of fluorescent Pseudomonas BRL-1 against A. niger in dual liquid culture

To test the antifungal activity of the fluorescent *Pseudomonas* BRL-1, dual liquid culture method was employed. 1 ml of freshly grown fluorescent *Pseudomonas* BRL-1 culture (containing 10^7 cfu/ml) and an agar block (5 mm diameter) of 5 days old culture of *A.niger* was inoculated onto 50 ml of Peptone Glucose medium in 250 ml of conical flask. The culture was incubated on a rotary shaker at 30°C for 72 h. For the control experiments the fungal pathogen were grown alone. Experiments were performed thrice taking triplicate for every set (Basha and Ulaganathan, 2002). A drop of fungal culture form each flask was taken out and placed on a clean glass slide and stained with lactophenol and cotton blue and observed under Leica DMLS Research Microscope.

Differences in dry weights between the fungal cultures grown with fluorescent *Pseudomonas* BRL-1 strain or the control culture grown without any bacterium were recorded (Basha and Ulaganathan, 2002). For this 72 h. old dual cultures were filtered through the pre weighted Whatman No. 1 filter paper. It was dried for 24 h at 70°C and weights were measured.

Inhibitory effect of fluorescent Pseudomonas BRL-1

To investigate the inhibitory effect on fluorescent *Pseudomonas* BRL-1 for the production of volatile compound 'inverted plate technique' was followed (Dennis and Webster, 1971). Spores of *A.niger* was point inoculated on the centre of the Petriplate containing 20ml of PGA. A loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture was strike on Petriplate having 20 ml of PGA then inverted over the plates inoculated with the fungal pathogen. Two plates were sealed together (mouth to mouth) with parafilm, control plate consist of *A.niger* inverted over uninoculated PGA plate. The plates were incubated at 30°C and three replicates were maintained for each treatment. After 5 days of incubation colony diameter of the test pathogen was measured and compared with the control.

Production of hydrogen cyanide was tested qualitatively according the method of Wei *et al.*, (1991). The fluorescent *Pseudomonas* BRL-1 was inoculated in TSA medium supplemented with amino acid glycin (4.4 g/l of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30°C for 4 days. A change of colour of the filter paper strip from yellow to light brown, brown or reddish brown was recorded as weak (+), moderate (++) or strong (+++) cyanogenic potential, respectively.

To test the chitinolytic property of fluorescent *Pseudomonas* BRL-1 it was inoculated on LB medium (Gunasekaran, 1995) supplemented with 0.5% colloidal chitin as principal source of carbon. Plates were incubated at 30°C for three days. Formation of a clear halo region around the colonies indicated chitinase activity of the strain (Basha and Ulagnathan, 2002).

Chrome Azurol S (CAS) agar medium was prepared as described by Schwyn and Neilands (1987) to detect the siderophore production. CAS agar (blue agar) was inoculated at the center of the plate with 24 h old fluorescent *Pseudomonas* BRL-1 and kept for incubation at 30°C for 72h. The change of the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore. Siderophore excreted into the culture medium was determined by spectophotometry. Concentration was calculated using absorption maximum and the molar absorption coefficient (λ_{max} = 400 nm and ε = 20,000 M^{-1} cm^{-1}) according to the method of Meyer and Abdallah (1978).

Production of hydrolytic enzyme was qualitatively assayed in minimal medium containing gelatin, starch, pectin and carboxymethyl cellulose (CMC)

for protease, amylase, pectinase and cellulase respectively. Plates were incubated for 48 h. at 30°C and formation of clear zone around bacterial colonies was read as positive (Gaur, *et al.*, 2004).

RESULTS AND DISCUSSION

In dual culture, significant growth inhibition of *A.niger* occurred due to antagonistic effect of fluorescent *Pseudomonas* BRL-1, which was evident with clear inhibition of colony diameter. At 48h of incubation, *A.niger* was strongly inhibited by fluorescent *Pseudomonas* BRL-1 and simultaneous increase in incubation time corresponded escalation in percent growth inhibition (Fig. 1). After 96h of incubation the mycelia growing toward the interaction zone, which was prominently evident in semicircular/U-shaped streak stopped, and the mycelia gradually lost vigor. Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching and finally resulting into lysis (Fig. 2). The difference in dry weights between the fungal culture grown with and without fluorescent *Pseudomonas* BRL-1 strain was recorded. There was more than 50% reduction in dry weight of the culture grown with fluorescent *Pseudomonas* BRL-1 strain when compared to the control.

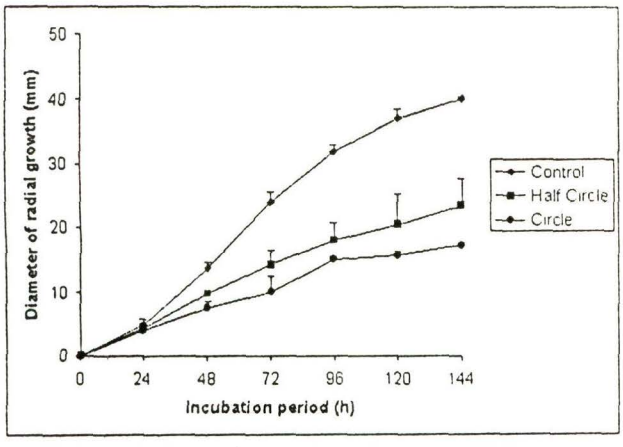


Fig. 1 : *In vitro* antagonistic activity of Fluorescent *Pseudomonas* BRL-1, cultured as semi-circular and circular streaks, on *Aspergillus niger* point-inoculated in the centre of PGA plates incubated at 30°C.

