PLANT GROWTH PROMOTING RHIZOBACTERIA MEDIATED INDUCED SYSTEMIC RESISTANCE AGAINST BACTERIAL WILT IN GINGER

By

RESHMY VIJAYARAGHAVAN

THESIS

submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University

Department of Plant Pathology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR-680 656
KERALA, INDIA

2007

DECLARATION

I, hereby declare that the thesis entitled **"Plant growth promoting rhizobacteria mediated induced systemic resistance against bacterial wilt in ginger"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Vellanikkara,
30-11-07

RESHMY VIJAYARAGHAVAN

CERTIFICATE

Certified that the thesis entitled **"Plant growth promoting rhizobacteria mediated induced systemic resistance against bacterial wilt in ginger"** is a record of research work done independently by Ms. Reshmy Vijayaraghavan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara,
30-11-07

Dr. Koshy Abraham

(Major Advisor, Advisory Committee)
Professor & Head
Department of Plant Pathology
College of Horticulture
Vellanikkara.

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Reshmy Vijayaraghavan (2003-21-13)** a candidate for the degree of **Doctor of Philosophy in Agriculture**, with major field in Plant Pathology, agree that the thesis entitled **"Plant growth promoting rhizobacteria mediated induced systemic resistance against bacterial wilt in ginger"** may be submitted by Ms. Reshmy Vijayaraghavan in partial fulfilment of the requirement for the degree.

Dr. Koshy Abraham
(Major Advisor)
Professor & Head
Department of Plant Pathology
College of Horticulture
Vellanikkara.

Dr. M.V. Rajendran Pillai
Professor
Department of Plant Pathology
College of Horticulture
Vellanikkara.

Dr. E.V. Nybe
Professor and Head
Dept. of Plantation Crops & Spices
College of Horticulture
Vellanikkara.

Dr. A. Augustine
Professor, CPBMB
College of Horticulture
Vellanikkara.

Dr. D. Girija
Associate Professor
CPBMB, College of Horticulture
Vellanikkara.

External Examiner

ACKNOWLEDGEMENT

It is with great respect and devotion, I place on record my deep sense of gratitude and indebtedness to my major advisor ***Dr. Koshy Abraham****, Professor & Head, Department of Plant Pathology, for his sustained and valuable guidance, constructive suggestions, unfailing patience, friendly approach, constant support and encouragement during the conduct of this research work and preparation of the thesis. I gratefully remember his knowledge and wisdom which nurtured this research project in right direction without which fulfillment of this endeavor would not have been possible.*

I place a deep sense of obligation to ***Dr. M.V.Rajendran Pillai****, Professor, Department of Plant Pathology, member of my Advisory Committee for the help and co-operation received from him during the entire programme.*

I am deeply indebted to ***Dr. E.V.Nybe,*** *Professor and Head, Department of Plantation Crops and Spices and member of my Advisory Committee for his unbounded support, valuable suggestions and whole- hearted co-operation towards the satisfactory fulfillment of this endeavor.*

I am extremely delighted to place on record my profound sense of gratitude to ***Dr. A. Augustine****, Professor of CPBMB, for his unstinted support, critical comments and valuable advice during the preparation of this manuscript. He inspite of his busy schedule has offered constructive suggestions for the betterment of my thesis work.*

I am deeply obliged to ***Dr. D.Girija,*** *Associate Professor, CPBMB, for the generous and timely help she has always accorded to me during the course of this study.*

I am very thankful to ***Dr. Sally K Mathew****, Professor, Department of Plant Pathology, College of Horticulture for kindly providing facilities, timely help and valuable suggestions at various stages of my work.*

My sincere thanks are due to ***Dr. S. Beena, Dr. Rehumath Niza, Dr. Sheela Paul, Dr. K, Surendra Gopal,*** *Department of Plant Pathology for the valuable help and advice rendered during the course of my study.*

I am genuinely indebted to ***Shri. S. Krishnan****, Assistant Professor, Department of Agricultural Statistics for his valuable assistance and guidance during the statistical analysis of the data.*

Words cannot really express the true friendship that I relished from Smitha, Deepthy, Alphonsa and Sindhu for the sincere help, timely suggestions and

encouragement which gave me enough mental strength to get through all tedious circumstances.

Let me express my sincere thanks to Elisa lincy, Sincy, Baburaj, Vijini, Deepa Sebastian, Seenath, Cinu, Nisha and Kochurani for their prompt help during the thesis work.

I wish to express my sincere thanks to all the non-teaching staff members, and labourers of the Department of Plant Pathology for the timely and whole hearted co-operation and assistance they always accorded me during the course of investigation.

I am happy to place on record my sincere thanks to my juniors of Dept. of Plant Pathology for their innumerable help and support during the course of this study.

I thankfully acknowledge Mr. Santhosh for his valuable help in computer work.

I shall be failing in my duty, if I forget to place on record the facilities offered by my alma-matter, College of Horticulture, Vellanikkara.

I am deeply indebted to my loving parents and sisters without whose moral support, blessings and affection this would not have been a success.

Words can't express my soulful gratitude to my caring husband for without his selfless sacrifice, boundless patience and unflagging interest this study would never have seen the light.

*Finally I blow my head before **THE ALMIGHTY** whose blessings enabled me to undertake this venture successfully.*

RESHMY VIJAYARAGHAVAN

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Dedicated
to my
beloved
family

Introduction

1. INTRODUCTION

Ginger [*Zingiber officinale* (Rosc.)], belonging to the family Zingiberaceae, is one among the most important and valued spice crops in the world after black pepper and cardamom. It is valued throughout the world for its aroma, flavour and pungency. Ginger, being considered as a native to tropical Southeast Asia, is now being cultivated in almost all tropical countries. The word 'Ginger' is derived from a Sanskrit word '*Singabera*' meaning shaped like deer's antlers / horn, an obvious reference to the 'hand' and 'finger' of ginger rhizomes and this can be traced back to Latin word *'Zingiber'* which was in turn a loan from the Greek word '*Zingiberis*'. It was the English botanist William Roscae who gave the plant the name *'Zingiber officinale*' in 1807.

An underground rhizome, ginger is grown in India for centuries both as a fresh vegetable and as a dried spice. India, the world's largest producer, consumer and exporter of ginger, has a predominant position in the global market and accounts for 50 per cent of world's total production. India contributes 33 per cent of the world production of dry ginger accounting for substantial foreign exchange earning. India production of dry ginger rhizomes was about 2, 30,000 million tonnes from an area of 70,000 ha and had earned foreign exchange to the tune of Rs. 5950 lakhs through the export of 13,000 million tonnes in the year 2004-2005 (FAO, 2005). Though grown in most parts of India, the premium quality ginger comes from Kerala as the state is blessed with a congenial climate and a rich earthy soil. Kerala occupied the largest area of 8418 ha with a production of 2086 tonnes during 2002-2003 (Spices Board, 2005). Ginger which is extensively used as a spice is also accredited with many medicinal properties. Indian ginger, known in the world market as 'Cochin Ginger' and 'Calicut Ginger' has been acclaimed world wide for its characteristic taste, flavour and aroma.

One of the important factors which limit the production of ginger is the occurrence of diseases. Bacterial wilt disease, incited by *Ralstonia solanacearum*

Yabuuchi (Smith), is at present considered as a very serious threat in most of the ginger growing areas. It causes significant losses each year to commercial ginger industry. The pathogen infects over 450 plant species including many economically important crops other than ginger. Because of its destructive potential to various crops, the control of *R. solanacearum* has attracted much research attention (Hayward, 1994). This soil and seed borne pathogen is not amenable to any of the conventional methods of management. Moreover, none of the varieties and cultivars of ginger is known to possess any absolute resistance against the disease.

Currently, much importance is given to ecofriendly management strategies such as the use of bioagents due to the ecological hazards inflicted by the excessive use of plant protection chemicals. It is widely accepted that a group of beneficial bacteria popularly termed as 'plant growth promoting rhizobacteria' (PGPR), are ideal for use as biocontrol agents, as they can provide first hand defense for plant roots against the attack by various plant pathogens (Kloepper *et al.,* 1991). Studies involving these rhizobacteria as biocontrol agents have revealed the potential usefulness of these organisms in managing many soil borne pathogens. Apart from suppressing soil borne inoculum by production of inhibitory metabolites, the application of PGPR can also improve plant growth and vigour. They are also known to induce systemic resistance in the host plant, which may account for less disease and increased yields (Haas and Defago, 2005). In this context, it is pertinent to explore the potentiality of PGPR associated with rhizosphere of ginger in improving the growth and vigour of the plant and also their effect in the management of bacterial wilt disease, which are viewed as a novel tool to impart substantial benefit to the farmers.

Considering the above facts, this research project was undertaken to study the role of PGPR on the management of bacterial wilt disease of ginger which encompasses the following objectives:

- Isolation of the pathogen.
- Isolation of rhizobacteria of ginger.

- Study of the i*n vitro* antagonistic effect of rhizobacteria against the pathogen.
- Evaluation of selected rhizobacterial isolates for growth promotion in ginger.
- Induction of systemic resistance in ginger due to PGPR against *R. solanacearum* by spectronic assay of phenolics, proteins and enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL). Native PAGE analysis of proteins, PO and PPO were also assessed.
- An attempt to elucidate the molecular mechanism of induction of systemic resistance by synthesizing cDNA (complementary DNA) and amplifying it by polymerase chain reaction (PCR) with random primers.
- Compatibility of the selected antagonists with common plant protection chemicals, fertilizers and *Trichoderma* spp. apart from the compatibility among the isolates.

Review of literature

2. REVIEW OF LITERATURE

Bacterial wilt of ginger, incited by the ubiquitous soil borne pathogen, *Ralstonia solanacearum* is considered as a serious threat to ginger cultivation next to the destructive rhizome rot disease and at times it causes severe damage in the field. This disease assumes serious proportions especially in the high ranges during warm humid season which is one of the factors for low production of ginger in India. Of late, the disease has become a serious obstacle to ginger cultivation in other parts of the State also, resulting in total crop loss under conducive climatic conditions (Mathew and Peter, 2004).

Bacterial wilt was reported for the first time from Italy in 1882 (Walker, 1952). Later, it was Smith (1896), at the end of the 19th century, first described the disease and its causal agent on potato, tomato and brinjal, southern USA and South America. Bacterial wilt of ginger (*Zingiber officinale* Rosc.) was first reported from Mauritius (Orian, 1953). Subsequently, the disease was reported from Hawaii by Ishii and Aragaki (1963), Malaya by Jamil (1964), Queensland by Hayward *et al.* (1967), Phillipines (Zehr, 1969), Malaysia (Chew, 1969), Srilanka (Gunawardena *et al.,* 1980), Indonesia (Mulya *et al.*, 1990 and Supriadi*.,* 2000), Japan (Tsuchiya *et al*., 2002), China (Yu-Zhang *et al*., 1997), Uganda (Sonko *et al*., 2005) and from Thailand (Thammakijjawat *et al.,* 2006).

In India, Thomas (1941) made the first authentic report on the occurrence of bacterial wilt of ginger. Later, it was reported from Kerala by Sarma *et al*. (1978) and Mathew *et al.* (1979). Srivastava *et al.* (1998) reported bacterial wilt of ginger from Sikkim. The disease was observed in 80 per cent of 310 ginger fields surveyed in Himachal Pradesh (Sharma and Rana, 1999). Nath *et al.* (2004) noticed a severe outbreak of wilt in ginger fields causing a destruction of 60-70 per cent yield loss in the fields of Jorhat, Assam during 1998-1999. Mondal *et al.* (2004) noticed the occurrence of the disease in West Bengal also.

2.1 THE PATHOGEN

The wilt bacterium was described for the first time as *Bacillus solanacearum* by Smith (1896). Later on, he described the bacterium causing wilt disease as *Pseudomonas solanacearum* (Smith, 1914). The bacterial wilt pathogen exhibits great degree of phenotypic and genotypic diversity and in the following years, at least five pathogenic races based on the host range and five biovars based on utilization of sugars have been discriminated (Buddenhagen *et al*., 1962; Hayward, 1964). Samuel (1980) characterized the isolates of ginger and reported that they belonged to race and biovar III of *P. solanacearum* where the ginger isolate caused wilting in tomato but tomato isolate failed to infect ginger. Later, Persley *et al.* (1985) grouped the bacterial wilt pathogen of ginger under Race 4. Jyothi (1992) characterized *P. solanacearum* from ginger as biovar III. In a taxonomic study of certain non - fluorescent species of the genus *Pseudomonas*, the genus *Burkholderia* was projected, so as to encompass the variation found in this group and the name *Burkholderia solanacearum* was proposed (Yabuuchi *et al.,* 1992). Subsequent study of this genus based on sequencing of 16s rRNA genes and polyphasic taxonomy led to the proposal of the genus '*Ralstonia*' which were sufficiently distinct from other members of the genus and the bacterial wilt pathogen was finally renamed as *Ralstonia solanacearum* by Yabuuchi *et al*. (1995). Nath *et al.* (2001) noticed that the strains of *R. solanacearum* of ginger from Assam belonged to race 4. Mathew *et al*. (2002) identified biovar III and IIIA infecting ginger. Likewise, Boonsuebsakul *et al.* (2003) noticed that all the 10 strains of *R. solanacearum* of ginger belonged to race 1 and some to biovar 3 and 4 whereas Sambasivam (2003) reported that all the 15 isolates of *R.solanacearum* of ginger belonged to biovar IIIA. However, Kumar and Sarma (2004) observed that all the isolates of *R. solanacearum* of ginger belonged to biovar 3.

2.1.1 Characteristics

Ralstonia solanacearum is rod shaped, non spore forming, non capsulate, gram negative, small bacteria with polar flagella, 1 to 4 in number. Stanford and

Wolf (1917) observed that the bacterium formed circular, glistening white colonies, slightly raised with smooth margin and appeared within 36-48 h. Kelman (1954) distinguished colony variants on Tetrazolium medium. The normal or wild type were irregularly round, entire, white or white with light pink centre and the wild type colonies are highly virulent and producing wilt in 14 days whereas the mutant type were round, translucent, smooth, deep red with a narrow light bluish margin and is either weakly pathogenic or non pathogenic. The virulence could be retained by preserving the culture in mineral oil (Kelman and Jenson, 1951) or in sterile water at room temperature (He *et al,* 1983 and Prior and Steva, 1994).The aerobic nature of the bacterium was well established by many workers (Smith, 1914 and Prior *et al*., 1990). However, Kelman and Jenson (1951) opined that it could grow anaerobically. Devi (1978) also noticed both aerobic and anaerobic growth of the bacterium. Kumar and Sarma (2004) also noticed that all the ginger isolates were highly fluidal with characteristic spiral pink centre on TZC medium.

Biochemical studies conducted by various workers revealed that different isolates of *R. solanacearum* produced a non-fluorescent but diffusible brown pigment. Gelatin hydrolysis of the bacterium was negative or weak; starch and esculin not hydrolysed, nitrate reduced by nearly all strains, many produce gas (denitrification), oxidative metabolism of glucose only, no growth at 4°or 40°C; growth weak at pH 8 with no growth at pH 4 or 9; oxidase and catalase positive; arginine dihydrolase, lecithinase (egg yolk) and lipase negative (He *et al.,* 1983; Swanepoel and Young, 1988; Rani, 1994; Paul, 1998; James, 2001; Mathew *et al.,* 2002). Characterization of isolates into biovars was made through test for utilization of carbon sources (Hayward, 1964). Most strains produce tyrosinase, the main exceptions being those isolated from the family *Musaceae*. Growth in 1 per cent NaCl broth but little or no growth in 2 per cent NaCl. This species belongs to r RNA group II. It is readily distinguished from other members of the group by failure to grow at 40°C (Jyothi, 1992; Prior and Steva, 1994; Mathew, 2001; OEPP / EPPO, 2004).

2.1.2 Ecology, survival and disease spread

Ralstonia solanacearum, the agent of bacterial wilt, infects over 450 plant species, including many economically important crops (Hayward, 1991). It has an extremely wide host range, but different pathogenic varieties / races within the species may show very limited host ranges. The pathogen infects tomato, potato, capsicum, brinjal, banana, tobacco, mulberries, geranium and many other solanaceous crops which are all susceptible. A number of non-solanaceous weed hosts are also known to harbour *R. solanacearum* as symptomless carriers (Hayward, 1994).

Entry into plants is by way of injured roots, stem wounds or through stomata. Within the plant, the bacteria move in the vascular bundles, a process which is accelerated by higher temperature. Blocking of the vessels by bacteria is the major cause of wilting (Ono *et al*., 1984). Disease severity mostly increases if *R. solanacearum* is found in association with root nematodes. In tobacco, nematode infestation changes the physiology of the plants causing susceptibility to bacterial wilt (Chen, 1984). Hayward (1991) reported that epiphytic phase was important in the life cycle of the bacterium and a high level of wilting occurred in soils previously planted with susceptible plant cultivars. The natural spread of most of the races of the pathogen is very limited and slow. However, the main path for international spread is by infected rhizomes (latent) and other vegetative planting materials (Kelman *et al.,* 1994). Although the disease is primarily soil borne, there is increasing evidence of long range dispersal of the pathogen on planting material. The dipteran flies feeding on diseased ginger rhizomes may carry the infection to plants on which it subsequently feed. Irrigation water and use of contaminated farm equipment are other means of carrying the bacteria from one area to another. The route of infection is usually through the roots. (Dohroo, 2001). *R. solanacearum*, being seed rhizome borne, the low level of inoculum is very difficult to be detected and monitored by conventional methods, which is a prerequisite for production of healthy crop of ginger in the field (Kumar *et al.,* 2002). Therefore, they evaluated the suitability of NCM-ELISA kit which indicated that the antibodies developed at CIP, Lima, Peru

for potato strain of *R. solanacearum* was sensitive enough to detect *R. solanacearum* from ginger, chilli, tomato and *Chromolaena* where the sensitivity of the kit was determined to be 42 cells per ml of ginger extract. *R. solanacearum* is listed as a quarantine organism in European Union, where new legislation has been introduced to control and eradicate the organism (OEPP / EPPO, 2004).