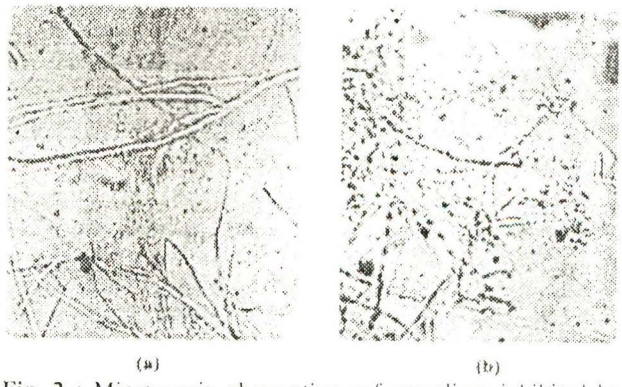


Fig. 2 : Microscopic observation sof mycelium inhibited by Fluorescent *Pseudomonas* BRL-1 strain. Mycelium of *A. niger* (a) grown on Peptone Glucose Agar (PGA) media (Control), (b) present in the inhibition zone, when grown with Fluorescent *Pseudomonas* BRL-1 on PGA.

Morphological abnormalities in hyphae of fungal pathogen, was clearly observed under microscope. Such abnormalities occurred due to secondary metabolites and diffusible lytic substances produced by the bacterium (Arora *et al.*, 2001). Further study was made to screened for the production of different secondary metabolites including siderophore, hydrogencyanide, antimicrobial compound, volatiles, hydrolytic enzymes investigated for their effect on *A.niger in vitro* (Table 1). The results (Table 1) showed fluorescent *Pseudomonas* BRL-1 produced mainly siderophore, protease and chitinase.

Table 1 : Showing different secondary metabolities production and enzymatic activity of Fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore production	+++
Portease activity	++
Chitinase activity	+
HCN production	—
Volatile substances production	—
Antibiotic substances	—

'+++' Stronger production, '++' Moderate production, '+' Low production, '—' No production

Mycolytic enzymes consisting of chitinase, protease etc. based formulations have been used to control fungal plant pathogens (Deshpande, 1999). Fluorescent *Pseudomonas* BRL-1 has been found to

produce chitinase and protease, when they were grown in chitin and gelatin media respectively, as a sole carbon source. As the fungal cell wall contains chitin as the major component and chitinase are well known to lyse the cell walls of both live and dead fungi (Ueno *et al.*, 1990). The reduction in dry weight of the fungal pathogen may be due to the proteolytic and chitinolytic activity of fluorescent *Pseudomonas* BRL-1. CAS agar (blue agar) plate assay indicate siderophore production by fluorescent *Pseudomonas* BRL-1. The strain forms colonies with an orange halo. This colour change is based on the principle that the blue colour of the CAS medium is due to the Fe-dye complex and when siderophore is produced by the fluorescent *Pseudomonas* BRL-1 the iron is released from the Fe-dye complex resulting in the change in colour to orange which indicate the hydroxamate nature of siderophore. The isolate did not produce catecholate or carboxylate siderophore, the positive result for pyoveridine nature of the hydroxamate siderophore was also evident by their absorption maxima between 407 and 413 nm.

The isolate did not produce hydrocyanic acid (HCN). Infact, it was reported that production of HCN proved to be deleterious to the plant (Arora *et al.*, 2001).

On the basis of these studies it can be concluded that the fluorescent *Pseudomonas* BRL-1 isolate is showing significant antagonistic property through combined and/or individual effect of siderophore, production of proteolytic enzyme, and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen (Remeshkumar *et al.*, 2002). These observations and further studies might help in developing this fluorescent *Pseudomonas* BRL-1 as potential rhizospheric biocontrol agent against *A.niger.*

REFERENCES

Arora, N. K., Kang, S. C. and Maheshwari, D. K. (2001). Isolation of siderophore producing strains of *Rhizobium meloloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of ground nut. *Curr. Sci.* 81:673-677.

Basha, S. and Ulagnathan, K. (2002). Antagonism of *Bacillus* species (strain BC121) toward *Curvularia lunata*. *Curr. Sci.* 82 : 1457-1463.

Bilgrami, K. S., Jamaluddin, S. and Rizwi, M. A. (1991). In *Fungi of India List and References.* Todays and Tomorrow's Printing Publishers. New Delhi. pp. 454-456.

Dennis, C. and Webster, J. (1971). Antagonistic properties of species group of *Trichoderma* II. Production of volatile antibiotics. Transaction of British Mycological Society. 82 : 41-48.

Despande, M. V. (1999). Mycopesticide production by fermentation : Potential and chanllenges. *Crit. Rev. Microbiol.* 25 : 229.

Gaur, R., Shani, N., Kawaljeet, Johri, B. N., Rossi, P. and Aragno, M. (2004). Diacetylphloroglucinol producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Curr. Sci.* 86 : 453-457.

Gunasekaaran, P. (1995). In *Laboratory Manual In Microbiology.* New Age International (P) Limited, New Delhi. pp. 125.

Gupta, C. P., Dubey, S. C., Kang, S. C. and Maheshwari D. K. (2001). Antibiosis-mediated necrotrophic effect of *Pseudomonas* GRC2 against two fungal plant pathogen. *Curri. Sci.* 81 : 91-94.

Holt, G. J., Krieg, R. N., Sneath, P. H. A., Staley, J. T. and Williams, S. T. (1994) In *Bergy's manual of Determinative Bacteriology.* Willims & Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp. 151-168.

Meyer, J. M. and Abdallah, M. A. (1978). The florescent pigment of *Pseudomonas fluorescens* Biosynthesis, purification and physical-chemical properties. *J. Gen. Microbiol.* 107 : 319-328.

Rameshkumar, N., Thirumalai Arasu, V. and Gunasekaran, P. (2002). Genotyping of antifungal compounds producing plant growth-promoting rhizobacteria *Pseudomonas fluorescens. Curr. Sci* 82 : 1463-1466.

Randhawa, H. S., Sandhu, R. S. and Kowshik, T. (2002). *In vitro* inhibition of *Aspergillus fumigatus* by *Candida* species, especially *C. albicans* and *C. glabrata. Curri. Sci.* 87 : 860-865.

Schwyn, B. and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Annal. Bichem.* 160 : 47-56.

Ueno, H., Miyashita, K., Swada, Y. and Oba, Y. (1990). Purification and some properties of extracellular chitinase from *Streptomyces* sp. S-84. *J. Gen. Appl. Microbiol.* 36 : 377.

Wei, G., Kloepper, J. W. and Sadik, T. (1991). Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth promoting rhizobacteria. *Phytopathology.* 81 : 1508-1512.

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IV. 2 : Special dispersion statistics and sequential sampling plan for frog eye leaf spot disease caused by *Cercospora capsici* in chilli

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Iwao's patchiness regression technique was used to study the spatial distribution of *Cercospora capsici* infecting chilli leaves in its five growth stages *viz.*, nursery, vegetative, flowering, podding and fruit ripening stages. The dispersion statistics indicated that the basic components of the disease lesion population aggregate and they were contagiously distributed in chilli at all growth stages. Botanical extracts spray did not alter the basic distribution pattern of disease. The dispersion statistics obtained from pooled data enabled to arrive at sequential sampling plan that requires a maximum of 58 leaves sample for assessing the threshold level of diseases in order to initiate control measures. A preliminary economic threshold of one lesion per leaf was used to prepare the sequential sampling plan for timing the initiation for using of plant extract to control the disease. It is a cost effective ecofriendly quantitative method in plant disease management.

IV. 3 : Integrated management approach against sigatoka leaf spot of Banana

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Leaf spot or sigatoka disease of banana causes considerable yield loss in every year and it may goes up to 50% or more in severe cases. The disease has a great importance in West Bengal as the commercial cultivation of the crop increases rapidly during recent years. Different management approaches have been taken in this investigation including chemical fungicides, botanical oils and a bacterium inoculants (*Pseudomonas fluorescens*). Among the 10 fungicides assessed, propiconazole showed best performance against the disease followed by chlorathalonil and tridemorph. Five botanical oils tested, of which neem oil was found to be the best to reduce the disease severity. Performance of the oil was as good as some chemical fungicide (eg. propineb) when applied @ 0.1% level. The bacterium *Pseudomonas fluorescens* when applied @ 5 g formulation (10^8 cfu/g) per litre, per cent disease index (PDI) was estimated as 17.83 as compared to 27.47 in the control sets. Yield of the crop was recorded during harvest where propiconazole gave the best result (30.82 t/ha). Productivity per ha of the neem treated plot and *P. florescens* plot were 27.12 t and 25.80 t respectively, whereas yield of the control plot was 21.66 t/ha. Yield increases over control were calculated 42.88%, 25.20% and 19.11% from propiconazole, neem oil and *Pseudomonas fluorescens* treated plot respectively.

IV. 4 : Management of an epidemic disease of *Cymbidium* rot by fluorescent *Pseudomonas*

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Orchid is an important crop for earning foreign exchange as it is one of the most beautiful ornamental plant, and also play a significant role in agricultural economy. In Darjeeling and its adjoining area it has been found that the commercially important part i.e., pseudobulb and roots of *Cymbidium* are attacked by a combination of two fungal pathogens namely *Fusarium oxysporum* Schlecht. and *Mucor hiemalis* Wehmer.f. *hiemalis*, and one pathogenic bacteria *Erwinia*. These three pathogens might play a synergistic effect to increase the severity of this disease. It was estimated by the Himalayan Orchid Society that the loss is more than million rupees during this last five years. Furthermore we have isolated an antagonist PGPR fluorescent *Pseudomonas* BRL-1 which showed significant inhibition over the growth of pathogens *in vitro* by the production of siderophore, chitinase, protease and IAA. An application of this antagonist in the nursery level protected plants upto 80%. Routine application of this biocontrol agent in the field might help the horticulturist to protect this commercially important plant and might boost up the economy of this area.

IV. 5 : Diseases of Oak Tasar host plants (*Quercus* spp.) in Manipur

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Quercus serrata and *Quercus acutissima* are the two common oak plants for rearing Oak Tasar silkworm (*Antheraea proylei*) in Manipur. These plants are occasionally attacked by fungal diseases. In the present investigation three fungal diseases namely Sooty mold (*Chaetophoma quircifolia*), Powdery mildew (*Phyllactenia corylea*) and Rust disease (*Cronartium quercum*) were found to attack mostly from March to November in a year. Survey data from five plantation sites of Oak tasar plantation farm revealed that disease incidence and disease severity increase from May to July and gradually decrease in the winter season. These diseases are mostly air borne as screening of seeds didn't reveal the presence of any of these pathogens on Oak tasar seeds. Effect of epidemiological factors on the development of these diseases on Oak Tasar host plants is discussed in the full paper.

IV. 6 : Effect of pre and post emergence herbicides on fibre yield and soil microbial population under a jute crop in an alluvial soil

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A field experiment was conducted in an alluvial soil (*Typic Ustocrept*) to study the effect of two pre (Trifluralin and Fluchloralin) and one post emergence (Quizalofop ethyl) herbicides on fibre yield and soil microbial population in jute crop. The fibre yield of jute increased significantly with herbicides application over control. The maximum fibre yield (37.9 q/ha) was recorded with pre emergence herbicide "Trifluralin" @ 0.75 kg a.i./ha which was significantly superior over other treatments. The total population of bacteria, fungi and actinomycetes decreased drastically after 7 days of application of these herbicides compared to initial status as well as control. The microbial population increased gradually after 15 days onwards and reached to the initial status at harvest i.e. after 120 days.

IV. 7 : Leaf rot of Betelvine : host range of, medium for isolation and technique for bioassay of fungicides against the pathogen

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Leaf rot of Betelvine caused by *Phytophthora nicotianae* is prevalent in all betelvine growing areas of West Bengal. In laboratory condition the pathogen infected brinjal, tomato, sweet pepper, red pepper, cucumber and guava fruits and leaves of black pepper. On artificial inoculation it infected red pepper (chilli) causing foot rot symptom in field condition. *Phytophthora nicotianae* isolated from fruit rot of brinjal, guava, leaf rot of black pepper, foot rot of *Hibiscus subdariffa*, foot rot and leaf blight of sesamum and *Phytophthora capsici* from foot rot of chilli infected betelvine leaves in laboratory and in field conditions. *Phytophthora* spp. from all the hosts were successfully isolated in Oat meal agar medium amended with vancomycin (200 ppm), pimaricin (10 ppm) and carbendazim (25 ppm). An easy technique for bioassay of fungicides against *Phytophthora nicotianae* has been standardized. When agar disc with mycelial growth of *Phytophthora nicotianae* was put in water in half-submerged codition, the fungus produced good mycelial growth with abundant sporangia and the sporangia thus formed germinated in water. Sensitivity of this fungus towards fungicides was successfully tested by putting agar disc mycelial growth in different concentration of a particular fungicide and subsequently recording the extent of mycelial growth, sporangia formation and sporangial germination. Copper oxychloride, copper hydroxide, mancozeb, thiram, combination product of mancozeb + metalaxyl, copper oxychloride + metalaxyl, cymoxanil + mancozeb showed good inhibition against all *Phytophthora* species.