2.1.3 Pathogenicity

Pathogenicity, a capacity of the pathogen to induce malfunctions or interfere with the physiological activities of the plant, can be determined by inoculating a suspension (10^6 cells ml^{-1}) of a 48 h culture into 5-10 susceptible plants at preferably the third true leaf stage or slightly older. Incubation should be for upto two weeks at 25-28^{o}C under high relative humidity conditions. The bacterium should be re-isolated from plants by taking a section above the inoculation point and placing it in a small volume of sterile distilled water or 50mM phosphate buffer and plating on agar media for observing typical colonies (European Union, 1998).

Okabe (1949) while studying *P. solanacearum* in Japan, related colony character with pathogenicity and noticed that weakly pathogenic or avirulent mutants differed in colony morphology from the fluidal wild type. To test the pathogenicity of *R.solanacearum*, several inoculation techniques have been attempted by earlier workers. Winstead and Kelman (1952) tested four different inoculation methods to test the pathogenicity of *R. solanacearum* strains infecting solanaceous crops. Among the different methods used like stem puncturing, root injury, root dipping and soil drenching, they found stem puncturing to be most effective method. This method was later followed by several other workers (Hussain and Kelman, 1958; He *et al.,* 1983 and Prior and Steva, 1994). Several other workers also followed root inoculation (Khan *et al.,* 1979, Swanepoel and Young, 1988; Paul, 1998 and James, 2001). Mathew (2001) and James (2001) found leaf clipping as effective method to inoculate chilli and tomato. Samabsivam (2003) reported pseudostem injection as the best method for inoculation in ginger. According to Kumar and Sarma (2004), all ginger isolates except one from Assam induced cent per cent wilting in ginger cv.

Himachal within a week and they observed that plants were wilted even at 3.2 x 10^2 cfu ml^{-1} in stem inoculation while in soil inoculation it was 10^5 cfu ml^{-1}. Molecular analysis by REP-PCR, ITS-PCR and RFLP-PCR of 33 strains of *R. solanacearum* isolated from ginger, paprika, chilli, tomato, chromolaena and potato from Kerala, Karnataka, W. Bengal and Assam revealed that the genetic diversity of *Ralstonia* is very low within ginger, confirming that the pathogen population is of clonal lineage and is distributed through rhizome transmission of the inoculum between locations also between seasons within the locality (Kumar *et al.,* 2004).

2.2 SYMPTOMATOLOGY

The pathogen, *R.solanacearum*, in general causes a complex disease syndrome resulting in complete wilting of the plant in several crops in different parts of the world. According to Hayward *et al.* (1967), Mathew *et al.* (1979) and Sharma and Rana (1999), the first conspicuous symptom was mild drooping and curling of leaf margins of the lower leaves which spread upwards. Yellowing starts from the lower leaves which gradually progresses upwards until all the leaves take on a wilted, golden appearance. In the advanced stage, the plants exhibit severe yellowing and wilting symptoms. The vascular tissues of the affected pseudostems show dark streaks. The affected pseudostem and rhizome when pressed gently extrudes milky ooze from the vascular strands and ultimately the rhizome rots. Dohroo (2001) and Anandaraj *et al*. (2005) noticed that as the disease progress, the stem becomes water soaked, slimy to touch and readily breaks away from the rhizome and the entire plant died within 2-3 weeks. Diseased rhizomes are usually darker than the healthy ones and have water soaked areas with pockets of milky exudate visible beneath. Under cool growing conditions, wilting and other foliar symptoms may not occur.

2.3 FACTORS AFFECTING DISEASE DEVELOPMENT

The soil factors are known to exert a greater influence on the pathogen population in the soil. Mathew and Peter (2004) noticed that the disease occurs in all soil types of Kerala as well as in both acidic soils of pH 5.0 to 6.9 and alkaline soils

of pH 7.4 to 8.5 as well. High soil temperature of 28-36ºC and high soil moisture (50 to 100 % water holding capacity) are the main factors associated with high wilt incidence.

According to Quinon *et al*. (1964), disease development in susceptible cultivars was favoured by high moisture and temperature of 30-35ºC. The disease was reported to be serious on sandy, loam, clay and peat soils but is never found in marshy soils (Vijaykumar *et al.,* 1985). The disease was most severe at 24-35ºC which was seldom found in temperate climates where the mean temperature for any winter months falls below 10ºC (Swanepol, 1990). High soil moisture and periods of wet weather or rainy seasons are associated with high disease severity. Soil moisture is also one of the major factors affecting reproduction and survival of the pathogen; the most favourable soil moisture is -0.5 to -1 bar while -55.0 to -15.0 bar is unfavourable (Nesmith and Jenkins, 1979). Radhakrishnan *et al.* (1997) reported that the disease incidence was reduced by increasing the shade intensity. According to Nath *et al.* (2004), the disease was particularly severe after the high rainfall when high humidity prevailed for a considerable period. High temperature favoured rapid disease development resulting in rapid multiplication of the bacteria in the host and its successive increase released the inoculum from roots of diseased plants which caused infection of neighbouring plants. Maximum wilt incidence is recorded during warm humid season (September - November). The bacterium can survive more temperate, cooler temperatures thereby posing a serious threat to ginger cultivation in the high ranges. According to Hepperely *et al*. (2004), ginger wilt is a complex and difficult disease to control infecting the ginger crop through all phases of production cycle. It is present systemically in seed rhizomes as both an active and latent infection that contaminates seed pieces when they are cut and prepared for field planting.

2.4 MANAGEMENT OF THE DISEASE

From a historical point of view, there have been many methods developed and attempted to control bacterial wilt of ginger including cultural which includes

host resistance, biological and the use of chemicals which are directed towards reducing the inoculum level in the field.

2.4.1 Cultural control

The pathogen being seed rhizome borne, heated air treatment with 75 per cent RH for 30 and 60 min proved to be a convenient method to disinfect ginger seed pieces of the bacterial wilt pathogen (Tsang and Shintaku, 1998). Hepperly *et al.* (2004) observed that surface sterilizing the seed pieces in 10 per cent solution of household bleach for 10 minutes could reduce the incidence of bacterial wilt disease. Procurement of healthy rhizome seed from a bacterial wilt-free source and planting the same in clean land where proper drainage is provided are some of the recommended practices (Kumar and Sarma, 1999; Indo Swiss Project-Sikkim, 2005). Since, the disease occurs when ginger is grown on the same piece of land year after year, rotation using tomato, potato, capsicum, chillies, brinjal and peanut are avoided as these plants act as hosts for the wilt organism. Crops such as bean, cucurbits and strawberry provide a suitable rotation. Weeds susceptible to bacterial wilt should be suppressed. Parthasarathy (2005a) reported that the survival of *R. solanacearum* on ginger rhizomes was severely affected by rhizome solarization for a period of two hours.

Crop rotation of 5-7 years without susceptible crops has been recommended (Vijaykumar *et al.,* 1985). Anith *et al.* (2000) noticed that soil solarization after irrigation, 45 days prior to planting was found effective in reducing bacterial wilt incidence in a wilt sick field in Waynad district of Kerala. Dohroo (2001) reported that all the crop residues, especially roots and decomposed rhizomes at harvesting, should be destroyed by burning and all tools should be disinfected before proceeding from one farm to another, especially at the time of weeding and earthing up. Moreover, the implements should be disinfected by dipping in formalin solution for at least five minutes and washing the same with water.

Amendment of soils with organic and inorganic substances has been practiced for managing soil borne plant pathogens which showed a reduction in disease incidence in different crops including ginger by *R. solanacearum*. Vudhivanich (2002) noticed that the population of *R.solanacearum* causing bacterial wilt of ginger in the soil amended with urea and calcium oxide decreased substantially within three weeks of planting which may due to the toxicity of ammonium, ammonia and nitrate degraded from urea in high pH of 7.0 to 7.2. According to Hepperly *et al.,* (2004) addition of gypsum at the rate of 300 ml per cu. ft. of the growing medium consisting of peat moss, perlite and vermiculite along with triple super phosphate at the time of planting ginger rhizomes helped in production of bacterial wilt free ginger rhizomes.

The combined application of organic amendments like oil cakes or saw dust or coir pith with antibiotic treatment was effective in reducing wilt incidence in tomato (Jayaprakash, 1977). The disease may also be controlled by application of fertilizers to change soil pH where in USA, the pathogen was eradicated by covering the soil pH to 4-5 in summer and raising it to pH 6 in the autumn (Vijaykumar *et al.,* 1985). Similarly, Devi (1978) observed that sawdust along with urea combined with Agrimycin (200 ppm) showed reduction in wilt incidence of tomato. Claiorn (1984) recommends liming with calcium oxide @ 1 t ha^{-1} or calcium carbonate @ 10 t ha^{-1} one month before urea application for reducing bacterial wilt of egg plant.

Ho (1988) and Kishun and Chand (1988) observed that application of bleaching powder @ 15 $kgha^{-1}$ was effective against bacterial wilt of tomato. Chilli plants treated with cow dung and two applications of Streptocycline (1000 ppm) showed a tendency to reduce the wilt incidence (Jyothi, 1992). Dhital *et al.* (1997) reported the use of stable bleaching powder (SBP) at the rate of 25 kg ha^{-1} for the control of bacterial wilt in potato both under green house and field conditions. Similarly, Mazumdar (1998) noticed that 5 and 10 g l^{-1} of bleaching powder could also reduce disease incidence and increase the yield in tomato. Application of bleaching powder in soil before planting also reduced wilt incidence in solanaceous vegetables (Mathew, 2004). Combination of Actigard (acibenzola-S-methyl) with the

S-H mixture (bagasse, rice husk, oyster shell powder, urea, KNO_3, calcium super phosphate and mineral ash) significantly reduced bacterial wilt of tomato caused by *R. solanacearum* (Anith *et al*., 2004).

Rani (1994) observed that botanicals *viz., Ocimum* spp. and garlic ($50gl^{-1}$) exhibited maximum inhibition of the *R. solanacearum* of ginger under *in vitro* conditions. Coffee leaf mulch and bacterial antagonist were effective to control the ginger bacterial wilt disease (Idris and Nasrun, 2000). The aqueous extract of *Aloe vera* followed by *Psidium guajava* was found to be the most effective in suppressing the growth of *R. solanacearum* causing wilt of ginger (Devanath *et al.*, 2002).

2.4.2 Chemical control

Wilt disease being systemic in nature, it is difficult to have total control of the disease using plant protection chemicals. However, the use of antibiotics and some fungicides were found to be effective in managing the disease to some extent.

2.4.2.1 Antibiotics

Moorgan and Goodman (1955) found aureomycin and terramycin to be effective to inhibit *P. solanacearum*. Likewise, Hindaka and Murano (1956) found Streptomycin to inhibit the pathogen. The *in vitro* effectiveness of Ambistryn-S and Agrimycin-100 against ginger isolate of *P. solanacearum* was noticed by Samuel (1980). He *et al.* (1983) and Prior and Steva (1994) reported that several strains of *R. solanacearum* were susceptible to ampicillin but resistant to chloramphenicol. *In vitro* studies revealed that, antibiotics *viz*., Ambistryn-S and Chloromycetin 1000 ppm exhibited maximum inhibition of *R.solanacearum* of ginger under *in vitro* conditions (Rani, 1994). The effectiveness of 500 and 1000 ppm concentrations of Streptomycin and Streptopenicillin over other antibiotics against wilt pathogen of ginger both under *in vitro* and *in vivo* conditions has been well elucidated by Singh *et al*. (2000). Whilst Penicillin G, tetracycline and Plantomycin did not inhibit the pathogen at any of the concentrations tested. According to Sambasivam (2003),

various isolates of *R.solanacearum* infecting ginger were resistant to Ampicillin and Rifampicin but sensitive to Chloramphenicol and Kanamycin. However, Carbenicillin, Nalidixic acid, streptomycin sulphate and Tetracycline showed a varied response with the isolates.

Under *in vivo* conditions, a Chinese bactericidal preparation of 95 Kekuling WP diluted at 1:500-1200 sprinkled onto the rhizosphere of ginger controlled the bacterial wilt incidence by 70.5 to 87.5 per cent (Zhang *et al*., 1993). Rani (1994) observed that plants treated with Ambistryn-S, terramycin and chloromycetin showed minimum wilt incidence. Similarly, pre-planting and pre-storage treatment in Streptocycline (200 ppm) along with Dithane M-45 (0.25%) and Bavistin (0.1%) delayed the disease development. The crop sprayed with Streptocycline solution (100 ppm) at regular intervals of 15 days also reduced the incidence of bacterial wilt in ginger (Dohroo, 2001). Likewise, seed rhizomes treated with Streptocycline 200 ppm for 30 min and shade dried before planting was found effective in reducing the wilt incidence (Anandaraj *et al*., 2005).

Attempts have been by many scientists to test the *in vitro* sensitivity of *R. solanacearum* infecting crop plants other than ginger to antibiotics. The inhibitory effects of Streptomycin and Streptocycline on *P. solanacearum* have been observed by many workers (Rangarajan and Chakravarti, 1969; Shivappashetty and Rangaswami, 1971). Several antibiotics like Oxytertacycline, Tetracycline, Penicillin G, Streptomycin were reported to inhibit the pathogen (Goorani *et al*., 1978). He *et al.* (1983) reported that all the strains of *P.solanacearum* from China showed susceptibility to many antibiotics but were resistant to penicillin, Viomycin and chloramphenicol. Gunawan (1989) found that optimum concentration for suppression of bacterial multiplication *in vitro* was 175 and 450 ppm of streptomycin sulphate. Paul (1998) observed that 250 and 500ppm concentration of Ambistryn-S, oxytetracycline and Streptocycline inhibited *R.solanacearum* under *in vitro* conditions. Likewise, Akbar (2002) reported that among the different concentrations of antibiotics tried, Ampicillin, streptomycin sulphate and Kanamycin at 100 and 200 ppm inhibited the pathogen whereas rifampicin, chloramphenicol and oxytertacycline

at the same concentration failed to restrict the growth of the pathogen. Kumar and Sarma (2004) noticed that all the ginger isolates tested were resistant to tetracycline, Polymixin B sulphate and chloramphenicol.

2.4.2.2 Fungicides

Fungicides with a broad spectrum activity, low mammalian toxicity and low residues would be the choice to manage disease in spices and plantation crops in India (Mouli *et al*., 2000). A perusal of the literature revealed that few reports on the *in vitro* and *in vivo* studies with fungicides on *R.solanacearum* of ginger. However, there are reports on the effect of fungicides on the bacterial wilt pathogen infecting different crops other than ginger.

Ojha *et al.* (1986) reported that complete control of bacterial wilt of ginger was obtained by rhizome teratmnt with Emisan 6 + Plantomycin for 30 minutes followed by three sprayings. Rani (1994) observed that, Bordeaux mixture, both under *in vitro* and *in vivo* conditions showed the maximum inhibition of wilt pathogen of ginger with minimum wilt incidence. According to Anandaraj *et al*. (2005) at the onset of wilt incidence of ginger is noticed in the field, all beds should be drenched with Bordeaux mixture 1 per cent or copper oxychloride 0.2 per cent to prevent the further spread of the pathogen.

Severin and Kupferberg (1977) reported that Bordeaux mixture, copper oxy chloride and Kocide were effective in controlling bacterial blight of walnut. Inhibitory action of Nabam (Dithane A-40) Maneb (Dithane M-22) and Dithane M-45 on bacterial wilt pathogen was studied by Goorani *et al.,* (1978). Leandro and Zak (1983) observed the inhibitory effect of Captan, Maneb, Mancozeb and Thiram on *R.solanacearum.* Jyothi (1992) reported that among the three fungicides *viz.,* Thiride, Blue copper and Bordeaux mixture, the latter recorded the maximum inhibition of *R.solanacearum*. Inhibition of *R.solanacearum* by Kocide (copper hydroxide) 0.15 per cent was reported by Akbar (2002). The incidence of bacterial wilt in tomato was

significantly less with Actigard (Acibenzola-S-methyl) treated plants than with non treated plants (Anith *et al.,* 2004).

2.4.3 Biological control

Biological control, an important component of the IDM package, has emerged as one of the important methods in the management of soil borne pathogens. Biological control reduces the dependence on high risk chemicals for disease management and is ecologically sound and environmentally friendly (Bowen and Rovira, 1999).

Biological control can be defined as reduction of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook and Baker, 1983a). There has been a remarkable increase of interest and research on biological control since 1960s because of the concern about environmental pollution, development of pesticide resistance in pathogens, lack of adequate or reliable resistance in crops to many important pathogens and the trend towards more intensive farming with less crop rotation (Cook and Baker, 1983b).

To date, antagonistic interactions have been exploited in the area of biological control of plant pathogens. Potential agents for biocontrol activity are rhizosphere competent fungi and bacteria which in addition to their antagonistic activity are capable of inducing growth responses by either controlling minor pathogens or producing growth stimulating factors (Cook and Weller, 1986). Moreover, biocontrol agents being ecofriendly, it is more attractive proposition of crop protection especially, where products are export oriented. In contrast to agrochemicals which get leached off during incessant rains, biocontrol agents gets stabilized once efficient strains that fit into the concerned ecological niche are introduced into a given environment. Also, biocontrol agents fit well with organic farming, a proposition which is gaining popularity in recent times (Harman *et al.,* 1989).

2.4.3.1 Screening and selection of efficient bioagents

Rhizosphere is an important ecological niche for isolation of antagonistic or hyperparasitic microorganisms (Schmidt, 1979). Selection and identification of growth promoting and disease suppressive biologically efficient microbes through *in vitro* and *in vivo* assays is crucial and is the first step towards the development of effective biological control before launching field evaluation (Weller*,* 1988). For biocontrol to be implemented on a practical level, the antagonists must be ecologically fit to survive, become established and function within the particular conditions of the ecosystem. To accomplish this, additional information regarding the mechanisms of action, ecological fitness, interactions, conditions and requirements responsible for effective biocontrol agent should be studied. Slininger *et al.* (2003) recently developed a relative performance index based on growth of bioagents and their antagonistic activity under different conditions.