In vivo studies revealed that all the antagonists reduced the root rot of sesame significantly, when applied as soil drenching or in combination of seed soaking and soil drenching. Combination of seed soaking and soil drenching gave better results than the soil drenching alone. Here among the antagonists maximum disease control was observed by the *T. harzianum* which gave up to 66.17% of sesame root rot disease control followed by isolate BS-12 (up to 65.27%) and BS-17 (up to 65.25%).

P-VIII. 4 : EFFECT OF DIFFERENT LEVELS OF IRRIGATION AND SOURCES OF SULPHUR ON NODULE NUMBER, WEIGHT AND YIELD OF SUMMER GROUNDNUT (*ARACHIS HYPOGAEA*)

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Groundnut is one of the leading oilseed crops in the global agricultural scenario. India, though ranks 2nd in groundnut production, but its productivity is far lower than most of the groundnut producing countries like China, America and Argentina. The main reasons advocated for such low productivity are lack of irrigation facilities and inadequate fertilizer application. Among the chemical fertilizers, sulphur is a major one.

Keeping in mind the above view points, the experiment was conducted during pre-kharif season of 2003 and 2004 at Jagulia Instructional Farm, B.C.K.V., Mohanpur, Nadia on sandy loam soil under irrigated upland condition. The experiment was conducted in a split-plot design having 4 levels or irrigation (Rainfed, one irrigation at flower initiation, two irrigations at flower initiation + pegging, and three irrigations at flower initiation + pegging + pod development) in the main plot and 4 sources of S (Elemental S, single super phosphate, ammonium sulphate and gypsum) applied at 40 kg/ha in the subplot. The experiment was replicated thrice. Spacing adopted were 30 cm (row to row) and 10 cm between plants. The seed rate and variety were 60 kg/ha and SB-XI respectively. Crop received 20 kg N, 40 kg P_2O_5 and 20 kg K_2O/ha applied as basal.

Experimental results revealed that number of nodules, dry weight of nodules and yield of groundnut crop were greatly improved by different levels of irrigation. Three irrigations level recorded the maximum nodule number and dry weight with a corresponding highest seed yield of groundnut. Different sources of sulphur influenced the number and dry weight of nodules and the seed yield of groundnut significantly. Gypsum proved to be the best performer in this regard. The interaction between irrigation levels and sources of sulphur was found to be significant on groundnut yield and three irrigation with gypsum was found to be the best.

P-VIII. 5 : INTEGRATED MANAGEMENT OF BLACK-ROT DISEASE OF CAULIFLOWER IN ORISSA

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The efficacy of bacterial antagonists *Pseudomonas fluorescens* and *Azotobacter chroococcum*, chemical like strptocycline and a botanical neem cake was studied for the management of black-rot disease of cauliflower. Field trials were conducted for three consecutive Rabi seasons 2001-02, 2002-03 and 2003-04 in the experimental field of All India Coordinated Vegetable Improvement Project, OUAT, Bhubaneswar with the variety Himlata-2. The trial was laid out in randomized block design with four replications. The row to row spacing of 60 cm and plant to plant spacing of 40 cm was adopted in the plot size of 8.1 sq.m. (3.0 m × 2.7 m) with the fertilizer dose of 150 : 75 : 75 kg N : P_2O_5 : K_2O/ha. Among the different treatments, seed treatment with sterptocycline (100 ppm) for 15 minutes followed by seedling dip (100 ppm) for 15 minutes before planting and three sprays of streptocycline (200 ppm) at 10 days intervals starting from 15 days after planting proved very effective in management of black-rot with mean PDI of 4.55 %, maximum curd yield of 220.23 q/ha and registering 81.84 % disease control and 75.71 % increase in curd yield over control. The same treatment gave maximum cost benefit ratio of 1:10.85 with net return of Rs.27,505.00. Seed treatment with *Azotobacter chroococcum* (1.5 g/kg), soil application of the culture (250 g/50 kg FYM/ha) and slurry culture drenching (after 15 days of planting) was found to be next best in respect of disease control (54.69 %) followed by use of *Pseudomonas fluroescens* as seed treatment (10 g/kg), seedling dip (0.2 % spore suspension for 30 minutes before planting) and foliar spraying (three sprays of 0.2 % spore suspension at 10 days interval starting from 15 days after planting) registered 37.71 % disease control with mean cost benefit ratio of 1:5.68. However, the control plots recorded maximum disease incidence of 25.6 % with lowest curd yield of 125.47 q/ha.

P-VIII. 6 : OPTIMIZATION OF DIFFERENT PHYSIOLOGICAL FACTORS ON GROWTH FOR THE DEVELOPMENT OF A COST EFFECTIVE MEDIA FOR LARGE SCALE PRODUCTION OF AN ANTAGONISTIC PGPR FLUORESCENT *PSEUDOMONAS* BRL-1

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Considerable research efforts are underway globally to exploit the potential of fluorescent Pseudomonads in maintenance of soil health and as crop protectants, since they represent not only a dominant bacterial group of rhizosphre ecosystem but are also metabolically and functionally most versatile. Our isolate, fluorescent *Pseudomonas* BRL-1, showed significant antagonistic activity against several phytopathogens like *Fusarium oxysporum, Sclerotium rolfsii, Myrothecium roridum, Colletotrichum capsici* and *Curvularia lunata* and also having PGPR activity. Correlation of antifungal activity of these isolates have been found to be linked with the production of siderophore, protease, chitinase and IAA. The objective of this study was to standardize a suitable cost effective fermentational technique for the large-scale production and powder formulation of the said strain. Various factors influencing the growth of the strain were worked out by keeping all the factors constant except the one was varied. The factors considered were pH of the medium, time period, temperature, aeration, inoculum volume and selection of suitable carbon and nitrogen sources.

Survival of the fluorescent *Pseudomonas* BRL-1 monitored at room temperature in a talc based formulation showed viable population up to 120 days. The population was drastically reduced after 120 days of storage. The results indicate that this efficient rhizospheric bacteria could be commercially produced in this cost effective media composition and could be maintained in talc based powdered form for at least five months.

P-VIII. 7 : EVALUATION OF BIOCONTROL POTENTIALITY AND PLANT GROWTH PROMOTING CAPABILITY OF A FLUORESCENT *PSEUDOMONAS* BRL-1 AGAINST SEVERAL PHYTOPATHOGEN

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Considerable research efforts are underway globally to exploit the potential of fluorescent Pseudomonads in maintenance of soil health and as crop protectants, since they represent not only a dominant bacterial group in the rhizosphere ecosystem but are also metabolically and functionally most versatile.

In this study we have evaluated the biocontrol potentiality and PGPR activity of an isolate, fluorescent *Pseudomonas* BRL-1. In dual culture, significant growth inhibition of several phytopathogens like *Fusarium oxysporum, Mucor hiemalis, Sclerocium rolfsii, Myrothecium roridum, Colletotrichum capsici, Alternaria alternata* etc. occurred due to the antagonistic effect of fluorescent *Pseudomonas* BRL-1. Microscopic study of the mycelia from the interacting zone showed hyphal shriveling, mycellial deformities, swelling, fragmentation, short branching and finally resulting into lysis.

Morphological abnormalities of the hyphae occurred due to the production of secondary metabolites mainly siderophore which is of hydroxamate in nature. The strain produce mycolitic enzyme like chitinase and protease, which are well known to lyse the cell wall of fungal pathogen. Furthermore the isolate produces significant amount of IAA which functions as a plant growth promoter as well as prevent the growth of pathogen.

The fluorescent *Pseudomonas* BRL-1 showing antagonistic activity through combined and/ or individual effect of siderophore, production of mycolitic enzyme and IAA activity. These observation and further studies might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential rhizospheric biocontrol agent against different pathogens.

P-VIII. 8 : ISOLATION, CHARACTERIZATION AND SCREENING OF FLUORESCENT PSEUDOMONADS POPULATION OF MEDICINAL PLANT'S RHIZOSPHERE AGAINST *ALTERNARIA ALTERNATA*

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In recent years fluorescent Pseudomonads have drawn attention world wide because of production of secondary metabolites such as siderophores, antibiotics, volatile compound, HCN, enzymes and phytohormons. Here, the phenotypic diversity of 100 fluorescent *Pseudomonas* strain isolated from the rhizosphere of different medicinal plants were compared. Vast ranges of biochemical, morphological, cultural and physiological homology were evaluated. The following tests were performed: proteolysis of gelatin, nitrate reductase, oxidase test, arginine dihydrolase production, lipase production, production of gas from carbohydrates (such as glucose, sucrose, lactose), urease production, levan formation from sucrose, H_2S formation, catalase, methyl red and Voges-Proskawer test. Morphological and cultural characteristics were examined on 4 days old culture. All the isolates were rod shaped, aerobic, gram negative; as regard to their enzymatic activity they were oxidase positive, nitrate positive and arginine dihydrolase positive they were levan positive and showed negative result in Voges-Proskauer test. Among all the Fluorescent Pseudomonad native isolates, 26 different isolates showed antagonistic activity against *Alternaria alternata*, out of which GS9, GS11, CA9, CA17, SR9, SR14, WS10, WS11 and RS11 were found to be most potent antagonist.

P-VIII. 9 : BIOLOGICAL CONTROL OF RHIZOME ROT DISEASE OF TURMERIC

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Turmeric (*Curcuma longa* Linn.) an important rhizomatous spice crop being cultivated in India, since ancient times, is affected by some diseases of which rhizome rot disease caused by *Pythium* sp is most important. The three biocontrol agents viz., *Trichoderma viride*, *Pseudomonas fluorescence* and *Bacillus subtilis* and Farm Yard Manure (FYM) were used in different combinations both as seed treatment and soil application to develop an effective biological control method against rhizome rot disease of turmeric. Combination of seed treatments with both *Trichoderma viride* and *Pseudomonas fluorescence* along with FYM showed highest disease reduction (63.65% less disease) as compared to check than other treatments. The soil application of *Trichoderma* and *Pseudomonas* along with FYM as well as combination of seed treatment and soil application with these two bio-control agents also found to give very good result in terms of per cent reduction in rot over control. Regarding rhizome yield, combined seed and soil treatment with both *Trichoderma* and *Pseudomonas* along with FYM was the best, recording the highest rhizome yield. *Bacillus subtilis* had less pronounced effect on disease incidence and rhizome yield.

P-VIII. 10 : POTENTIAL DETERRENCE OF *TRICHODERMA* SPP. AGAINST FUSARIAL WILT OF TOMATO

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Wilt of tomato (*Lycopersicon esculentum*) is a most dreadful disease caused by *Fusarium oxysporum* leading to an enormous economic loss to the growers as a consequence. An attempt was made to control the disease biologically with the application of antagonists viz. *Trichoderma viride, T. lignorum, T. harzianum, T. hamatum and T. reesei*. The effect of volatile and non-volatile antibiotics of *Trichoderma* origin on growth inhibition of the wilt pathogen was studied. *T. harzianum* showed maximum growth inhibition (91%) of the pathogen through mycoparasitism followed by *T. viride* and the non-volatiles produced by these antagonists exhibited their excellent antagonism to the growth of the pathogen (100%) under *in vitro* condition. *Trichoderma* spp produced siderophore, iron-chelating compounds that contribute much towards enhancement of their competitive behaviour for nutrition with the target pathogenic fungi and as such offer their greater antagonistic potentiality. Overall experimental studies clearly indicate *T. harzianum, T. viride* and *T. hamatum* are capable of retarding the growth of the pathogen while *T. lignorum* and *T. reesei* are comparatively less efficient to this effect.