2.4.3.2 Rhizobacteria as biocontrol agents

Bacteria are active colonizers of the rhizosphere and rhizoplane of several crop plants. Some of these are plant growth promoting rhizobacteria (PGPR) which are free living soil borne bacteria, isolated from the rhizosphere which when applied to seeds or crops enhance the growth of the plant as well as reduce the damage from soil borne pathogens (Kloepper *et al.,* 1980a). The term 'rhizobacteria' is used to describe a subset of rhizosphere bacteria able to colonize the root environment (Kloepper *et al.,* 1991). Beneficial root colonizing rhizosphere bacteria, the PGPR, are defined by three intrinsic characteristics (i) they must be able to colonize the root (ii) they must survive and multiply in microhabitats associated with the root surface in competition with other microbiota (iii) they must promote plant growth. PGPR competitively colonize plant roots, and stimulate plant growth and/or reduce the incidence of plant disease. 'Suppressive soils' contain rhizobacteria that are able to control plant diseases that are caused by fungi or bacteria (Bloemberg and Lugtenberg, 2001). The PGPR concept has been vindicated by the isolation of many bacterial strains that fulfill at least two of the three criteria described above

(aggressive colonization, plant growth stimulation and antagonism / biocontrol) (Kloepper *et al*., 1991; Weller *et al*., 2002; Vessey, 2003; Preston, 2004; Lucy, *et al.* 2004; Haas and Défago, 2005).

According to Kloepper and Schroth (1978), PGPR colonize the root surfaces as well as enter root interior and establish endophytic populations which reflects the ability of bacteria to selectively adapt to these ecological niches. Consequently, intimate associations between bacteria and host plants can be formed without harming the plant. Despite their different ecological niches, the beneficial effects of PGPR strains in suppressing pathogens and increasing nutrient availability, promoting direct growth as well as in inducing systemic resistance have been reported by Kloepper (1992) and Mahaffee *et al.* (1994). Some of the mechanisms by which rhizobacteria exhibit biological control are antibiosis, competition for space and nutrients, parasitism, production of iron-chelating siderophores, cell wall degrading enzymes and hydrogen cyanide (Wei *et al.,* 1996; Kumari and Srivastava, 1999; Zehnder *et al*., 2001, Mayak *et al.,* 2004).

Plant growth promotion dominates in some PGPR which occasionally are termed as 'biofertilizers'. The mechanisms that are involved in this process includes nitrogen fixation, phosphate solubilization and production of phytohormones and volatile growth stimulants (Vessey, 2003 and Ryu *et al.,* 2003). In certain other cases PGPR, are sometimes called biopesticides, where the biocontrol aspect is most conspicuous. Bacteria thought to have potential for biocontrol may be *Pseudomonas* (Burr *et al*., 1978), *Agrobacterium* (Kerr, 1980), *Erwinia* (Sneh *et al*., 1984), *Xanthomonas* (Kwok *et al.,* 1987) *Azetobacter*, *Bacillus* (Chanway *et al*., 1988) etc. *Agrobactreium radiobacter* strain 84 is the first bacterium successfully used worldwide for biocontrol. Fluorescent and non-fluorescent pseudomonads are of great importance nowadays and have been successfully used for biocontrol of several plant pathogens (Howell and Stipanovic, 1979; Wei *et al*., 1991). The role of fluorescent pseudomonads in disease suppression has been established in several pathogens including *Erwinia carotovora* (Xu and Gross, 1986), *Colletotrichum orbiculare* (Wei *et al*., 1991), Fusarium (van Peer *et al*., 1991), *Xanthomonas oryzae*

pv. *oryzae* (Gnanamanickam, 1999), *R..solanacearum* (Anith *et al*., 2000). Fluorescent pseudomonads have emerged as the largest and potentially most promising group of PGPR for plant disease control and growth promotion (Kloepper and Schroth, 1978; Yeole and Dube, 2000 and Sivaprasad, 2002).

The present work on the biocontrol of bacterial wilt of ginger concentrates on the efficacy of these rhizobacteria and their mechanism of disease suppression in addition to their growth enhancement role in ginger.

2.4.3.3 Mechanism of action of PGPR

A perusal of the literature revealed that not much work has been carried out on this line with bacterial antagonists in ginger. However, an effort has been made to include some of the available literature on crop plants other than ginger.

(i) Growth promotion and disease suppression

PGPR has both direct and indirect impact on plant growth. The indirect plant growth promotion may occur by preventing some of the deleterious effects of phytopathogenic organism or by reducing the deleterious effects of minor pathogens by one or more mechanisms (Whipps, 2001). An additional hypothetical theory is that, PGPR might enhance plant growth by excluding so-called 'deleterious rhizobacteria', which are thought to inhibit plant growth without causing root invasion and disease. In retrospect, the evidence for the existence of these deleterious rhizobacteria in nature is not convincing (Kloepper, 2003).

On the other hand, direct plant growth promotion generally entails the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment (Glick *et al*., 1999). The addition of PGPR may benefit plant growth by increasing germination rate, root growth, yield, leaf area, chlorophyll, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weights and delayed leaf senescence (Lucy *et*

al., 2004). However, according to Kurek and Scise (2003), growth promotion and pathogen inhibiting efficiencies were not directly dependent on the sole ratio of *Pseudomonas* strains to pathogen number in the rhizosphere but were likely dependent on environmental conditions.

Rajan *et al.* (2000) noticed various endophytic bacterial strains isolated from different ginger tracts could enhance tillering, overall growth of plants and suppressed the various pathogens and thereby disease incidence. Anith *et al*. (2000) reported the effectiveness of seed rhizome treatment with *P. fluorescens* strain EM 85 in reducing bacterial wilt incidence and increased yield along with 45 days soil solarization. Fluorescent pseudomonads isolate P-1 was found effective for the control of bacterial wilt of ginger and solanaceous plants (Sivaprasad, 2002). Devanath (2002) reported efficacy of *P. fluorescens* and *B. subtilis* in suppressing the growth of *R.solanacearum* causing bacterial wilt of ginger. Tong *et al.* (2002) observed that *B. megatherium* treated rhizome reduced ginger bacterial wilt by 75 per cent. Anith *et al.* (2004) reported that *P. putida* 89B61 significantly reduced bacterial wilt in tomato by *R. solanacearum* when applied to the transplants at the time of seeding and one week prior to inoculation with the pathogen. Paul (2004) reported that seven rhizobacterial strains significantly enhanced growth in treated black pepper in terms of shoot and root biomass and also in terms of height of the plant and among them five strains significantly reduced the root rot upon challenge inoculation.

In vitro toxicity of *P. fluorescens* to *R. solanacearum* was reported by Sivamani *et al*. (1987). Opina and Valdez (1987) observed that compared to *B. polymyxa*, *P. fluorescens* reduced wilt incidence of tomato when the seedlings were dipped in the bacterial suspension. Schmidt (1979) obtained control of bacterial wilt of Italian rye grass by pre-inoculation with *P. fluorescens*. Ciampi-Panno *et al.* (1989) established that the antagonistic *P. fluorescens* BC8 isolate induced significant reduction in severity of potato bacterial wilt. Furuya *et al.* (1991) noticed that all strains of *P. glumae* showed inhibition of *R. solanacearum* of tomato wilt in *in vitro* and even suppressed the disease under *in vivo* conditions. Piexoto *et al.*

(1995) reported that *P. aeruginosa* inhibited *R. solanacearum* with maximum inhibition zones of 14.3mm in Kings'B and Nutrient yeast extract dextrose agar media. JianHua *et al.* (1996) noticed that *in vitro* inhibition test against *R. solanacearum* combined with assays of root colonization provided an effective means of screening and selecting bacterial antagonists against plant diseases. Silveire *et al.* (1996) observed that *P. aeruginosa, P. fluorescens* and *B. cereus, B. coagulans* and *B. megatherium* were effective against *R. solanacearum* in *in vitro*. According to Karuna *et al.* (1997), seed bacterization with *P. fluorescens* was the most effective in reducing wilt incidence of tomato by 50 per cent under field conditions compared to *P. aeruginosa* and *B. subtilis*. Strains of fluorescent pseudomonads isolated from rhizosphere of healthy egg plants inhibited *R. solanacearum* as well as increased plant height, total weight and root weight (Yungchun *et al*., 1997).

Das *et al.* (2000) also observed that *P. fluorescens* applied 14 days prior to inoculum showed the least disease incidence and highest yield of tomato. Significant reduction in wilt incidence in tomato was obtained due to incorporation of antagonistic bacteria, *P. fluorescens* in soil (Kumar and Sood, 2001). Akbar (2002) reported that seed treatment + soil drenching with *P. aeruginosa* reduced the wilt incidence to 11. 1 per cent in variety Pusa Ruby of tomato under pot culture experiments. Seed coating with *P. fluorescens, B. subtilis* and *T. harzianum* resulted in increased seed germination and enhanced growth and vigour as expressed by increase in seedling height, root length and mean leaf area of cardamom (Thomas and Vijayan, 2003). The efficacy of bacterial antagonists such as *P. aeruginosa* and *P. fluorescens* in promoting growth and suppressing *R.solanacearum* of tomato bacterial wilt has been reported by various workers (Manimala, 2003; LiQuiQin, 2003 and JianHua *et al.,* 2004).

van Peer and Schippers (1988) documented the increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Several studies showed that growth promotion effects are early in plant development and these subsequently translate into higher yields. (Kloepper *et al.,* 1988 and Polyanskaya *et al*., 2000). Inoculation of strains of *P.fluorescens* and

B. polymyxa significantly promoted growth of spruce seedlings in the green house where the performance was evaluated in the field using relative growth rates (RGR). *Pseudomonas* strain Ss2-RN increased both shoot and root RGR by 10-234 per cent but increase of 28-70 per cent were most common. In contrast, *Bacillus* strain S20-R was ineffective at all planting sites (Shishido and Chanway, 2000). Seed bacterization with fluorescent *Pseudomonas* strain GRC2 reduced charcoal rot disease in groundnut by 99 per cent (Gupta *et al.,* 2002b). Kumar (2002) noticed that seed inoculation of *Pseudomonas* strain RRLJ 130 enhanced of seed germination, shoot height, root length, fresh and dry weight in chick pea and pigeon pea. Kumar *et al.* (2002) observed that seed treatment with *P. fluorescens* antagonistic against rice blast pathogen increased germination, seedling vigour and reduced blast disease incidence. They also reported that efficacy in disease reduction was significantly higher when seed treatment was followed by a foliar application than seed treatment alone.

Ramanathan *et al.* (2002) observed that all the 27 strains of *P. fluorescens* promoted plant growth and reduced ragi blast incidence under glass house conditions. Seed bacterization with *Pseudomonas* strain RRLJ 008 improved germination, shoot height, root length, fresh and dry mass and enhanced the yield and chlorophyll content of leaves in the five test crops (aubergine, *Phaseolus vulgaris,* cabbage, Kohlrabi and tomato) under field conditions in Assam (Boruah and Kumar, 2002). Zhang *et al*. (2004) elucidated that all PGPR strains applied as seed treatment and root drench enhanced tobacco growth compared to the non treated control as well as seed treatments alone at seven weeks after planting. Seed bacterization with fluorescent *Pseudomonas* improved germination per cent compared to control or treatment with carbendazim which showed an increase in length of epicotyl and hypocotyl of mustard and thereby improved the vigour index (Samanta and Dutta, 2004). Dhoke and Kurundkar (2005) reported that compared to control, all the fluorescent *Pseudomonas* isolates increased seed germination, root and shoot length and vigour index with the maximum seed germination in plants treated with *P. aeruginosa.* Bhatia *et al.* (2005) observed that sunflower seeds bacterized with fluorescent *Pseudomonas* PS I and PS II showed early and enhanced

seed germination, root length, shoot height, fresh and dry weight of roots and shoots and yield of sunflower and reduced incidence of collar rot substantially.

(ii) Growth regulators

Direct influence of growth promotion by PGPR is attributed to the production of phytohormones *viz.,* cytokinins, IAA, gibberellins and regulatory molecules like ACC deaminase and fixation of atmospheric nitrogen (Patten and Glick, 1996). Many rhizobacteria, especially fluorescent pseudomonads are known to produce several hormones like auxins, cytokinins etc. This leads to certain morphological changes in plants like increased root growth, leaf expansion, shoot growth etc.

Barea *et al*. (1976) observed that among the 50 isolates of phosphate solubilizing bacteria which were positive for IAA, gibberellins and cytokinin production, 17 isolates belonged to the genus *Pseudomonas*. According to Suslow (1982), PGPR such as *P. fluorescens* and *P. aeruginosa* promote plant growth by secreting plant hormones like gibberellic acid like substances. IAA which enhance plant growth, is a phytohormone involved in root initiation, cell division and cell enlargement, commonly produced by PGPR enables the plant to access more nutrients from soil (Salisbury, 1994; Barazani and Friedman, 1999). Rubio *et al*. (2000) noticed *Pseudomonas* spp. *viz., P. putida*, *P. aeruginosa*, *P. fluorescens* and *P. cichorii* produced extracellular IAA at varying concentrations, of which, *P. putida* produced 28.7 to 14.8 mg l^{-1} and *P. aeruginosa* 21.2 mg l^{-1}. They also found that *Pseudomonas* spp. produced siderophores and recorded good colonization behaviour.

Patten and Glick (2002) demonstrated that IAA produced by *P. putida* played a major role in the development of host plant root system. Gupta *et al.* (2002a) observed that *P. aeruginosa* were able to promote root and shoot elongation in soyabean as the strain produced IAA of 5.6 µg ml^{-1}. PGPR influenced the growth and yield of inoculated *Brassica juncea* plants by production of auxins (Asghar *et al.,* 2002). The biosynthesis of IAA production by fluorescent pseudomonads was

enhanced by increasing the concentration of tryptophan added to the growing medium (Khan and Zaidi, 2002; Bano and Mussarat, 2003a). The seed inoculation with these isolates increased plant height, stem diameter, number of branches, number of pods per plant and 1000 grain weight, grain yield and oil content over the uninoculated control.

Khalid *et al.* (2004) observed that PGPR strains inoculated wheat plants exhibited increase in grain yield, root length, root dry weight, shoot length and shoot dry weight under field conditions which is due to the production of auxin and they also observed that L-trytophan amended culture media stimulate auxin biosynthesis. The major auxins in the cultures filtrates of these rhizobacteria subjected to HPLC analysis were IAA and indole acetamide (IAM). Dey *et al.* (2004) observed that fluorescent Pseudomonads were the best in production of IAA and siderophore which resulted in higher pod yield, haulm yield, nodule dry weight, root length and pod number of peanuts than control. Paul (2004) reported that the selected strains of *Pseudomonas* spp. produce IAA and GA as was detected in chromatographic studies. Likewise, Samanta and Dutta (2004) reported that all the rhizobacterial fluorescent *Pseudomonas* isolates produced IAA with the maximum amount of IAA by MPf-1. One isolate of *Pseudomonas synxantha* and three isolates of *B. subtilis* produced IAA, Gibberellins, siderophores and HCN which showed antagonistic activity against *R. solani* and *F. solani* (Ebstam *et al.,* 2005). Bhatia *et al.* (2005) reported that production of IAA was confirmed by development of pink colour upon addition of phosphoric acid to culture supernatant of *Pseudomonas*.

(iii) Antibiosis

Antibiosis plays an active role in the biocontrol of plant disease and often acts in concert with competition and parasitism. Antibiosis is the inhibition or destruction of one organism by a metabolite produced by another organism. Antagonists may produce powerful growth inhibitory compounds that are effective against a wide array of micro organisms and such compounds are referred to as broad

spectrum antibiotics (Ownley and Windham, 2003). There are numerous reports on the evidence of antibiosis in biocontrol of plants pathogens.

Antibiotics encompass a chemically heterogenous group of organic low molecular weight compounds produced by microbes which at low concentrations are deleterious to the growth or metabolic activities of other micro organisms (Thomashow *et al.,* 1997). It was Roberts in 1894 who first noted antibiotic action in cultures and introduced the term 'antagonism' into microbiology (Cook and Baker, 1983c). Antibiosis can be an effective mechanism to protect germinating seeds. As the seeds germinate, the bacteria multiply in the rhizosphere, using exudates from the roots as a food source. The antibiotic 2,4 DPAG produced by *P. fluorescens* Q2-87 is effective against the take-all-pathogen in minute quantities and has been recovered from the wheat rhizosphere (Bonsall *et al.,* 1997). However, the effectiveness of antibiotics in soil can be variable as they can become bound to charged clay particles, degraded by microbial activity or leached away from the rhizosphere by water. There are numerous reports that metabolites produced by bacteria *in vitro* are involved in disease suppression *in vivo*. The metabolites include oomycin A (Howie and Suslow, 1991), hydrogen cyanide (Flaishman *et al*., 1996), kanosamine (Milner *et al*., 1996a), phenazine-1-carboxylic acid (PCA) (Delaney *et al*., 2001), 2,4-diacetyl phloroglucinol (2,4 DAPG) (Mavrodi *et al*., 2001), cyclic lipopetides (Raaijmakers *et al*., 2002), pyrrolnitrin (Prn) (de Souza and Raaijmakers, 2003), pyoluteorin (Plt) (Brodhagen *et al*., 2004), tensin, trpolone etc produced by pseudomonads.

The ability to produce phenazines is limited almost exclusively to bacteria *viz*., *Pseudomonas, Streptomyces, Nocardia, Sorangium, Brevibacterium* and *Burkholderia* spp. (Turner and Messenger, 1986). Tambong and Hofte (2001) pointed out that phenazines are produced by a considerable number of *Pseudomonas* strains. Delaney *et al*. (2001) stated that despite the phenazine biosynthetic locus being highly conserved among fluorescent *Pseudomonas* spp., individual strains differ in the range of phenazines compounds they produce. Phenazine-1, 6-carboxylic acid is the first phenazine formed and it is thought to be converted to PCA, a key intermediate in the synthesis of other phenazines by fluorescent

pseudomonads (Delaney *et al*., 2001). For rapid detection of pyrrolnitrin and 2, 4 DAPG, colonies of *P. fluorescens* were directly pasted on TLC plate and run with different solvents. (Tazawa *et al.,* 2000). They observed that a clear red spot of Rf 0.74 appeared at ethyl acetate mixture when sprayed Ehrlich's reagent which indicated pyrrolnitrin. A blue spot was detected on the plate with Rf 0.68 after spraying with Gibb's reagent after running first with acetone and second with chloroform-methanol which indicated 2,4 DPAG. The mode of action of phenazines has been studied extensively and several strains have already been developed into commercial biocontrol products (Woeng *et al.*, 2003). Antagonistic fluorescent *P. aeruginosa* PUPa3 strain reported the production of phenazine-1-carboxamide (PCN) as well as IAA besides enzymes like protease and phosphatase, as a result of which, the strain can be used as a biofertlizer and antagonist against a wide range of phytopathogens (Kumar *et al.,* 2005).

The antibiotic 2, 4 DAPG is a major determinant in the biocontrol activity of PGPR (Gardener *et al*., 2001). Numerous studies have demonstrated that 2, 4 DAPG produced by *Pseudomonas* spp., is a phenolic metabolite with antibacterial, antifungal, antihelminthic and phytotoxic properties and. can suppress a wide variety of plant pathogens including nematodes. (Duffy and Defago, 1997). Root inoculation of *P. fluorescens* produced 2, 4 DPAG which was responsible for the induction of ISR to *Perenospra parasitica* infecting *Arabidopsis thaliana* (Iavicoli *et al.*, 2003). Ahmadzadeh *et al*. (2004) noticed that among the 19 strains of *P. fluorescens* tested, only 15 produced detectable levels of 2,4 DPAG, among which, strain CHA0 produced 11.4 mg 2,4 DPAG. The *P. fluorescens* strains 29 and 31 produced very low amount of antibiotic while strains 3 and 21 did not produce any amount at all.

Several studies have demonstrated that many of the antibiotics produced by bacterial biocontrol agents have a broad spectrum activity. For example, the broad spectrum activity of pyrrolnitrin 'Prn' produced by *Pseudomonas* spp. is already noticed in the1960's by Japanese scientists. Pyoluteorin 'Plt' and pyrrolnitrin 'Prn' were reported to be isolated from several *Pseudomonas* and *Burkholderia* spp., and both of them play and important role in the suppression of multiple plant diseases (de

Souza and Raaijmakers, 2003). Interestingly some bacteria can produce more than one antibiotic. Several *P.fluorescens* strains produce both Prn and Plt. *Pseudomonas* spp. strain PHZ-48 produces both phenazine and pyorrolnitrin (de Souza and Raaijmakers, 2003). *P. fluorescens* Pf-5 was reported to produce 2, 4-DAPG, pyoluteorin and pyrrolnitrin (de Souza and Raaijmakers, 2003). Biological agents with ability of producing multi antibiotics might have greater potential for broad spectrum disease suppression. Paul (2004) observed that all *Pseudomonas* strains produced pyrrolnitrin but only strains IISR-6 and IISR-8 only produced pyoluteorin indicating their role in disease suppression of *Phytophthora* rot of black pepper.

Fluorescent pseudomonads owe their fluorescence to an extracellular diffusible pigment called pyoverdin (Pvd) or pseudobactin. This pigment has high affinity for Fe^{3+} ions and is a siderophore (iron-carrier) of the producer strain (Meyer and Abdallah, 1978). Another pseudomonad siderophore, pyochelin, has been identified as an antifungal antibiotic in a screening programme (Phoebe, 2001.). However, it has not yet been investigated whether iron deprivation is the antibiotic mechanism that is involved. As pyochelin is a relatively weak Fe^{3+} chelator, but a good Cu^{2+} and Zn^{2+} chelator (Visca, 1992) it might be able to deprive some fungi of copper and / or zinc. Audenaert *et al*. (2002) reported that *P. aeruginosa* 7NSK2 produces secondary metabolites such as pyochelin (Pch), its precursor salicylic acid and the phenazine compound, pyocyanin (Pyo), which induces resistance to *Botrytis cinerea* in tomato. It is also observed that 'Pch' in turn increases the presence of L-cysteine which is not sufficient to induce resistance and also the salicylic acid produced was probably converted to 'Pch'. The synergistic effect of 'Pyo' and 'Pch' is responsible for ISR against *B. cinerea* in tomato plants inoculated with *P. aeruginosa*.

Most biocontrol strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds that are unrelated to typical siderophores. *In vitro*, these antibiotics inhibit fungal pathogens, but they can also be active against many bacteria and, in some cases, against higher organisms. Pal *et al.* (2000) observed that the fluorescent pigment and the antibiotics of fluorescent

Pseudomonas spp. EM85 might be involved in the suppression of *Rhizoctonia* induced damping off of cotton rather than the siderophores and HCN produced. Comprehensive lists of antibiotics that are involved in biocontrol, producer strains, target pathogens and host plants have been compiled by Raaijmakers *et al*., (2002) and Morrissey *et al*., (2004). In this review, we focus on the six classes of antibiotic compounds for which the experimental evidence most clearly supports a function in the biocontrol of root diseases: phenazines, phloroglucinols, pyoluteorin, pyrrolnitrin, cyclic lipopeptides (all of which are diffusible) and hydrogen cyanide (HCN) which is volatile. The modes of action of these secondary metabolites are partly understood. According to Samanta and Dutta (2004), antibiosis property might be the most important parameter for judging the plant growth promoting potential of indigenous rhizobacteria.

The significance of antibiotics in microbial antagonism in natural disease suppressive soils often has been questioned in the past because of the indirect nature of the supporting evidence and constraints to the *in vitro* production of antibiotics (Gutterson *et al.,* 1986). Depending on the nature of the metabolite, recovery and detection may be hampered by chemical instability of the compound, irreversible binding to the soil colloidal organic matter or microbial decomposition (Thomashow and Mavrodi, 1997). Sensitive methods such as the use of reporter gene systems have been developed to detect the *in vitro* production of antibiotics; however, it does not provide an accurate measure of the amount of antibiotics produced (Loper and Lindow, 1997). Bio-analytical techniques like thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are used to detect and quantify antibiotics, of which HPLC makes it one of the best direct methods to study the production of antibiotics *in situ* (Bonsall *et al.,* 1997).

(iv) Hydrogen cyanide (HCN) / Volatile compounds

Several volatile substances have a role in biocontrol of plant pathogens which include ammonia, alkyl pyrones and hydrogen cyanide. Production of HCN by certain strains of fluorescent pseudomonads has been involved in the suppression of

soil borne pathogens (Voisard *et al.,* 1989). Suppression of black root rot of tobacco and take all of wheat (Defago *et al*., 1990) by *P. fluorescens* strain 'CHA0' was attributed to the production of HCN which accounted for about 60 per cent of the biocontrol activity. They suggested that CHA0 was found to colonize the root cortex which might produce a stress effect in the plant leading to cyanide respiration and possible modification of tobacco metabolism resulting in enhanced host resistance mechanisms. YongHoon *et al*. (2001) observed that HCN was not produced by *P. fluorescens* compared to that of *P. putida* and *Pseudomonas* sp. Mondal *et al.* (2000) stated that among the strains of *P. fluorescens*, *P. putida* and *P. alcaligenes* found antagonistic to *Xanthomonas axonopodis malvaceraum*, *P. fluorescens* CRb-17 was the most effective producer of HCN and siderophore. Fluorescent *Pseudomonas* strain GRC2 produced HCN, IAA as well as siderophore in iron deficient medium (Gupta *et al.,* 2002a).

Bano and Mussarat (2003b) observed low HCN under iron limiting conditions and *vice versa* which showed that secondary metabolite producing *P. aeruginosa* strain NJ-15 exhibited innate potential of plant growth promotion and biocontrol activities *in vitro. P. fluorescens* 2P24 strain produces several antibiotic secondary metabolites such as HCN, siderophore, 2, 4 DPAG and proteases which showed inhibitory activity against *R. solanacearum* and *Rhizoctonia solani* (XiaoXue *et al.,* 2004). Nagarajkumar *et al.* (2004) observed a significant relationship between antagonistic potential of *P. fluorescens* MDU2 against *R. solani* causing sheath blight in rice, as the strain was able to produce HCN, siderophore, salicylic acid besides the production of chitinase and ß-1, 3 -glucanase. Few isolates of *P. fluorescens* produced salicylic acid in succinate medium or HCN in *in vitro* assay (Blanco *et al*., 2004). Samanta and Dutta (2004) reported that all the efficient native rhizobacterial isolates were found to be non-cyanogenic in nature. Different strains of *Pseudomonas* produced different intensities of colour indicating different amounts of HCN produced and the highest was by strain IISR-6 (Paul, 2004). Ahmadzadeh *et al*. (2004) and Bhatia *et al.* (2005) observed that only few strains of fluorescent *Pseudomonas* were able to produce HCN which was confirmed by the change in colour from yellow to reddish brown of filter paper.

(v) Siderophores

Iron is a vital element required by virtually all living organisms, including bacteria (Archibald 1983, Posey and Gherardini, 2000). It is an important element in many cellular processes including the electron transport chain and in deoxyribonucleotide synthesis and it acts as a cofactor for many enzymes, such as nitrogenase, peroxidase, catalase, and succinic dehydrogenase (Litwin and Calderwood, 1993). Iron limitation therefore poses a serious threat to microorganisms, as they are unable to survive without adequate supplies of iron. To overcome this iron deficiency, bacteria have adapted to aerobic environments by evolving several mechanisms for acquiring iron. Siderophore production is one such mechanism. Many Gram-negative bacteria are known to produce one or more siderophores and the components for their transport (Wandersman and Delepelaire, 2004). Gram-positive bacteria also secrete siderophores under iron stress, but mechanisms for their transport are not as well understood.

The term 'siderophore' is a Greek word meaning "iron carrier" and is so named because these molecules produced by microorganisms have an extremely high affinity for ferric iron (Lankford, 1973); thus, siderophores bind ferric iron and transport it into the bacterial cell. They are low molecular weight (350-1500 Daltons) iron chelating organic molecules, which can compete for ferric iron in ferric hydroxide complexes (Postle, 1990). There are over 500 described siderophores (Wandersman and Delepelaire, 2004) that are classified based on their chelating group specific for ferric iron. They are secondary metabolites produced virtually by all bacteria and fungi under iron limiting conditions, selectively chelates iron and make it unavailable to other deleterious micro organisms and soil borne pathogens, thus reducing their population (Schippers *et al.,* 1987).

The effect of siderophores produced by PGPR on rhizosphere interactions and biocontrol of plant pathogens was first demonstrated in 1980 (Kloepper *et al.,* 1980a). Fluorescent pseudomonads are characterized by the production of yellow green pigments termed 'pyoverdines' and 'pseudobactins' which fluoresce under UV

light and also pyochelin and pseudoamine which function as siderophores (Demange *et al.,* 1987). Audenaert *et al.* (2001) noticed that *P. aeruginosa* 7NSK2 induces resistance to *Botrytis cinerea* on bean and tomato as it produces three siderophores *viz*., pyoverdine, pyochelin, salicylic acid, a precursor of pyochelin. The isolate produced only nanogram amounts of salicylic acid which are sufficient to induce phenyl alanine ammonia lyase (PAL) expression in the roots and leaves. Gupta *et al.* (2002a) similarly noticed that *P. aeruginosa* (NBRI 4014) produced significant levels of siderophore of 143.87 μg ml^{-1}.

YongHoon *et al.,* (2001) observed that pyoverdine production was reduced in the standard succinate medium by increasing the concentration of $FeCl_3$, however iron binding ability on CAS media was observed only in *P. fluorescens* and *P. putida* which slightly inhibited the growth of pathogen at low concentration of $FeCl_3$. However, *Pseudomonas* sp. WR9-11 showed antagonism in the concentration of $FeCl_3$ from 0 to 1000 μM. Salicylic acid was not detected in all the strains of *Pseudomonas*. According to Bakker *et al.* (2002) competition for iron and ISR are the effective mechanisms of siderophore mediated disease suppression by *P. fluorescens*. They reported that *P. fluorescens* WCS374 produces the siderophore pseudobactin and salicylic acid (SA) at low iron availability and both these compounds are involved in ISR in radish. However, the strain cannot induce resistance in *A. thaliana* as it was unable to produce SA and instead it produces a second siderophore, 'pseudoamine' which indicates that disease suppression by the same strain varies with plant pathogen systems.

Boruah and Kumar (2002) noticed that *P. fluorescens* strains produced antibiotics / secondary metabolites like phenazines (Phe), 2, 4 DPAG and siderophore pyoverdine (Pyo) where 'Pyo' was identified by comparing the UV spectra and moss green colour development after diazotized sulphanillic acid (DSA) spray in TLC. 'Phe' and 2, 4 DPAG were identified by comparing the standard compounds on TLC with orange colour development immediately after DSA spray. A significant increase in plant growth was recorded in siderophore amended plantlets whereas 'Phe' and 2, 4 DPAG did not show any growth promoting activity. These

results support the importance of secondary metabolites by the strain *P. fluorescens* in enhancing plant growth and in controlling fungal and bacterial pathogens. They also observed that the *Pseudomonas* strain RRLJ 008 produced both a yellowish green siderophore in standard succinate medium and a yellow viscous antibiotic compound in King's B medium where the results confirmed that plant growth promotion was due to siderophore production whereas disease suppression was due to antibiotic substances. The increase of Fe^{3+} concentration had a negative effect in siderophore production especially above 10μM and they found that the production of this metabolite in glutamic media by *P. aeruginosa* without the addition of iron (Villegas *et al.,* 2002).

All the isolates of *P. fluorescens* and *P. putida* produced green fluorescent siderophore pseudobactin *in vitro* (Blanco *et al*., 2004). Fluorescent pseudomonads produced siderophores by formation of yellow halo zones in CAS plates (Storey, 2005). Siderophore production was observed on the reverse side of Petri plates as green dots and also the change of colour of the medium to fluorescent green by growing in King's B medium (Reddy *et al.,* 2004). Though siderophores are part of primary metabolism, at occasions they also behave as antibiotics which are commonly considered to be secondary metabolites (Haas and Defago, 2005). For example, production of antibiotic, phenzaine-1-carboxylic acid by the strain, *Pseudomonas* 2-79 and other phenazines appear to respond to iron. Siderophores supplies essential quantities of iron needed for production of the antibiotic. All the strains of fluorescent *Pseudomonas* were able to chelate Fe^{3+} from chromeazurol S agar medium (Bhatia *et al.,* 2005). Radheyshyam *et al.* (1990) confirmed the antibiotic property of *Pseudomonas fluorescens* using $FeCl_3$.

(vi) Rhizosphere colonization and competence

Microbes which grow in the rhizosphere are ideal for use as biocontrol agents since the rhizosphere provides the front-line defense for roots against attack by pathogens (Lugtenberg *et al*., 1999).

The PGPR, despite their potential as low input practical agents of plant protection has been hampered by inconsistent performance in field tests (Gaskins *et al*., 1985) which is usually attributed to their poor rhizosphere competence (Weller, 1988). Rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period in the presence of indigenous microflora (Nautiyal, 1997; Bais *et al.,* 2004; Chatterton *et al*., 2004).

Competition is the result of two or more organisms trying to utilize the same food (carbon and nitrogen) or mineral source or occupy the same niche or infection site. The successful competitor excludes the others due to a faster growth or reproductive rate or is more efficient in obtaining nutrients from food sources. Competition for root niches and bacterial determinants directly involves root colonization. The root surface and the surrounding rhizosphere act as significant carbon sinks where root surfaces serve as suitable nutrient rich niches attracting a greater diversity of microorganisms including phytopathogens (Rovira, 1965). Root mucilage also offers a utilizable carbon source for PGPR to use for competitive colonization (Knee *et al.,* 2001). Competition for these nutrients and niches is a fundamental mechanism by which PGPR protects plants from phytopathogens (Duffy, 2001). The introduced fluorescent *Pseudomonas* strain RRLJ 130 was colonized on the roots of the plants and were able to multiply along with the microflora (Kumar, 2002). PGPR reach root surfaces by active motility facilitated by flagella and guided by chemical attractants present in root exudates which include organic acids, amino acids and specific sugars (Nelson, 2004; Welbaum *et al*., 2004) and a few of these exudates can also serve as effective antimicrobial agents and thus give ecological niche advantage to organisms that have adequate enzymatic machinery to detoxify them. The quantity and composition of chemo attractants and antimicrobials exuded by plant roots are under genetic and environmental control (Bais *et al.,* 2004). According to Dey *et al*. (2004), studies on rhizosphere competence of PGPR isolates evaluated on the basis of spontaneous Rifampicin resistance indicated that among the four fluorescent pseudomonads, PGPR-7 was

considered as the best rhizoplane colonizer and PGPR-1, the best rhizosphere colonizer.

According to Kloepper and Schroth (1981), it is not possible to extrapolate results obtained in sterile soils to those expected under field conditions. However, Scher *et al.* (1985) in turn observed that, two fluorescent pseudomonads strains (SS3 and 3K) failed to colonize roots in raw soil but successfully colonized in autoclaved soil, indicating that microbial competition had a negative effect on colonization by these strains in raw soil. *Pseudomonas* spp. are a widely studied group of beneficial rhizobacteria that possess successful colonization, including motility, chemotactic responses and fast growth rate (Howie *et al.,* 1987).