P-VIII. 11 : EVALUATION OF ANTAGONISTIC POTENTIAL OF SOME WILD AND MUTANT ISOLATES OF *TRICHODERMA HARZIANUM* AGAINST *MACROPHOMINA PHASEOLINA* (TASSI) GOID CAUSING STEM ROT OF JUTE

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Six gamma irradiated mutant isolates of *Trichoderma harzianum* and their two wild biotypes were evaluated for antagonistic potential against *Macrophomina phaseolina* causing stem rot of jute. All the mutant isolates were more effective in inhibiting the mycelial growth of the pathgogen than their corresponding wild biotypes in dual culture and two mutant isolates namely $75^{Th}4IV$ and $125^{Th}41$ were most effective in inhibiting pathogen growth by 78%. The highest production of volatile compound was recorded in case of $150^{Th}31$ which was evidenced by 49% inhibition of mycelial growth of the pathogen over control. The maximum inhibition of mycelial growth of the pathogen through production of nonvolatile antibiotic was recorded in case of Th4, $150^{Th}31$ and $75^{Th}41V$. The mutant isolate $75^{Th}41V$ was also found to produce greater quantity of extracellualr enzymes like β-1,3 glucanase, β-1,4 endoglucanase and chitinase when Czapek Dox broth was partially induced with mycelial powder of *M. phaseolina*. *In vivo* trial was conduced to determine the biocontrol potential against *M. phaseolina* incited stem rot of jute and observations on disease incidence were taken at fortnightly interval up to 8 weeks. At 56 days after sowing all the mutants were superior to their respective wild biotypes in non-sterilized soil, but they were highly variable in their disesase management potential. However, the mutants $75^{Th}41V$ and 125th 41 were found most promisoing in reducing stem rot infestation in both-sterlized and unsterilized soil.

P-VIII. 12 : DIVERSITY OF WILD EDIBLE MUSHROOMS AND THEIR ECONOMIC VALUES IN SHEPOUMARAMTH NAGA AREAS UNDER SENAPATI DISTRICT OF MANIPUR

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Out of the total twenty-four different edible fungi collected and identified in local dialects, the present work focused on nine locally well known mushrooms. Detail information's in regard to habit, habitats,

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In vitro antagonistic effect of fluorescent *Pseudomonas* BRL-1 against *Sclerotium rolfsii*

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Key words: Fluorescent *Pseudomonas, Sclerotium rolfsii*

Sclerotium rolfsii Sacc. is an important soil-borne plant pathogen distributed throughout the world, having broad host range and persists for longer period in soil by resistant resting structures (sclerotia) (3). Use of synthetic fungicides and crop management strategies were unsuccessful in eradicating this pathogen completely, and the former causing environmental hazards and chronic health problem (2).

In recent years, florescent Pseudomonads have drawn attention worldwide owing the ability of production of secondary metabolites such as siderophore (16), antibiotics (12, 17), volatile compound, HCN (24), enzymes and phytohormones (12, 17). These have been implicated in reduction of plant pathogenic fungi and harmful rhizobacteria with simultaneous induction of growth of crop plants. Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, because chemical control is expensive and hazardous to ecosystem.

Taking it as an objective, an attempt has been made to isolate a potential antagonistic organism having lethal effect on this phytopathogenic fungi *Sclerotium rolfsii.*

Soil bacterial isolates were made from the rhizosphere soil samples of potato following serial dilution technique in King's B media (13). All the isolates were subcultured and maintained on Tryptic Soy Agar (TSA) medium. Screening for the antagonistic activity was carried out by following the method of Huang and Hoes (1976) (11). An agar block of 5mm diameter was placed at the center of the PGA plate and allowed to grow for 48 hours. A loopful of bacterial culture was streaked at the periphery of the plate and incubated at 30°C for five days. Development of inhibition zone around the bacterial colony was observed. Out of the 96 isolates tested 6 showed inhibition against *S. rolfsii,* of which strain BRL-1 showed the highest level of inhibition and selected for further study. This strain was characterized morphologically and biochemically following Bergey's Manual of Determinative Bacteriology (10) and found to be a fluorescent *Pseudomonas* species.

The florescent *Pseudomonas* BRL-1 was subcultured and maintained on TSA medium for subsequent use. During *in vitro* antagonistic study, dual culture technique was performed on Peptone Glucose Agar (PGA) solid (23) and liquid (2) media. In the former experiment bacterial isolate was streaked on PGA plate in a circular / O shaped and semicircular / U shaped pattern (23). In the later case 50ml of PG broth was inoculated with 1ml the bacterial suspension (10^7 cfu ml^{-1}). Then the pathogen was subsequently challenged in the form of a single sclerotium at the center of O or U shaped region on the PGA plate and in dual liquid culture technique PG broth was reinoculated with mycelial mat (5mm diameter) of 5 days old culture of *S. rolfsii.* Sole inoculation with pathogen in both solid and liquid media was served as control. Plates were incubated for 6 days at 30°C and inhibition of colony growth was measured and compared with the control, where as broths were kept at 30°C for 72 h in a rotary shaker and the mycelial dry weight were estimated. The liquid

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culture was filtered through pre weighted Watman No. 1 filter paper and was dried for 24 h at 70°C and compared with the control set. Microscopic studies were also performed to detect physical and / or morphological changes of mycelia.