PGPR used for biocontrol must be present on the roots in sufficient numbers to have a beneficial effect on the plant. The crucial colonization level that must be reached has been estimated at 10^5 - 10^6 cfu g^{-1} of root in the case of *Pseudomonas* spp. Artificially introduced PGPR can initially colonize roots at 10^7 and 10^8 cfu g^{-1} but these levels always decline within a few weeks (Kuiper *et al.,* 2001). Tong *et al.* (2002) identified *Bacillus megatherium* with biological control capability from field soils of ginger and it was found to colonise the surroundings of ginger rhizome and reduced the population of pathogenic bacteria. The mode of action includes production of inhibitory substances and competition for space and nutrients. *P. fluorescens* J3 could colonise the root system strongly and controlled the bacterial wilt caused by *R. solanacearum* in capsicum (JianHua *et al.,* 2003). According to Paul (2004), population of introduced bacteria shoots up in sterile soil as there is no competition from other native micro organisms. Bhatia *et al.* (2005) observed that as a result of aggressive root colonization, population of fluorescent *Pseudomonas* strains increased in rhizosphere upto 60 days of sowing but slightly decreased thereafter.

(vii) Efficacy of rhizobacteria in mobilizing nutrients from the rhizosphere

Exploitation of PGPR as biofertilizers to enhance plant growth has been documented by Glick (1995). A large proportion of phosphorous in soil is present in

an insoluble form and therefore not available to the plant. The ability to convert insoluble ‘P’ to an accessible form like orthophosphate is an important trait for PGPR for increasing plant yields (Rossolini *et al.,* 1998). ‘P’ one of the key nutrient stimulates growth and development of roots making plant more resistant to drought and facilitates more nutrient absorption. The total ‘P’ availability is low (0.1 per cent) due to low solubility and fixation in soil. The introduction of ‘P’ solubilizer species of *Pseudomonas* in the rhizosphere of crop helps in increasing the availability of ‘P’ from insoluble sources of soil bound phosphate. The microbe mediated ‘P’ mobilization in plants may be through an increase in the surface area of roots or by increasing the soil mobility of organic forms of ‘P’ or through stimulation of metabolic processes that are effective in directly solubilizing and mineralizing ‘P’ from poorly available forms of inorganic and organic ‘P’ (Jones, 1998). The soil ‘P’ is also utilized by the microbes itself to meet their own requirement. Incubation studies using labelled phosphate have highlighted that microbial ‘P’ in turn gets into the soil solution and is available to the plant (Oberson *et al.,* 2001).

Apart form ‘P’ solubilization, biological nitrogen fixation, improvement of other plant nutrient uptake and phytohormone production like IAA are some mechanisms that directly influence plant growth. The main forms of nitrogen uptake by plants are as either ammonium or nitrate. Nitrogen, which is available to plants, may come from decomposition of organic matter, biological fixation of nitrogen and from addition of nitrogen in organic and inorganic fertilizers. The inorganic nitrogen taken up by the plants is converted to organic compounds as amino acids. The ability of *Pseudomonas* species to fix nitrogen is still debated. Vetiver could grow and survive without nitrogen and phosphorous application especially in the infertile soil with the help of diazotrophs including the genera of *Pseudomonas* (Sipirin, 2000). According to Gupta *et al.* (2002a), *P. aeruginosa* is a potent ‘P’ solubilizer which produced 284 µg ml^{-1}.

Katiyar and Goel (2003) reported that a high ‘P’ solubilization ability of *P. fluorescens* strains were observed to be good rhizosphere colonizer showing a significant increase in root and shoot length in mung bean whereas a low ‘ P’

solubilizer did not stimulate plant growth. Likewise, Bano and Mussarat (2003c) observed that the rhizobacterial strains NJ-15 and NJ-101 showed 'P' solubilization of 99 and 76.5 μgml^{-1}. Dey *et al.* (2004) noticed that *P. fluorescens* (PGPR-1) strain possessed characters like tricalcium phosphate solubilization and ammonification which resulted in an increase in all the growth parameters including yield. *Pseudomonas* strains solubilized complex forms of 'P' in the soil thus making it available to the plant. The intake of other minerals such as 'N' and 'P' was also found to be more with *P. fluorescens* treated black pepper plants (Paul, 2004). Samanta and Dutta (2004) elucidated that from among the six fluorescent rhizobacterial isolates tested, only four showed phosphate dissolution zones on Pikovaskya's TCP medium plates whereas the other two cultures, Pf W1 and P-2 showed phosphate solubilization in liquid medium. Phosphate solubilization by the bacterial strains was found positive for six strains from among the ten which was confirmed with a clear zone on Pikovaskya's solid medium (Bhatia *et al.,* 2005). Similarly, Parthasarathy (2005b) reported the phosphate solubilizing efficacy of *P. fluorescens* in suppressing *Phytophthora* rot of black pepper.

(viii) ***Induced systemic resistance (ISR)***

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated by microorganism or environmental stress (Zhu-Salzman *et al.,* 2005). The resulting elevated resistance due to biotic agents is referred to as ISR (van Loon *et al.,* 1998). Unlike animal immunization, plant induced resistance is generally non specific against plant pathogens. The pathogen related systemic acquired resistance (SAR) and rhizobacteria mediated induced systemic resistance (ISR) are the major components of plant induced resistance (Pieterse and van Loon, 1999 and Bakker *et al.,* 2003). The mechanism of SAR and ISR has been widely integrated into biological control of plant pathogens (Bakker *et al.,* 2003 and Woeng *et al*., 2003). In addition, the induced resistance usually persists for a relatively long time in plants and the level may change after initial elicitation (Kuc, 2001). Therefore, protection of plants through plant induced resistance has a great potential in biocontrol for a wide spectrum of plant pathogens. The term

'pathogenesis related proteins' (PR proteins) is a collective term for cell microbe induced proteins and their homologues to the extent that enzymes such as PAL, PO, PPO which are generally present constitutively and only increased during most infections are often also referred to as PR proteins. Inducible defense related proteins encompass both the known PR protein families and non-classified proteins where the term defense related refers to the fact that these proteins are induced in association with resistance responses but doesnot by itself imply a functional role in defense (van Loon *et al.,* 2006).

Induced systemic resistance (ISR) is defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation with rhizobacteria (van Peer *et al.,* 1991, Wei *et al.,* 1991) and once resistance is induced, it will afford non- specific protection against pathogenic fungi, bacteria and viruses as well as against insect pests and nematodes on occasion. A large number of defense enzymes have been associated with ISR which include phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), catalase (CAT), chitinase, ß-1,3-glucanase, super oxide dismutase (SOD), lipoxygenase, ascorbate peroxidase (APX) and proteinase inhibitors (van Loon, 1997). These enzymes also bring about liberation of molecules that elicit the first steps of induction of resistance, phytoalexins and phenolic compounds (Keen and Yoshikawa, 1983). Bacterial determinants that are claimed to produce ISRs include siderophores, the *O*-antigen of lipopolysacharide, jasmonic acid, ethylene and salicylic acid. The latter compound has even been indicated to cause an ISR when present in nanogram amounts (De Meyer *et al.,* 1999). Black pepper roots and leaves showed increased synthesis of PAL, PO, PPO and catalase which contributed to ISR against *Phytophthora* rot of black pepper (Paul, 2004). Studies with *A. thaliana* mutants indicate that the jasmonate / ethylene-inducible defense pathway is important for ISR, whereas the salicylate-inducible pathway mediating systemic acquired resistance (SAR) seems to be less important. ISR allows plants to withstand pathogen attack to the leaves or roots, without offering total protection (Haas and Défago, 2005).

Inducing the plants own defense mechanism by prior application of a biological inducer is a novel technique for plant protection (Kuc, 1995). The role of ISR in biocontrol of plant diseases is focused on non pathogenic rhizosphere colonizing *Bacillus* and *Pseudomonas* spp. (Whipps, 2001). PGPR strains can induce systemic resistance to foliar pathogens when used as a seed treatment (Wei *et al.,* 1991). *Pseudomonas* spp. can induce systemic biochemical and ultra structural changes in the roots that lead to greater ability of the host plant to defend itself against root infecting pathogens. Maurhofer *et al*., (1994) observed that ISR induced by *P.fluorescens* strain, CHA0 is associated with pathogenesis related protein (PRP) accumulation. Later, reports suggested that ISR might be controlled by SA independent pathway (van Wees *et al*., 1997).

PGPR elicited ISR was first observed on carnation with reduced susceptibility to wilt caused by *Fusaruim* sp. (van Peer *et al.,* 1991). Furuya *et al.* (1997) reported that strain ATCC of *P. aeruginosa* was highly antagonistic to *R. solanacearum* causing wilt of tomato and gave protection to tomato roots which may be due to induced resistance and competition and antibiotic production in suppression of the disease. Ramamoorthy *et al.* (2002) reported that in addition to direct antagonism and plant growth promotion, induction of defense related enzymes involved in phenyl propanoid pathway collectively contribute to enhanced resistance against invasion of *Pythium* in tomato and hot pepper. Senthil *et al.* (2003) observed that all the five PGPR strains of *Pseudomonas* spp. induced systemic resistance to *Colletotrichum falcatum* causing red rot of sugar cane. These PGPR strains were more effective than *Trichoderma viride* and Carbendazim in inhibiting disease incidence by 50 per cent apart from increasing germination percentage and yield. The PGPR mediated ISR was more pronounced in disease susceptible cultivars than in moderately susceptible and moderately resistant cultivars.

ISR by PGPR like *Pseudomonas* spp. has been achieved in large number of crops including carnation (van Peer *et al.,* 1991), Arabidopsis (Pieterse *et al.,* 1996), cucumber (Wei *et al.,* 1996), tobacco (Troxler *et al*., 1997), radish (Leeman *et al.,* 1996), bean (DeMayer and Hofte, 1997), sugarcane (Viswanathan and

Samiyappan, 1999), chilli, brinjal (Ramamoorthy and Samiyappan, 2001; Bharathi *et al.,* 2004), rice (Vidhyasekaran *et al.,* 1997; Nandakumar *et al*., 2001), mango (Vivekananthan *et al.,* 2004) and finger millet (Radjacommare *et al.,* 2004) against broad spectrum of pathogens including fungi (Leeman *et al.,* 1995), bacteria (Liu *et al.,* 1995 a,b) and viruses (Kandan *et al.,* 2005).

(a) Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase is the key enzyme involved in the synthesis of phenolics, phytoalexins and lignin compounds *via* phenyl propanoid pathway that accumulate in response to pathogen infection (Klessig and Malamy, 1994) and hence PAL is considered as the most important enzyme in inducing disease resistance. PAL as well, is the key enzyme in inducing synthesis of salicylic acid which induces systemic resistance in many plants. Induction of enzymes such as PAL and peroxidase (PO) leading to the accumulation of phenolics and lignin can occur in response to insect and pathogen attack, exposure to oxidizing pollutants, mechanical stimulation and are thought to function in the resistance of plants to damage by these stresses. Seed treatment and seedling root dip with *Pseudomonas* spp. induced early and enhanced levels of PAL in rice plants (Nayar, 1996). Plants treated with *Pseudomonas* strains initially showed higher levels of PAL compared to control (Chen *et al.,* 2000). Viswanathan and Samiyappan (2002) observed that *P. putida strain* KKM1 treatment systemically induced PAL enzyme significantly in all three sugarcane cultivars tested. Earlier and increased activities of PAL, PO and PPO were observed in *P. fluorescens* Pf1 pretreated tomato and hot pepper plants challenged with *P.aphanidermatum* (Ramamoorthy *et al*., 2002). Radjacommare *et al.,* (2004) reported that seedling dip with talc based formulation of *P. fluorescens* induced the activity of PAL in finger millet leaves against blast disease. Increased PAL and PO activities were observed in ragi plants treated with *P. fluorescens* 1 strain upto four days after treatment (Ramanathan *et al*., 2002). Increase in the PAL activity was observed in the roots of plants treated with strains of *Pseudomonas* spp. within two days of treatment. PAL showed a peak of maximum enzyme production on third day on inoculation (Paul, 2004).

(b) Peroxidase(PO)

Peroxidase is an important enzyme in the synthesis of lignin. It has been correlated with disease resistance in many plants. Increase in PO activity was observed in rice by Reimers *et al.,* (1992) during bacterial blight infection. Peroxidase are haeme containing glycoproteins that catalyze the oxidation of a variety of organic and inorganic substrates at the expense of hydrogen peroxide. Moreover, PO have a number of physiological function that may contribute to resistance, including oxidation of hydroxyl cinnamyl alcohols into free radical intermediates, phenol oxidation, polysaccharide cross linking, cross linking of extension monomers and lignification which may limit the pathogen ingress and spread in resistant infection (Vidhyasekaran *et al.,* 1997).

Plant root colonization by PGPR was associated with PO activity (Albert and Anderson, 1987). Schneider and Ullrich (1994) also demonstrated the increase in PO activity of tobacco plants treated with *P. fluorescens*. Increased activity of peroxidases elicited by rhizobacteria in different plants such as cucumber (Chen *et al*., 2000), groundnut (Meena *et al*., 2000), rice (Nandakumar *et al*., 2001) and tomato (Ramamoorthy, *et al*., 2002) has been reported against the infection of plant pathogens. Two peroxidase isoforms have been induced in the PGPR treated rice plants inoculated with sheath blight pathogen (Nandakumar *et al*., 2001). Treatment of *P. putida strain* KKM1 enhanced the PO levels in three sugarcane cultivars. However, manifold levels of PO induction in the PGPR treated canes after challenge inoculation with *C. falcatum* was observed. (Viswanathan and Samiyappan, 2002).

Significant increase in PO activity was detected in six to nine days after *P. fluorescens* treatment in banana plants, challenge inoculated with *Fusarium oxysporum* f sp. *cubense* (Thangavelu *et al.,* 2003). These enzymes are also part of the response of plant defense to pathogens (Hammerschmidt and Kuc, 1995) and they may decrease the quality of these plants as host for insects. High level expression of PO was reported in *P. fluorescens* treated chilli plants challenged with *C. capsici* (Bharathi *et al.,* 2004). A three fold increase in PO levels in black pepper

within two days of bacterial treatment was observed by Paul (2004) which was continued to be synthesized through out the study period. PO showed a peak of maximum production on third day after challenge inoculation of the pathogen, *P. capsici*. The groundnut plants treated with *P. fluorescens* and challenge inoculated with *A. alternata* recorded significant increase of PO isozyme. Expression of PO2 isoform was found in all plants treated with Pf1 while PO1 isoform was observed in Pf1 treated plants followed by challenge inoculation with the pathogen. There was a significant increase in induction of peroxidase (Chitra *et al.,* 2006).

(c) Polyphenol oxidase (PPO)

Polyphenol oxidase oxidizes phenolics to highly toxic quinones and hence it has been assigned a role in disease resistance as it accumulates upon wounding in plants. PPO, induced *via,* octadecanoid pathway, is a copper containing enzyme and is involved in terminal oxidation of diseased plant tissues, which was attributed for its role in disease resistance (Kosuge, 1969). Chen *et al.,* (2000) reported that PPO was stimulated by PGPR or by the pathogens, but the wounds on split roots did not influence PPO activity compared to intact control in 13 days. Expression of new PPO isoform was observed in *P. fluorescens* Pf1 treated tomato plants challenged with *Fusarium oxysporum* f.sp. *lycopersici* (Ramamoorthy *et al*., 2002). In tomato, PPO is induced by caterpillar feeding, jasmonates and mechanical damage but not by mites or leaf miners (Thaler *et al.,* 1996). Similarly increased activity of PPO was observed in tomato by fluorescent pseudomonads in response to infection by tomato spotted wilt virus (Kandan *et al.,* 2002). More induction of PPO activity in *P. fluorescens* Pf1 treated chilli plants was observed in response to *C. capsici* correlated with reduced infection of anthracnose disease (Bharathi *et al.,* 2004).

The induction of PPO was found to be gradual in the bacterized black pepper plants unlike other defense enzymes where a peak of maximum enzyme production appeared only after five days after challenge inoculation of the pathogen (Paul, 2004). High PPO activity was observed in groundnut plants treated with *P. fluorescens* and challenge inoculated with *A. alternata.* Expression of PPO1 and

PPO2 isoforms were found in all the groundnut plants treated with Pf1 while additional PPO3, PPO4 and PPO5 were noticed in Pf1 treated plants followed by challenge inoculation with the pathogen (Chitra *et al.,* 2006).

(d) Catalase

Catalase is a well studied antioxidant involved in a oxygen radical scavenging that play a critical role in determining the consequences of plant pathogen interactions (Bowler *et al.,* 1992). Catalase activity was more in the susceptible interaction and is a competitor for PO activity for the substrate hydrogen peroxide. The disease susceptible cultivar of sugarcane 'COC 671' recorded higher catalase enzyme as compared to other two canes after the addition of PGPR (Viswanathan and Samiyappan, 2002).

(e) Phenolics

Plants have multiple defense mechanisms against invading pathogens including structural barriers, preformed and induced biochemical products. Among them, phenolics are well known fungitoxic and antibacterial substances and these are most important group implicated both in constitutive and induced resistance (Vidhyasekaran, 1988). Presence of phenols and their oxidation products in the plant tissue is toxic to the growth and development of pathogen.

Phenolic compounds are a group of chemicals composed of one or more aromatic benzene rings with one or more hydroxyl groups (C-OH) (Armstrong, 2003). Several phenolics are found to be associated with plant defense mechanisms and the modes of action of these compounds include direct toxic effects and the active and rapid deposition of barriers such as lignin (Bennett and Wallsgrove, 1994). In the presence of hydrogen peroxide, peroxidases could carry out the oxidation of phenolics and some other compounds to yield lignin (Nicholsoln and Hammerschmidt, 1992). The accumulation of phenolics is observed in different cases of disease suppression (Prats *et al.,* 2003; Benhamou and Belanger, 1998; Ongena *et*

al., 2000; Paul and Sharma, 2002 and Benhamou *et al*. 2000). Mondal *et al.* (2000) observed four major phenolics compounds (two fluorescent and two non fluorescent) produced by *Pseudomonas* sp. *P. putida* and *P. alcaligenes* which inhibited the growth of the pathogen, *Xanthomonas axonopodis*. In association with phenol biosynthesis, the activity of phenyalalnine ammonia lyase (PAL) and other biosynthetic enzymes might be enhanced such as tyrosine ammonia lyase (TAL) (Goodman *et al.,* 1986), cinnamic acid (Shiraishi *et al.,* 1989) and peroxidases (Southerton and Deverall, 1990).

Wei *et al.* (1991) observed that seed bacterization with PGPR results in greater accumulation of phenolic compounds or mediates induced systemic resistance (ISR) in host plants, which offers a practical way of immunizing plants against pathogen ingress. Higher accumulation of phenolics was noted in tomato and hot pepper plants pretreated with *P. fluorescens* isolate P.f1 challenged with *P. aphanidermatum* (Ramamoorthy *et al.,* 2002). A recent study by Singh *et al* (2003) showed that resistance in chickpea plants induced by *Pseudomonas* strains involved the increase induction of phenolic compounds as well as induced systemic resistance *via* SA dependent pathway. Singh *et al.* (2003) observed that *P. fluorescens* and *P. aeruginosa* protected the seedling s from the attack of *S. rolfsii* showing only 16 to 17 per cent mortality compared to 44 and 24 per cent mortality in control treatments. The two PGPR also induced the synthesis of phenolic acids, like salicylic acid and total phenolics with varying amounts at different growth stages of seedlings. Gallic, ferulic, chlorogenic and cinnamic acids were the major phenolic acids detected by HPLC analysis. It was observed that foliar spray of *Pseudomonas* strains enhanced the phenolic acid content as well as total phenolics within 24 h of application. Salicylic acid was induced frequently during only the first three weeks of growth of chickpea seedlings. The enhanced quantity of phenolics produced in black pepper is supposed to contribute to the disease suppression obtained where the induced production was found both in leaves and roots which demonstrates the involvement of signaling mechanism contributing to systemic resistance (Paul, 2004). Guleria and Kumar (2006) observed a change in difference in the number and type of phenolics

after benzothiadiazole treated and control plants indicating the resistance inducing activity of BTH which probably relies on the activation of a natural defense pathway.

2.5 COMPATIBILITY OF RHIZOBACTERIA WITH PLANT PROTECTION CHEMICALS AND FERTILIZERS

Very few workers have examined the compatibility of bacterial antagonists with plant protection chemicals or fertilizers.

Elkins and Lindow (1999) reported that mancozeb had no detrimental effect on *P. fluorescens* A506 when applied at least five days before or after application of antagonist. However, the studies elsewhere also showed that there was no obvious detrimental effect of fertilizers and chemicals on survival and establishment of *Serratia entomophila* (Townsend *et al.,* 2003). Mathew (2003) noticed that *P. fluorescens* (P11) was compatible with Mancozeb and Carbendazim. Nallathambi and Thakore (2003) observed that combined treatment using fungicides and *P. fluorescens* (CIAH-196) resulted in per cent control efficacy of more than 60 per cent when mixed with Thiophenate methyl, Captan and Alcidine at 50 ppm. The compatibility of fungicides to *P. fluorescens* were studied by Bhavani (2004). According to him, Akomin-40, Indofil M-45 and Bavistin were compatible with the bacterial antagonists whereas among the copper fungicides, Bordeaux mixture was more inhibitory to the antagonists followed by Kocide and Fytolan. The *in planta* studies with *Pseudomonas* strains confirmed the compatibility with the fungicides tested *viz*., metalaxyl, mancozeb, potassium phosphonate and carbendazim. However, the bacterial strains were not compatible with copper oxychloride (Paul, 2004).

Five rhizobacterial isolates of fluorescent *Pseudomonas* spp. were tested for their sensitivity to various antibiotics by Samanta and Dutta (2004) and they found that the strain MPf-1 was found to be insensitive to all the tested antibiotics *viz.,* Pencillin G (10 units), Stretopmycin (10 µg), Gentamicin (10 µg), Norfloxacin (10 µg), Kanamycin (30 µg) and Nalidixic acid (30 µg) whereas the strain MPf-2

was highly sensitive to only Norfloxacin and the isolate P-2 was highly sensitive to Norflaxicin, Gentamicin and Streptomycin

Mathew (2003) also noticed the compatibility of insecticides with *P. fluorescens*. According to him, Imidachlorprid, Etofenprox, Chlorpyriphos and Triazophos at the recommended doses were compatible with *P. fluorescens*. Thankamani *et al.* (2003) noticed that application of *P. fluorescens* + VAM along with Phorate and CoC spray resulted in significantly higher number of leaves, maximum length of roots, leaf area and total biomass of black pepper which indicated the compatibility of Phorate with *P. fluorescens*. Among the insecticides, the lower two concentrations of Sevin, Ekalux, Nuvacron and Endosulfan were compatible with the *Pseudomonas* strains compared to their higher concentrations. Phorate at all concentrations was found compatible with the isolates (Bhavani, 2004). According to Paul (2004) the selected *Pseudomonas* strains were found compatible with chlorpyriphos, quinalphos, dimethoate and phorate.

With respect to compatibility with fertilizers, Rajphos and MoP were compatible compared to urea which restricted the growth of the antagonists. In addition, ammonium chloride and ammonium sulphate showed varying levels of inhibition of growth indicating their partial compatibility (Bhavani, 2004).

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled, 'Plant growth promoting rhizobacteria mediated induced systemic resistance against bacterial wilt in ginger' was carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara, from December 2003 to December 2006. The experimental materials and the methodologies of the study are given below.

3.1 ISOLATION AND CHARACTERISATION OF THE PATHOGEN

The pathogen causing bacterial wilt of ginger was isolated from naturally infected plants. The infected pseudostems and rhizomes were washed thoroughly with running tap water to remove soil particles, air dried and subjected to ooze test. Such infected plant parts were cut into small pieces, surface sterilized with 0.1 per cent mercuric chloride and washed in three changes of sterile water. The pieces were gently crushed/teased and dipped in five ml sterile distilled water in a test tube and observed for exudation of the ooze. When the water turned turbid, a loopful of the suspension was streaked on Triphenyl Tetrazolium Chloride (TTC) medium (Appendix I) in a Petri dish (Kelman, 1954). The plates were incubated at room temperature for 48 h. When the bacterial growth was visible, the typical colonies were selected and subcultured on Nutrient agar (NA) (Appendix I) slants. The cultures were purified by repeated streaking on NA and later transferred to slants of the same medium and preserved at 4°C. The isolate was also preserved by taking few loopful of bacteria from a freshly streaked agar plate and suspended in sterile tap water in eppendorf tubes and stored both at room temperature and refrigerated conditions for subsequent use as a stock culture to prevent the loss of virulence.

The pathogenicity of the isolate was proved in three month old ginger plants by inoculating fresh bacterial ooze collected from wilted plants from which isolation was made by leaf axil puncturing method. This method of proving pathogenicity was adopted due to the loss of virulence of the bacterium when

brought into pure culture. The inoculated plants were covered with polythene bags and incubated. Observations were recorded upto 7-10 days for the development of typical symptoms of the disease. The pathogen was re-isolated from the artificially inoculated plants and compared with the original isolate. The isolate was subjected to various cultural, morphological, and biochemical characters *viz*., colony characters, Grams reaction, pigment production, oxidase, catalase, arginine hydrolase, lecithinase, lipase, urease testes and levan production, gelatin liquefaction, starch hydrolysis, citrate utilization, lysine decarboxylase, ornithine decarboxylase, phenylalanine deamination, nitrate reduction, H_2S production and mode of utilization of sugars *viz*., glucose, dextrose, sorbitol, dulcitol, inositol, mannose, maltose, mannitol, fructose, sucrose, lactose, cellobiose, adonitol, arabinose and glycerol following the methods as suggested in the Manual of Microbiological Methods, published by the Society of American Bacteriologists (1957) for appropriate identification as well as using the Laboratory Guide for Identification of Plant Pathogenic Bacteria, Schaad (1992).

3.2 ENUMERATION OF RHIZOSPHERE MICROFLORA OF GINGER AND ISOLATION OF RHIZOBACTERIA

The rhizosphere soil of ginger from major ginger growing tracts of Wyanad, Thrissur and Palakkad districts were collected. A total of 20 soil samples were collected from the rhizosphere of healthy ginger plants adjacent to the infected ones. Four samples each from ginger fields of Kalpetta and Mananthavady areas of Wyanad district, from Pattikkad and Vellanikkara areas of Thrissur district and from Alathur areas of Palakkad district were collected. Similarly, four samples of the rhizosphere soils of other members belonging to family Zingiberaceae adjoining to the Silent Valley tracts of Western Ghats of Palakkad district were also collected. Thus, a total of 24 soil samples were collected for the study as given below:

Wyanad : Manathavady - M-1, M-2, M-3, M-4
Kalpetta - K-1, K-2, K-3, K-4
Thrissur : Vellanikkara - VK-1, VK-2, VK-3, VK-4

Pattikkad - P-1, P-2, P-3, P-4

Palakkad : Alathur - A-1, A-2, A-3, A-4

Silent Valley - SV-1, SV-2, SV-3, SV-4

The samples were pooled separately, shade dried and the total microflora were quantitatively estimated by serial dilution plate technique (Johnson and Curl, 1972). Martins Rose Bengal Streptomycin Agar, Thornton's standardized Agar and Kenknights Agar media (Appendix I) were used for estimating the total microflora *viz.,* fungi, bacteria and actinomycetes at dilutions of 10^{-2}, 10^{-4} and 10^{-4} respectively. Representative rhizobacterial colonies from the medium used for enumeration of bacteria were selected, purified and maintained for further studies.

In addition, isolation of rhizobacteria using Nutrient agar (NA) and Kings'B (KB) for isolation of *Pseudomonas* spp., Soil Extract agar (SEA), Methyl Red agar (MRA) for isolation of Gram positive bacteria and Crystal Violet agar (CVA) for isolation of Gram negative bacteria (Appendix I) were also carried out for selecting appropriate bacterial isolates. The typical bacterial colonies developed in the dilution plates from each medium were picked up and purified. Altogether 163 rhizobacterial isolates were selected and the cultures were maintained on NA slants by subculturing at fortnightly intervals and also in sterile tap water and preserved at 4°C for further use.

3.3 PRELIMINARY SCREENING OF RHIZOBACTERIA AGAINST THE PATHOGEN

The *in vitro* antagonistic effect of 163 isolates of rhizobacteria against the bacterial wilt pathogen, *Ralstonia solanacearum* was tested by dual culture method (Dennis and Webster, 1971). For preliminary screening, NA seeded with 48 h old culture of the pathogen in Petri dishes was spot inoculated with rhizobacterial isolate. In each plate, four different rhizobacterial isolates were inoculated at equidistant points, 2 cm away from the periphery of the plate. The plates were incubated at room temperature and observed for inhibition of the pathogen after 48 h. The plates with

pathogen alone served as control. Forty-five rhizobacterial isolates which showed zone of inhibition of the pathogen were selected for further studies.

The 45 bacterial isolates which showed antagonism in the preliminary screening were tested individually. These selected antagonists were again spot inoculated at the centre of the NA plate seeded with the pathogen. Three replications were maintained for each antagonist. The plates with pathogen alone served as control. Antagonistic effect of two reference cultures of *Pseudomonas fluorescens* [from KAU (Pf1) and TNAU (Pf2)] and *Bacillus subtilis* against the pathogen was also tested. Observations on the zone of inhibition were recorded after 48h and isolates were grouped into three categories as given below:

Prominent zone : +++ (>15mm)
Good zone : ++ (>5 <15mm)
Slight zone : + (<5 mm)

Thus, 20 rhizobacterial isolates which showed prominent zone of inhibition were selected for further studies, the details of which are given below:

Sl.No.	Isolate No.	Location	S.No.	Isolate No.	Location
1.	RB-4	SV-1	11.	RB-66	K-3
2.	RB-7	SV-2	12.	RB-67	VK-2
3.	RB-11	K-4	13.	RB-69	M-3
4.	RB-17	K-2	14.	RB-70	A-2
5.	RB-18	SV-2	15.	RB-71	SV-3
6.	RB-22	SV-4	16.	RB-76	M-3
7.	RB-31	SV-3	17.	RB-77	M-1
8.	RB-33	SV-3	18.	RB-82	K-2
9.	RB-36	VK-1	19.	RB-144	SV-1
10.	RB-64	K-1	20.	RB-151	K-3

A-Alathur, SV- Silent Valley, K-Kalpetta, M-Mananthavady, VK-Vellanikkara

3.4 *In vivo* EVALUATION OF SELECTED RHIZOBACTERIAL ISOLATES FOR GROWTH PROMOTION IN GINGER

A pot culture experiment was laid out to assess the growth promoting effect of 20 potential antagonistic rhizobacterial isolates in comparison with the reference cultures of *P. fluorescens* and *B.subtilis.* The experiment was carried out during June-December 2004 at College of Horticulture, Vellanikkara. The details of the experiment are as follows:

Design : CRD

Treatments : 25

Replications : 5

Variety : Himachal

Method of application : Seed bacterization, soil drenching and foliar application (60 and 90 DAP)

The treatment details of the experiment are given below:

Treatments	Isolate No.	Treatments	Isolate No.
T_1	RB-144	T_{14}	RB-33
T_2	RB-22	T_{15}	P.f 1
T_3	RB-82	T_{16}	RB-77
T_4	RB-66	T_{17}	*B.subtilis*
T_5	RB-11	T_{18}	RB-7
T_6	RB-36	T_{19}	P.f 2
T_7	RB-151	T_{20}	RB-18
T_8	RB-67	T_{21}	RB-69
T_9	RB-31	T_{22}	RB-71
T_{10}	RB-17	T_{23}	RB-76
T_{11}	RB-70	T_{24}	PoP
T_{12}	RB-4	T_{25}	Absolute control
T_{13}	RB-64		

3.4.1 Preparation of potting mixture and planting

The potting mixture consisting of sand : soil : cowdung in the ratio of 1:1:1 was prepared and filled in earthen pots of size 12″x 12″. Ginger variety, Himachal procured from Wyanad area was used for the study. The seed rhizomes weighing 15-20g were treated with rhizobacterial suspension (x 10^7cfuml^{-1}) prepared from 48 h old cultures grown in NA. Carboxy methyl cellulose (CMC) @ 0.5 per cent was added to increase the sticky property. Rhizomes were tretaed for a period of 30 min and later shade dried. A single rhizome was planted per pot and mulched with Glyricidia leaves. All the cultural operations except fungicidal application were carried out as per the Package of Practices Recommendations, 'Crops' 2000 (KAU, 2000). The rhizobacterial inoculum was again prepared in sterile distilled water containing 10^7 cfu ml^{-1} and was given as soil drench and foliar spray at 60 and 90 days after planting (DAP). Fifty ml of the inoculum suspension was also used for soil drenching.

3.4.2 Observations recorded

Observations on the germination percentage, plant biometric characters and yield at different intervals were recorded.

3.4.2.1 Germination percentage

The number of rhizomes germinated in each treatment was counted 30 days after planting (DAP) to calculate the germination percentage. Also observations on the pre emergence and post emergence rot were recorded.

3.4.2.2 Biometric observations

The following observations were taken at monthly intervals from two to five months after planting (5 MAP).

- Number of tillers– Number of tillers was recorded by counting the number of fully emerged ones.
- Number of leaves per plant – Number of leaves was recorded by counting the number of fully opened leaves of each tiller.
- Height of plants – Distance from the base of the tiller to the tip of plant was taken as the height and expressed in centimetre (cm).

3.4.2.3 Yield of rhizomes

The fresh rhizome yield per pot from each treatment was recorded at the final harvest and was expressed in g pot^{-1}.

3.5 SELECTION OF POTENTIAL RHIZOBACTERIA

Based on the above pot culture experiment, 11 potential antagonists including the two reference cultures of *P.fluorescens* were selected and further subjected to various analysis for understanding the parameters that contribute to the growth promoting effect in ginger as described below. The following rhizobacterial isolates selected for the study are:

1. RB-144	3. RB-66	5. RB-77	7. RB-22	9. RB-11
2. RB-69	4. RB-82	6. RB-151	8. RB-71	

3.5.1 Antagonistic index (AI) of potential antagonists

The 11 potential isolates selected were screened again for their *in vitro* inhibitory effect against the pathogen and a modified antagonistic index (AI) suggested by Kasinathan (1998) was calculated. The isolates were screened as per 3.3 and the diameter of the inhibition zone was recorded and the antagonistic index was calculated using the formula:

$$AI=PI \times IZ$$

where, AI – Antagonistic index, PI – Per cent inhibition, IZ – Inhibition zone (mm)

The per cent inhibition was calculated using the formula suggested by Vincent (1927). The inhibition zone (IZ) produced by each isolate was further scored following the scale as:

IZ zone of >1 <10 mm = 1; >10 <20 mm = 2; >20 <30 mm = 3 and > 30 mm = 4

3.5.2 Vigour index

The selected rhizobacterial isolates along with reference cultures were bioassayed for the ability to promote or inhibit seedling growth using the method as described by Shende *et al.* (1977) and Elliot and Lynch (1984) with a few modifications. Sorghum seeds were surface sterilized with 0.1 per cent mercuric chloride for 3 min followed by successive washing with sterile water. Water was decanted and the seeds were soaked for 30 min in 48 h old cultures grown in nutrient broth containing atleast 10^7 cfu ml^{-1}, after which, the medium was decanted. The Perti plates were poured with 0.8 per cent sterile plain agar and 10 seeds were placed equidistantly on this soft agar and incubated at 30°C for 72 h. Three replications were maintained and the seeds treated with sterile nutrient broth alone served as control. Germination percentage, length of epicotyl and hypocotyl were measured after 72 h. The vigour index (VI) was calculated using the formula:

VI = (Mean root length + Mean shoot length) x Germination percentage.

The vigour index (VI) was scored as follows:

VI of >1 <2 = 1; VI of >2 <3 = 2; VI of >3 <4 = 3 and VI >4 = 4

3.5.3 Production of hydrogen cyanide

Production of hydrogen cyanide (HCN) by the potential isolates was detected by following the method of Wei *et al.* (1991). Log phase of bacterial culture (25 µl) were inoculated to 25 ml of Kings'B broth supplemented with 4.4 g l^{-1} of glycine taken in a sterile Petri plate. Sterile filter paper strips soaked in picric acid

solution (2.5g picric acid + 12.5g Na_2CO_3 in 1000 ml of water) were placed in the lid of each plate. Petri dishes were sealed with parafilm and incubated for 72 h in a slow shaking platform. Change in colour of filter paper strips from yellow to brown and to red indicates the production of hydrogen cyanide. The reaction was scored on a 1-4 scale depending on the colour gradation.