Some biochemical studies regarding secondary metabolites were performed to evaluate the nature of antagonism of this strain. Production of volatile compound was assayed by 'inverted plate technique' (5) where two lids of separate petriplates were taken and poured with 20 ml of PGA. One was inoculated with a sclerotium and the other was streaked with a loopful of 2 days old fluorescent *Pseudomonas* BRL-1 culture. The petriplates were sealed mouth to mouth with parafilm. Control set consisted of only *S. rolfsii* inverted over uninoculated PGA plate. Plates were incubated at 30°C for 5 days as triplicate. After incubation period colony diameter of the fungal pathogen was measured and compared with the control set. Estimation of hydrogen cyanide production was done following the method of Wei *et al.* (24). Bacterial culture was inoculated in TSA medium supplemented with amino acid glycine (4.4g/l of medium). A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% (yellow) and sodium carbonate (2%) was placed in the upper lid of the petridish. The petridishes were then sealed with parafilm and incubated at 30°C for 4 days. A change of colour of the filter paper strip from yellow to brown indicates the production of HCN. Production of diffusible antibiotic was detected by an 'agar layer technique' (7). PGA plates covered with a cellophane membrane were inoculated in the center with 100 μl of the antagonist suspension (2×10^7 cfu/ml). After incubation for 48 h at 30°C, the membrane with the grown bacterial isolate was removed, and the plates were inoculated in the middle with a sclerotium of *S. rolfsii.* Controls were run with sterile water instead of bacterial suspension on the cellophane membrane. Plates were further incubated at 30°C for 4 days and the growth of the pathogen was measured and compared with the control set. Protease activity was performed by inoculating the antagonist on minimal media (5X: $(NH_4)_2SO_4$ -5g; KH_2PO_4-22.5g; K_2HPO_4-52.5g; Na-citrate-2.5g; distilled water-1L) containing 3% of skim milk and 1% agar; incubate at 30°C for 24-48 h and testing with 1% Tri Carboxylic Acid (TCA) solution. Presence of clear zone around the colonies indicated protease activity (8). To test the chitinololytic activity, the bacterium was inoculated on Luria Bertani (LB) medium (9) supplemented with 0.5% colloidal chitin and incubated at 30°C for three days. Formation of a clear halo region around the colonies indicated chitinase activity of the strain (7). IAA production was quantified spectrophotomertically growing the fluorescent *Pseudomonas* BRL-1 in 10 ml of minimal salt media supplemented with 100mg/ml of tryptophan, and incubated at 30°C under shaking for 48h. Broth culture was centrifuged at 7500 rpm for 10 min. To 1ml of culture supernatant, 2ml of Salkovsky reagent was added and incubated at 30°C for 25 min. Absorption was read at 530 nm and levels quantified from standard curve of IAA (8). Chrome Azurol S (CAS) agar (blue agar medium) was used to detect siderophore production (21). Fluorescent *Pseudomonas* BRL-1 was inoculated at the center of the plate and incubated at 30°C for 72 h. Appearance of yellow to light orange halo surrounding the bacterial colony indicates the production of siderophore.

In dual culture, significant growth inhibition of *S. rolfsii* by fluorescent *Pseudomonas* BRL-1 was observed both in solid and liquid culture. In solid culture, mycelial growth was restricted near bacterial streak and continued away from it as evident from figure 1A-1C. Increase in incubation period was proportionate to growth inhibition of *S. rolfsii* upto 6 days (Fig. 1F). Microscopic study of mycelia form interacting zone showed hyphal shriveling, mycelia deformities, swelling, fragmentation, short branching, and finally resulting into lysis (Fig. 1E). Co-culture of *S. rolfsii* and fluorescent *Pseudomonas* BRL-1 in liquid media resulted in 55 percent reduction in the mycelial dry weight. Among different biochemical tests performed to detect the cause of inhibitory effect of fluorescent *Pseudomonas* BRL-1, the isolate showed significant protease, chitinase, IAA and siderophore producing activity (Table 1). Lorito *et al.* (15) and Dunne *et al.* (6) reported earlier that the exposure of selected phytopathogenic fungi to lytic enzymes such as chitinase, protease can result in the degradation of the structural matrix of the fungal cell wall. So the reduction of mycelial dry weight might be coincided by the chitinase and protease activity. In general Indoleacetic acid is universally accepted as a plant growth promoter. Chirst and Mosinger (1989) (4) reported that this

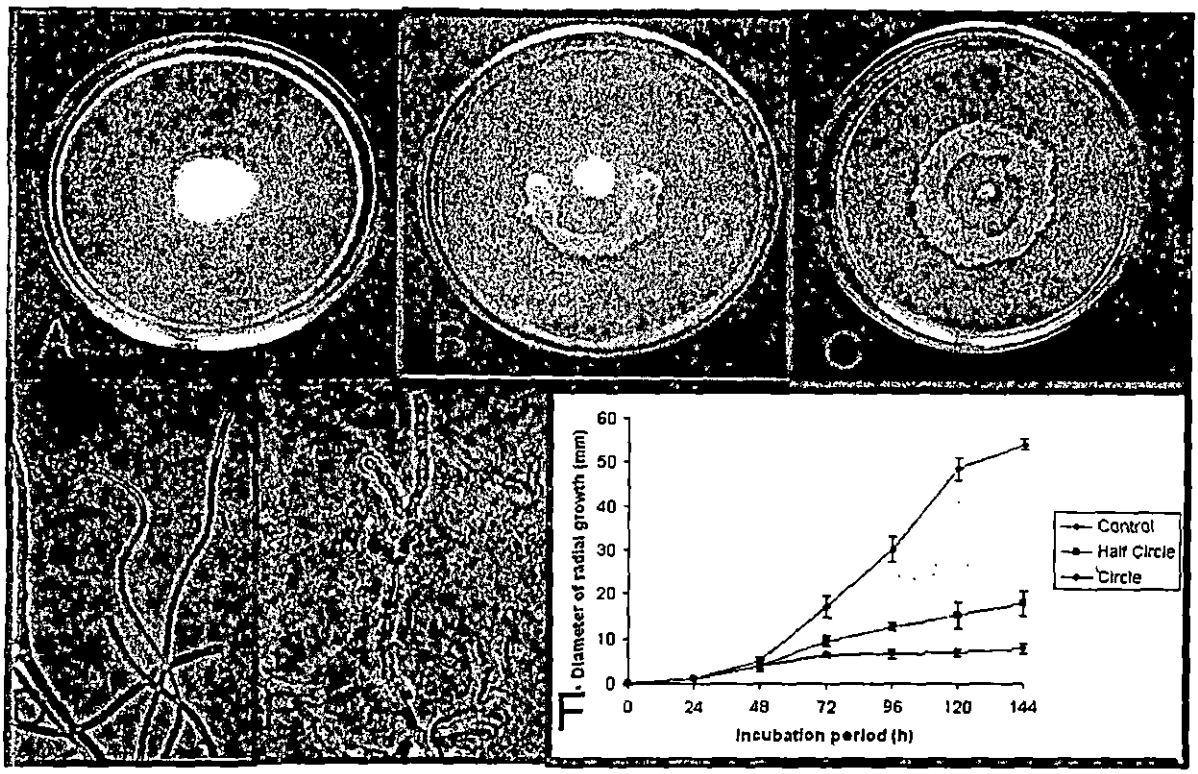

Fig. 1. Inhibitory effect of fluorescent *Pseudomonas* BRL-1 on *S. rolfsii*. (A) Control; *S. rolfsii* colony in pure culture showing more rapid growth at 30°C; (B) Colony of *S. rolfsii* showing restricted growth, but growing freely towards the top end away from the U shaped streak culture of the antagonist and (C) Colony of *S. rolfsii* showing restricted growth due to the bacterium inoculated as a circular streak. (D) Microscopic observations of mycelium from set 'A' (control) (E) Microscopic observations of mycelium from set 'C' (treated) (F) Incubation period verses radial growth of the fungi in control and treated sets. Data represents the mean of triplicate sets of experiments