3.5.4 Production of ammonia

The qualitative estimation of production of ammonia was done following the method of Dye (1962). The selected rhizobacterial isolates were grown in 25 ml of peptone water (Appendix I) and incubated at 30°C for four days. Three replications were maintained for each isolate. After incubation, 1 ml of Nessler's reagent was added to the broth. The presence of faint yellow to deep yellow or brown colour indicated production of ammonia. The reaction was scored as nil, low, medium and high in 1-4 scale based on the intensity of colour.

3.5.5 Phosphorous (P) solubilization

The phosphate solubilizing capacity of the potential rhizobacterial isolates was tested *in vitro* using Pikovskaya's agar (Appendix I) as well as in its broth (Pikovskaya, 1948). Ten µl of log phase of the isolates were spot inoculated at the centre of the plate containing the medium and incubated at 28°C for five days. Plates were observed for clearing zone around the colony and its diameter measured. Three replications were maintained for each isolate.

For quantification of phosphorus solubilization, the bacterial isolates were inoculated to 25 ml of Pikovskaya's broth and incubated for 48 h at 28°C at 150 rpm in an orbital shaking incubator. Medium without inoculation served as control. The cultures were centrifuged at 7000 rpm for 10 min at 4ºC. Supernatant was collected and pellet discarded. One ml of supernatant was taken in a test tube and diluted by adding 6 ml of distilled water. Then two ml of chloromolybdic acid (to 15g of ammonium molybdate in 400 ml of warm distilled water, added 342 ml of 12 N HCl,

cooled and made upto one litre) and one ml of chlorostannous acid (2.5 g of the $SnCl_2$. $2H_2O$ in 10 ml of conc. HCl, heated gently and volume made upto 100 ml after cooling) were added to the mixture. Absorbance of the mixture was read at 660 nm using a spectrophotometer (Spectronic-20 D+). From the standard graph (for 100 ppm 'P' solution, 0.439 g of dried KH_2PO_4 in 400ml of distilled water and to this added 25 ml of 7 N H_2SO_4 and made upto one litre), amount of phosphate released from tricalcium phosphate by bacteria were calculated. The 'P' solubilization capacity of the isolates was also scored following the scale based on the 'P' solubilization as:
>1<3 mg $50ml^{-1}$ = 1; >3<6 mg$50ml^{-1}$= 2; >6<9 mg $50ml^{-1}$ =3 and > 9 mg $50ml^{-1}$ =4

3.5.6 Assay of growth promoting hormones

3.5.6.1 Quantitative estimation of Indole Acetic Acid (IAA)

A modified protocol by Bric *et al.* (1991) was used to estimate the IAA produced by the selected PGPR isolates. A loopful of the bacterial culture was inoculated in 25 ml broth of Luria - Bertani medium (LB) (Appendix I) amended with 100 μgml^{-1} of tryptophan (100 μgml^{-1} tryptophan in 50 per cent ethanol) as precursor and incubated on a rotary shaker for 30 h. The supernatant from cultures were collected after centrifugation at 10,000 rpm for 10 min. To one ml of cell free culture filtrate (CFCF), two drops of o-phosphoric acid and 2 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 per cent $HClO_4$) was added and incubated at 28°C for 30 min and the absorbance was measured at 530 nm (Spectronic-20 D+). A standard curve was prepared with different concentrations of IAA and was used to quantify the IAA production and these were finally scored in a scale as: >0<15 μgml^{-1} =1; >16<30 μgml^{-1}=2; >31<45 μgml^{-1} = 3 and > 46 μgml^{-1} = 4

3.5.6.2 Detection of auxins and gibberellins by thin layer chromatography

The growth promoting hormones produced by the potential rhizobacterial isolates was assayed by thin layer chromatography (TLC) as per the protocol of

Hassan (2002). Log phase culture of isolates (100 µl) was inoculated to 100 ml Glucose mineral medium (Appendix I) in 250 ml conical flasks and incubated for 48h. The cultures were centrifuged at 10,000 rpm for 15 min to pellet the cells. The cell free culture filtrates (CFCF) were used for extraction of IAA and GA. The CFCF were adjusted to pH 2.5 with 1 M HCl and then extracted with equal volume of ethyl acetate. The ethyl acetate layer was dried over sodium sulphate and evaporated in a rotary evaporator (Heidolph WB 2000). The residue was taken in 5ml acetone and air dried to 2 ml and developed on TLC using solvent system, isopropanol - ammonia - water (10:1:1). The plates were sprayed with the reagent (3 per cent H_2SO_4 in methanol + 50 mg $FeCl_3$) heated in an oven at 80°C for 10 min. The plates were observed under a UV transilluminator for yellowish green and orange-red fluorescence, which indicated the presence of GA and IAA respectively. Plates were also observed in visible light for violet / red colour which indicated the presence of IAA.

3.5.7 Determination of PGPR index

All the qualitative and quantitative data of plant growth promoting parameters *viz*., antagonistic index, vigour index, HCN, ammonia, IAA production and phosphate solubilization were transformed into 1 to 4 scale and the PGPR index was calculated for each rhizobacterial isolate. The isolates which showed the higher index compared to that of control were considered as a potential antagonists. The PGPR index was calculated as suggested by Samanta and Dutta (2004) is as follows:

PGPR index = (Net PGPR score / Gross PGPR score) x 100

3.6 ASSAY OF SECONDARY METABOLITES

Secondary metabolites *viz*., salicylic acid (SA), antibiotics and siderophores produced by the rhizobacterial isolates were assed by various means.

3.6.1 Spectroscopy and thin layer chromatography analysis of salicylic acid

For both quantitative and qualitative analysis of salicylic acid (SA) production, the isolates were grown in Casamino acid broth (Appendix I) and incubated for 48 h at 30°C at 2000 rpm in an orbital shaker in the dark (DeMeyer and Hofte, 1997). Subsequently, 100 µl of this culture was transferred to 25 ml of Casamino acid broth and incubated for 36 h under the same conditions. Qualitative analysis of SA from the culture supernatant (after centrifugation at 2800 rpm for 15 min) was assayed by TLC after ethyl acetate extraction. To quantify SA production, the ethyl acetate extract was concentrated (1:3) under vacuum. The concentration of SA was determined by adding 5 µl of 2 M $FeCl_3$ and 3 ml of water to one ml of concentrated extract. The absorbance of the purple iron - SA complex, which developed in the aqueous phase was measured at 527 nm in a spectrophotometer (Spectronic 20D+) and was compared with a standard curve of SA dissolved in ethyl acetate.

3.6.2 Detection of antibiotics by thin layer chromatography (TLC)

Antibiotic detection was carried out as per the protocol of Howell and Stipanovic (1980) and Kraus and Loper (1992). The bacterial cultures were inoculated to Nutrient Broth Glucose media (NBG) (Appendix I) and incubated at 28°C for 5 days. Cells were pelleted at 7000 rpm for 10 min and the cell free culture filtrates (CFCF) were separated. Antibiotics were extracted from pelleted cells with 6 ml of 80 per cent acetone. The acetone was removed under a rotary evaporator (Heidolph WB 2000) and the residual aqueous phase was shaken with an equal volume of chloroform. The chloroform phase was dried under vacuum and the residue was dissolved in 50 µl of acetone. Silica gel plates were spotted with 5 µl aliquots of final acetone solution developed with chloroform / acetone (9:1) and observed under UV light (254 nm). The plates were also kept in iodine vapour chamber for 60 minutes for development of coloured bands and Rf values were calculated and compared with the standard / reference values of the antibiotics.

3.6.3 Detection of siderophores

The potential rhizobacterial isolates along with the reference cultures were tested for the production of iron-chelating siderophores by UV fluorescence method, CAS assay as well as by broth assay.

3.6.3.1 Detection of siderophores by UV fluorescence method

Log phase of the rhizobacterial isolates including the reference cultures were streaked on to a King'B plate and incubated at 28^{o}C for 48h. The plates were observed on a UV trans illuminator to view the fluorescence (Kloepper *et al.,* 1980c)

3.6.3.2 Chrome azurol (CAS) assay

Protocol of siderophore production by CAS assay was described by Vellore (2001). The bacterial cultures were grown in modified Fiss minimal medium (Appendix II) containing 0.5 µM added iron for 24 h on a rotary shaker at 27°C. For this, all glasswares used to store the stock solutions of the modified Fiss minimal medium were treated with concentrated hydrochloric acid (HCl) and then rinsed with Milli pore water, to remove traces of contaminating iron.

The CAS plates were employed to check the culture supernatant for the presence of siderophore. The CAS plates were prepared in three separate steps.

First step was the preparation of CAS indicator solution. For this, initially, 60.5 mg of chrome azurol S was dissolved in 50 ml of Millipore water. 10ml of Fe III solution (27mg $FeCl_3.6H_2O$ and 83.3µl concentrated HCl in 100ml Milli pore water) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40ml Millipore water. The HDTMA solution was added slowly while stirring, resulting in a dark blue solution (100ml total volume), which was then autoclaved.

The second step involved preparation of basal agar medium. In a 250 ml flask, 3g 3-(N-Morpholino) propane sulfonic acid (MOPS) (0.1 M), 0.05g NaCl, 0.03 g KH_2PO_4, 0.01 g ammonium chloride (NH_4Cl) and 0.05 g L-Asparagine were dissolved in 83 ml Milli pore water. The pH of the solution was adjusted to 6.8 using 6 M NaOH. The total volume was brought to 88 ml using Millipore water, and 1.5 g agar was added to the solution while stirring and heating until melted. The solution was then autoclaved.

The third step was the preparation of CAS agar plates. Here, the autoclaved basal agar medium was cooled to 50ºC in a water bath. The CAS indicator solution was also cooled to 50ºC, along with 50 per cent solution of glucose. Once cooled, 2ml of 50 per cent glucose solution was added to the basal agar medium with constant stirring, followed by addition of 10 ml of CAS indicator solution, which was added carefully and slowly along the walls of the flask with constant stirring, but at a speed so as not to generate any bubbles. Once mixed thoroughly, the resulting solution (100ml) was poured into sterile plastic plates, each plate receiving approximately 25 ml of blue agar. Under minimal iron conditions, siderophore is produced and released into the culture medium. To isolate and collect siderophore, the bacterial isolates were grown in iron-restricted (0.5µM added iron) modified Fiss minimal medium with a high concentration of iron (20µM). After 24 h of growth, the culture was centrifuged at 13,500 rpm for 2 min and the cell free culture filtrate (CFCF) was collected. A well was made on the CAS plate with 0.6cm cork borer and 60µl of CFCF was added to the well and the plate was incubated at room temperature. A maximum of 8 h was given for any colour change to develop. Siderophore was detected by the presence of an orange halo around the well. Control consisted of culture grown in high iron medium and uninoculated medium.

3.6.3.3 Iron dependent production of siderophores

The 11 potential rhizobacterial isolates including the reference cultures were tested for iron dependent production of siderophores following the standard protocol of Loper (1988) and Kloepper *et al.* (1980 b). Kings'B broth was amended

with different concentrations of iron (0, 100, 200, 300 µM $FeCl_3$). The medium was inoculated with 50µl of log phase culture of the bacterial isolates separately and incubated for 72h at 28°C. The cells were pelleted by centrifugation at 7000 rpm for 10 min and the cell free culture filtrates (CFCF) was collected. The concentration of siderophore in the CFCF was read at 420 nm.

3.7 POPULATION DYNAMICS OF THE POTENTIAL ANTAGONISTS

The potential rhizobacterial isolates were tested for their efficiency to colonize the rhizosphere following the method described by Nautiyal (1997) with slight modifications. The study was conducted with cowpea seeds in sterile potting mixture in plastic cups under controlled conditions. The seeds were surface sterilized with 0.1 per cent mercuric chloride and washed in three changes of sterile water. The seeds were soaked in the bacterial suspension of 10^7 cfu ml for 30 min, dried and sown in sterile potting mixture. The cups were drenched with 10 ml of bacterial suspension at the time of sowing and thereafter sprinkled with sterile water in order to maintain the required moisture level. Enumeration of rhizobacterial population in the soil was carried out at monthly interval for three months by serial dilution plate technique using NA medium.

3.8 *In vivo* EVALUATION OF POTENTIAL RHIZOBACTERIA FOR GROWTH PROMOTION AND DISEASE SUPPRESSION

An experiment was laid out to assess again the growth promoting effect of the 11 potential antagonistic rhizobacterial isolates selected from *in vitro* evaluation from 3.4 in comparison with the reference cultures of *P.fluorescens*. The experiment was carried out during June-December 2005 at College of Horticulture, Vellanikkara. The details of the experiment are as follows:

Design	: CRD
Treatments	: 25
Replications	: 10
Variety	: Himachal

The treatment details are as follows:

T_1 : RB-144	T_5 : RB-11	T_9 : P.f2
T_2 : RB-22	T_6 : RB-151	T_{10} : RB-69
T_3 : RB-82	T_7 : P.f1	T_{11}: RB-71
T_4 : RB-66	T_8 : RB-77	T_{12} : PoP
		T_{13} : Absolute control

Method of application : Seed bacterization, soil drenching and foliar plication (60 and 90 DAP)

The experiment was conducted as per 3.4. Apart from the observations recorded as per 3.4, data on early sprouting, pest and disease incidence, percentage of wilt incidence after challenge inoculation with the pathogen, number of roots, fresh root and dry shoot weight as well as total soil microflora was also taken. The changes in total phenol, protein and amino acid contents on challenge inoculation were estimated by colorimetric methods. Further, qualitative changes in phenols and amino acids were also estimated by thin layer chromatography.

3.8.1 Early sprouting of ginger plants

Observations on early sprouting of plants were taken at 20 and 25 days after planting (DAP). Data on the number of tillers and leaves as well as height of tillers were taken at both the above intervals.

3.8.2 Natural incidence of bacterial wilt, rhizome rot and shoot borer

Data on shoot borer infestation were recorded by counting the number of tillers showing holes and dead hearts and the percentage of tillers infested per pot was worked out. Similarly, incidence of bacterial wilt and rhizome rot were also recorded and their percentages were calculated.

3.8.3 Bacterial wilt incidence after challenge inoculation with the pathogen

For challenge inoculation, the inoculum of the pathogen was prepared from wilted ginger as bacterial ooze, in sterile water. During active tillering *i.e*., four months after planting (4 MAP), the plants in each treatment were challenge inoculated with the bacterial pathogen by placing a piece of cotton dipped in the inoculum on 3-4 leaf axils after giving pin pricks. The inoculated plants were kept under polythene covers for 24 h. Three replications were maintained in each treatment. The pots were irrigated profusely and observations on the incidence of bacterial wilt were taken both at 7^{th} and 14^{th} day after inoculation (DAI) and the percentage wilt incidence calculated.

3.8.4 Number of roots, fresh root weight and dry shoot weight

The number of roots of ginger in each treatment were counted and tabulated. The roots were separated from the plants and the fresh weight of roots were taken and expressed in grams per pot.

3.8.5 Population dynamics of soil microflora

Soil samples were collected from different treatments one, three and five months after planting. Population of fungi, bacteria and actinomycetes in different treatments were estimated by serial dilution plate technique as described in 3.2.

3.8.6 Estimation of phenols by spectroscopy and TLC after challenge inoculation with the pathogen

Total phenol was estimated as per the protocol of Malick and Singh (1980). One gram of leaf was weighed and ground using a pestle and mortar in 10 times the volume of 80 per cent of methanol. The homogenate was centrifuged at 10,000 rpm for 20 min. The supernatant was saved and the residue was re-extracted with five times the volume of 80 per cent methanol, centrifuged and the supernatants

were pooled together and this was used for qualitative estimation of phenols by thin layer chromatography (TLC) using the solvent system of acetic acid : chloroform (1:9). The plates were developed by spraying with dilute Folin- Ciocalteau reagent followed by spraying with 20 per cent Na_2CO_3 solution. The Rf values of individual phenols were calculated and in turn compared with the Rf values of the available standards run in the same solvent system.

The supernatant was evaporated to dryness and the residue was dissolved in 25 ml of distilled water. From the above residue dissolved, one ml was pipietted out into a test tube and the volume was made up with 3 ml distilled water. Folin-Ciocalteau reagent (0.5 ml) was added to the test tube. After 3 min, 2 ml of 20 per cent Na_2CO_3 solution was added to each tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one minute, cooled and the absorbance was measured at 650nm (Spectronic-20 D+) against a reagent blank. A standard curve was prepared with different concentrations of catechol.

3.8.7 Estimation of total protein after challenge inoculation with pathogen

Total protein was estimated as described by Lowry *et al.* (1951). One gram of leaf sample was extracted with 5 ml of 0.1M phosphate buffer, pH 7.5 using a pre-cooled pestle and mortar and the extract was transferred to a centrifuge tube. The homogenate was centrifuged for 15 min at 10,000 rpm at 4°C. The supernatant was decanted and discarded residue. Added equal volume of 15 per cent trichloro acetic acid (TCA) to the supernatant, which precipitated the protein and centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the precipitate dissolved in 0.1N NaOH and the volume was made upto 10 ml with 0.1N NaOH. This solution was further used for protein assay.

From the above prepared sample, 0.5 ml was pipetted into a test tube. Added 5ml of freshly prepared reagent comprising of 1 ml of 0.1per cent $CuSO_4.5H_2O$ + 1 ml of 0.2 per cent sodium potassium tartarate + 100 ml of 2 per cent Na_2Co_3 in 0.1N NaOH and mixed well and kept for 10 min. 0.5 ml of 1N phenol

reagent (2N Folin-Ciocalteau reagent diluted with equal volume of distilled water) was also added and the samples were vortexed thoroughly. The tubes were incubated at room temperature for 20-30 min. The absorbance of samples was determined at 640nm (Spectronic-20 D+) with a blank. A standard curve was prepared with different concentrations of bovine serum albumin (BSA).