phytohormone could also induce resistance through PR protein production; Sharaf and Farrag (2004) (22) reported that IAA reduce spore germination, mycelial dry weight and protein content of the pathogenic fungi and thus prevent significantly any chance for disease induction by soil pathogens. Our study complements their findings by demonstrating significant production of IAA by fluorescent *Pseudomonas* BRL-1. CAS agar (blue agar) plate assay indicated the hydroxamate nature of siderophore production by fluorescent *Pseudomonas* BRL-1 as indicated by orange halo around the colony. Siderophore have been shown to play a role in increased growth response of certain plants to treat the planting material with fluorescent pseudomonads (1, 14, 19). The response is thought to involve suppression of deleterious rhizospheric microorganism (14, 19, 20).

Table 1. Showing different secondary metabolites production and enzymatic activity of fluorescent *Pseudomonas* BRL-1

Different Metabolites	Rate of Production
Siderophore	+++
Antibiotic Substances	-
HCN	-
Volatile substances	-
Chitinase	+
Portease	++
IAA	+++

'+++' Stronger production, '++' Moderate production, '+' Low production, '-' No production

Thus the present study concluded that the fluorescent *Pseudomonas* BRL-1 showed significant antagonistic property through combined and / or

individual effect of siderophore, proteolytic enzyme, IAA and chitinolytic activity. Considerable attention has been paid to plant growth promoting rhizobacteria (PGPR), as the best alternative to chemicals to facilitate eco-friendly biological control of soil and seed borne pathogen (18). These observations and further study might help in developing this PGPR (fluorescent *Pseudomonas* BRL-1) as a potential biocontrol agent against *S. rolfsii.*

REFERENCES

1. **Ahl, P., Voisard, C.** and **Defago, G.** (1986). *Phytopathol.* **116:** 121-134.
2. **Basha, S.** and **Ulagnathan, K.** (2002). *Curr. Sci.* **82:** 1457-1463.
3. **Bilgrami, K.S., Jamaluddin, S.** and **Rizwi, M.A.** (1991) *Fungi of India List and References.* Todays and Tomorrow's Printing Publishers. New Delhi. pp. 454-456.
4. **Christ, U.** and **Mosinger, E.** (1989). *Physiol. Mol. Plant Pathol.* **35:** 53-65.
5. **Dennis, C.** and **Webster, J.** (1971). *Trans. Br. myco. Soc.* **82:** 41-48.
6. **Dunne, C., Crowley, J.J. Moenne-Loccoz, Y., Dowling, D.N., de Brunijn, F.J.** and **O'Gara, F.** (1997). *Microbiology.* **143:** 3921-3931.
7. **Frandberg, E.** and **Schnurer, J.** (1998). *Can. J. Microbiol.* **44:** 121-127.
8. **Gaur, R., Shani, N., Kawaljeet, Johri, B.N., Rossi, P.** and **Aragno, M.** (2004). *Curr. Sci.* **86:** 453-457.
9. **Gunasekaaran, P.** (1995). *Laboratory Manual in Microbiology.* New Age International (P) Limited, New Delhi. pp. 125.
10. **Holt, G.J., Krieg, R.N., Sneath, P.H.A., Staley, J.T.** and **Williams, S.T.** (1994). *Bergy's Manual of Determinative Bacteriology.* Willims & Wilkins, A Waverely Company. Baltimore, Philadelphia, Hong Kong, London, Munch, Sydney, Tokyo, pp.151-168.
11. **Huang, H.C.** and **Hoes, J.A.C.** (1976). *Can. J. Bot.* **54:** 406-410.
12. **Keel, C. U., Schnider, M., Maurhofer, C., Voisard, J., Laville, U., Burger, P., Wirthner, D.** and **Haas, G.** (1992). *Mol. Plant-Microbe Interact.* **5:** 4-10.
13. **King, E.O., Ward, M.K.** and **Raney, D.E.** (1954). *J. Lab Clin. Med.* **44:** 301-307.
14. **Kloepper, J.W.** and **Schroth, M.N.** (1981). *Phyotopathology* **71:** 1020-1024.
15. **Lorito, M., Peterbauer, C., Hayes, C.K.** and **Harman, G.E.** (1994). *Microbiology.* **140:** 623-629.
16. **Neilands, J.B.** (1981) *Annl. Rev. Biochem.* **50:** 715-731.
17. **O'Sullivan, M, Stephens, P.M.** and **O'Gara, F.** (1991). *Soil Biol. Biochem.* **23:** 623-627.
18. **Rameshkumar, N., Thirumalai Arasu, V.** and **Gunasekaran, P.** (2002). *Curr. Sci.* **82:** 1463-1466.
19. **Scher, F.M.** and **Bakker, R.** (1982). *Phytopathology* **72:** 1567-1573.
20. **Schippers, B., Bakker, A.W.** and **Bakker, P.A.H.M.** (1987). *Annu. Rev. Phytopath.* **25:** 339-358.
21. **Schwyn, B.** and **Neilands, J.B.** (1987). *Annal. Bichem.* **160:** 47-56.
22. **Sharaf, E.F.** and **Farrag, A.A.** (2004). *Pol. J. Microbiol.* **53:** 111-116.
23. **Skidmore, A.M.** and **Dikinson, C.H.** (1976). *Trans. Br. mycol. Soc.* **66:** 57-64.
24. **Wei, G., Kloepper, J.W.** and **Sadik, T.** (1991). *Phytopathology.* **81:** 1508-1512.

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