3.8.8 Spectroscopic and TLC analysis of total free amino acids

The protocol of Moore and Stein (1948) was followed for spectroscopic assay of amino acids. Leaves of 500mg were weighed and ground using a pestle and mortar with a small quantity of acid washed sand. To this homogenate, 5-10 ml of 80 per cent methanol was added and then centrifuged at 10,000rpm at 4^{o}C for 10 min. The supernatant was saved and the extraction was repeated twice with the residue with methanol and all the supernatants were pooled together. Finally, the volume of the extract was reduced by evaporation and this was used for the qualitative estimation of amino acids by thin layer chromatography using the solvent system of 1-butanol: acetic acid: water (80:20:20). The plates were developed by spraying with 0.1 per cent ninhydrin in acetone and were baked in oven for 5 min at 100^{o}C. The Rf values of individual amino acids were calculated and in turn compared with the Rf values of the available standards run in the same solvent system.

For quantitative estimation, to 0.1ml of original extract, added 1 ml of ninhydrin solution [0.8g stannous chloride in 500ml of 0.2M citrate buffer (pH 5.0) + 20g of ninhydrin in 500ml of methyl cellosolve]. The volume was made upto 2 ml with distilled water. The tube was heated in a boiling water bath for 20 min. Added 5ml of the diluent (equal volumes of water and n-propanol) and the contents were mixed thoroughly. After 15 min, the intensity of the purple colour was read at 570 nm against a reagent blank. The reagent blank was prepared as above by taking 0.1ml methanol instead of the extract. A standard curve was prepared with different concentrations of leucine.

3.9 ASSAY OF DEFENSE RELATED ENZYMES

Based on the previous experiment, selected eight most promising rhizobacterial isolates including reference cultures were further selected and a third pot culture experiment was laid out so as to assess the effect of these isolates in the induction of systemic resistance in ginger against bacterial wilt pathogen. The details of the experiment are furnished below:

Design : CRD
Treatments : 10
Replications : 15
Variety : Himachal

The treatment details are given below:

T_1: RB-144 T_5: RB-11 T_9: Chemical
T_2: RB-22 T_6: P.f1 T_{10}: Control T_3: RB-82 T_7: RB-77
T_4: RB-66 T_8: P.f2

Method of application : Seed bacterization, soil drench and foliar application (60 and 90 DAP)

Apart from the observations taken as per 3.3, estimation of chlorophyll, NPK as well as the oil and oleoresin contents of the rhizomes were also recorded. During active tillering *i.e.*, 4 MAP, the plants were challenge inoculated with the bacterial pathogen by leaf axil puncturing method as described in 3.2 and the activity of defense related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were estimated on the day of inoculation as well as three and five days after inoculation (DAI) by spectroscopy. The electrophoretic profile of soluble proteins was also studied by Native-PAGE besides the isozymes of PO and PPO.

3.9.1 Activity of defense related enzymes in ginger

500mg of leaf samples from each treatment was weighed and homogenized in 10mM of sodium phosphate buffer using a pre-cooled pestle and mortar and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was taken in 2ml eppendorf tubes and stored at -20°C and this forms the extract for estimation of PO, PPO, catalase and PAL.

3.9.1.1 Assay of peroxidase (PO)activity

The assay was carried out as per the protocol of Rathmell and Sequeira (1974). The crude enzyme (50 µl) was taken in one ml of 10 mM sodium phosphate buffer (pH 6.0) and mixed with one ml of pyrogallol and one ml of one per cent hydrogen peroxide. Initial rate of increase in absorbance was measured for 5 min at 1 min interval at 436 nm. Peroxidase activity was expressed as units of PO per $min^{-1}g^{-1}$ fresh tissue.

3.9.1.2 Assay of poly phenol oxidase (PPO) activity

As per the protocol of Mayer *et al.* (1965), 700 µl of sodium phosphate buffer (pH 6.0) was added to 200 µl of the crude enzyme. To this 100 µl of 0.2 M catechol was added and the initial rate of absorbance was measured to 5 min at 420. The enzyme activity was expressed as units of PPO $mt^{-1}g^{-1}$ fresh tissue using the formula as described in 3.9.3.1.

3.9.1.3 Assay of phenylalanine ammonia lyase (PAL) activity

Phenylalanine ammonia lyase was estimated as per the protocol of Brueske *et al.* (1980). To 500 µl of enzyme extract, 500 µl of 0.5 M Tris HCl buffer (pH 8.5) was added. To this 500 µl of 0.15 M L-phenylalanine was added and incubated at 37°C for 60 min. The reaction was stopped by adding 500 µl of 1 M trichloro acetic acid (TCA) and incubated at 40°C for 5 min. It was centrifuged to

remove any particles and the absorbance was read at 270nm in a UV visible spectrophotometer (SpectronicR Genesys5). The control tube contained L-phenyalanine added after TCA. The rate of the reaction was expressed as µmol of trans cinnamic acid formed g^{-1} fresh tissue. The standard was prepared with different concentrations of trans cinnamic acid.

3.9.2 Isozyme analysis

Fresh ginger leaves from various treatments weighing 500mg were homogenized in 0.1 M phosphate buffer (pH 7.0) using a pre-cooled mortar and pestle (Malick and Singh, 1980). The homogenate was centrifuged at 15,000 rpm at 4°C for 15min. The isozymes were analysed as per the protocol of Laemmli (1970) by Native Polyacrylamide Gel Electrophoresis (Native PAGE).

The gel was prepared with a Biorad vertical electrophoresis unit (Bio-Rad, USA). The resolving / separating gel mixture (8 per cent) (Appendix III) was prepared and poured carefully between the glass plates. A layer of distilled water was added above the gel layer and was allowed to polymerize for 30 min. Stacking gel mixture (6 per cent) (Appendix III) was prepared and poured over the separating gel after removing the layer of water. The comb was placed in the gel and allowed to set for 30 min. After polymerizing, the gel was installed in the electrophoresing apparatus. The electrode buffer (Appendix III) was poured slowly. The centrifuged supernatant (45µl) of different treatment samples were taken and mixed with the sample buffer (5µl) (Appendix III) and finally 50µl was loaded carefully into the wells with a micropipette. The isozymes were separated by electrophoresis run in a Mini-Protein system (Bio-Rad, USA). The electrophoresis was conducted at a constant current of 15 mA for 15 min or more until the samples moved through the stacking gel and then at 30 mA for 2-3 h approximately. After the completion of run, the gel was carefully removed and immersed in the respective staining solutions prescribed for each enzyme with uniform shaking. It was then destained by using the particular destaining solutions known for each enzyme to visualize the bands. The gel was photographed and documented in a gel doc machine. Analysis of isozyme

bands were designed by determining the relative mobility (Rm) values. Dendrograms were constructed for isoforms of various enzymes based on the clustering using the Unweighted Pair Group Method of Arithmetic Average (UPGMA) by Sneath and Sokel (1973) using NTSYS package.

3.9.2.1 Peroxidase (PO)

The isoforms of peroxidase on the gel after electrophoresis were visualized by incubating the gel at 37ºC for 20-30 min in the following staining solution comprising of benzidine (1.04 g), acetic acid (9 ml), hydrogen peroxide (3 per cent) (50 ml) and water (40ml) (Reddy and Gasber, 1971). The enzyme reaction was stopped by immersing the gel in large volume of seven per cent acetic acid solution for 10 min.

3.9.2.2 Poylphenol oxidase (PPO)

The isozymes of PPO after electrophoresis were visualized by incubating the gel for 30 min in 0.1 per cent p-phenylene diamine in 0.1 M sodium phosphate buffer (pH 7.0) followed by 10mM catechol in the same buffer (Jayaraman *et al.,* 1987). The enzyme reaction was stopped as described in 3.9.2.1.

3.9.2.3 Protein

The separated polypeptides on gels were visualized by staining with coomassive brilliant blue 250 (CBB) as per the protocol of Laemmli (1970). The gel was immersed in staining solution (0.1g of CBB dissolved in methanol and mixed with acetic acid: water in the ratio 10: 50) overnight with uniform shaking. When sufficient intensity of bands developed, the gel was transferred to a suitable container with atleast 200-300 ml destaining solution prepared with methanol, acetic acid and water in the ratio 40:10:50 and shaken gently and continuously. The destainer was changed frequently, particularly during initial periods, until the background of the gel turned colourless.

3.9.3 Estimation of chlorophyll

Chlorophyll is estimated as per the protocol prescribed by Vernon (1960). Fully opened matured top leaves were collected in the early hours of the day. 0.1g of the sample was weighed and finely cut into very small uniform segments and were put in 100ml beaker containing 10ml of dimethyl sulphoxide (DMSO) and shaken well. The beaker was kept in an oven for 20 min at a temperature of 45-60°C. DMSO was again added until the leaf turned white and kept in the oven at the same temperature. As the leaf turned white, the extract was transferred to 25 ml volumetric flask and the volume was made upto 25 ml with DMSO. The absorbance of the solution was read at 645 and 663 nm in a spectrophotometer against the solvent (DMSO) blank. The amount of chlorophyll present in the extract was calculated as mg chlorophyll per gram tissue using the following equation:

$$\text{mg chlorophyll a } g^{-1} \text{ tissue} = 12.7\,(A_{663}) - 2.69\,(A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b } g^{-1} \text{ tissue} = 22.9\,(A_{645}) - 4.68\,(A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{mg total chlorophyll } g^{-1} \text{ tissue} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

where, A- Absorbance at specific wavelengths, V- Final volume of chlorophyll extract in DMSO, W- Fresh weight of tissue extracted

3.9.4 Oil and oleoresin content of dry ginger rhizomes

The content of volatile oil and oleoresin was estimated from ginger rhizomes of different treatments. The fresh rhizomes after peeling were dried under sun for one week and was then kept in hot air oven at 70-80°C till constant weight was obtained. The rhizomes were powdered thoroughly and volatile oil from 25g sample each from different treatments was estimated by Clevenger distillation method (AOAC, 1980). Likewise, oleoresin was estimated by Soxhlet method (AOAC, 1980) using Socs Plus (Pelican equipments) from 5 g sample using acetone

as solvent. The contents in different treatments were expressed as percentage (v/w) on dry weight basis.

3.9.5 NPK analysis of ginger rhizomes

The total quantity of N, P and K were determined by wet digestion of a known quantity of plant material with single acid digestion. The powdered rhizomes weighing 0.5g was digested in K-Jel Plus at 480°C for 130 min with 10ml of H_2SO_4 and a pinch of catalytic mixture (200g of K_2SO_4 + 10g of $CuSO_4$). The tubes containing the mixture were cooled to room temperature and this digest were used for the estimation of NPK by following different protocols.

3.9.5.1 Estimation of nitrogen (N)

Nitrogen was estimated in a Kel Plus distillation unit using the protocol as described by Subbiah and Asija (1956). Ten ml of the digest taken in a K-Jel Plus tube was connected to the distillation unit and distilled for 5 min. The ammonia liberated was collected in 10 ml boric acid (4 per cent) containing two drops of mixed indicator (0.5g bromocresol green + 0.1g methyl red in 100 ml of 95 per cent ethanol) taken in a conical flask during which the colour of boric acid changed from red to green. This was titrated against 0.02 N H_2SO_4 until a faint red colour appeared. The per cent nitrogen was calculated from the titre value using the standard formula.

3.9.5.2 Estimation of phosphorous (P)

The phosphorus present was estimated by the vanado-molybdate method as described by Jackson (1958). Ten ml of the digested sample was pipetted into a 50 ml standard flask and 10 ml of Bartons reagent [(i) 22.5g ammonium molybdate in 400ml distilled water (ii) 1.25g ammonium metavanadate in 300 ml boiling water which was mixed with 250 ml conc. HNO_3. The solution (i) and (ii) were made upto 1 litre by constant stirring] was added to it and kept for 30 min until yellow colour developed. This was read in a spectrophotometer at 420 nm and the percentage of

phosphorous was calculated by plotting a standard curve with the standard solution (0.2195g KH_2PO_4 in 1000ml distilled water) of phosphorous.

3.9.5.3 Estimation of potassium (K)

Potassium was determined using an Atomic Absorption flame photometer following the method of Jackson (1958). The concentration of potassium was measured in the flame photometer and calculated using the standard curve.

3.10 MOLECULAR MECHANISM OF INDUCTION OF SYSTEMIC RESISTANCE

An attempt was made to elucidate the molecular mechanism of induction of systemic resistance by synthesizing complementary DNA (cDNA) through Reverse Transcription and amplifying it by polymerase chain reaction (PCR) with random primers so as to find out whether there was any additional band unique to the induced plant which in turn will be sequenced and homology could be compared with the database.

3.10.1 Primer screening

In order to select the random primers which show amplification with ginger variety, Himachal, selected for the entire pot culture experiment, a primer screening was carried out. For this, DNA was isolated from fresh ginger leavese and subjected to RAPD assay.

3.10.1.1 Extraction of total DNA

Tender leaf tissue of ginger plants were washed in running tap water and one gram sample weighed. This was homogenized in pre-frozen mortar and pestle in liquid nitrogen into a fine powder without allowing the samples to thaw (Doyle and Doyle, 1987). Then 3ml of extraction buffer (Appendix IV) followed by 50µl ß-

mercaptoethanol and a pinch of sodium metabisulphite were added to the extraction buffer just before transferring the leaf powder to the polypropylene tubes. The homogenate was transferred to a 50ml polypropylene tube containing 4ml of pre-warmed lysis buffer at 65°C. After dispensing the homogenate thoroughly using the spatula in the lysis buffer (Appendix IV), 1ml of sarcosin (5 per cent) was also added and the tubes were incubated for 10min at 65°C. Contents were gently shaken periodically. After incubation, equal volume of chloroform-isoamyl alcohol mixture 24:1 (v/v) was added and again mixed by gentle inversion to emulsify the contents. The aqueous phase was separated by centrifugation at 15,000 rpm in a high speed centrifuge (Kubota) for 15 min at 4°C. The upper aqueous phase was pipetted out and saved in a microcentrifuge tube. DNA was precipitated by adding 0.6 volume of chilled isopropanol for 30min at -20°C followed by a gentle inversion and later pelleted out by centrifugation at 15,000 rpm for 15min at 4°C. The pellet was washed in 1ml of 70 per cent ethanol for 5min at 15,000 rpm, dried and finally dissolved in 100µl of TE buffer (Appendix IV) / Milli Q water and stored at -20ºC for further use.

3.10.1.2 Agarose gel electrophoresis of DNA extract

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al*., 1989). 1X TAE buffer was prepared from the 50 X TAE (pH 8.0) stock solutions (Appendix IV). Agarose (Genei, Low EEO) (1 per cent) was weighed and dissolved in TAE buffer by boiling. Ethidium bromide prepared from a stock of 10mgml^{-1} was added to it, at a concentration of 0.5µgml^{-1} and mixed well. The open end of the gel casting tray was sealed with cellotape and the tray was placed on a horizontal surface. The comb was placed properly and the dissolved agarose was poured into the tray. The gel was allowed to set for 30min after which the comb was removed carefully. The gel was then placed in the electrophoresis unit with the well side directed towards the cathode. 1X TAE buffer was added to the buffer tank (Genei, Bangalore) so as to cover the gel with a few mm of buffer. 5µl DNA sample was mixed with 1µl tracking dye (6X) (Appendix IV) and carefully loaded into the wells using a micropipette. The λDNA / *Eco*RI / *Hind*III Double Digest (Genei, Bangalore) were used as the molecular weight

marker. The cathode and the anode of the electrophoresis unit were connected to the power pack (Hoefer, USA) and the gel was run at constant voltage of 70V (35mA) for 15min. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.10.1.3 Gel documentation

The gel was taken from the electrophoresis unit and viewed under UV light of 320nm in a transilluminator (Herolab^{R}). The DNA emitted orange fluorescence under UV light on account of intercalating with ethidium bromide dye. The image was documented and stored using the Gel documentation and analysis system, Alpha Imager TM-1200 (Alpha InfoTech, USA).

3.10.1.4 Estimation of quantity of DNA

The quantity of DNA present in the sample was measured using spectrophotometer. The DNA isolated was quantified by diluting 3 µl of DNA to 1.5 ml with sterile water and absorbance at 260 nm and 280 nm read against distilled water blank using UV visible spectrophotometer (Spectronic R Genesys 5). The purity of DNA was assessed from the ratio of OD value at 260 to OD value at 280. A ratio of 1.8 to 2.0 indicates pure DNA. The quantity of DNA in the pure sample was calculated using the formula:

OD_{260} = 1 is equivalent to 50µg double stranded DNA ml^{-1}

Hence, quantity of DNA present in the sample = OD_{260} x 50 x dilution factor (µg/ml)

3.10.1.5 Screening of random primers for RAPD assay

After isolation of good quality genomic DNA, RAPD analysis was carried out for screening of random primers so as to identify the best primers. The decamer primers obtained from 'Operon Technologies', USA, were used for the study. Thirty two decamer primers in the series OPA, OPE and OPF were screened with genomic

DNA and those primers that gave good amplification with more than 4-8 bands were selected. Genomic DNA of 20-50ng was subjected to amplification using these selected random oligonucleotide decamer primers. The PCR reaction mix was prepared in disposable micro-ampoule tubes of 0.2 ml capacity. The reaction mixture of RAPD assay (25µl) contained:

10x Assaybuffer for Taq DNA polymerase (15mM $MgCl_2$)	- 2.5 µl
$MgCl_2$	- 1.0 µl
d NTP mix (100µM each of dATP, dCTP, dGTP, dTTP)	- 1.0 µl
Decamer primer (5pM)	- 2.0 µl
Taq DNA polymerase (0.6 U)	- 2.0 µl
Genomic DNA (1: 10)	- 2.0 µl
Milli Q water	- 14.5µl
	25 µl

The template DNA was kept the same throughout the screening procedure. A master mix without the template DNA and primer was prepared using the reaction mixture for the required number of reactions. From this master mix, 21.0 µl was pipetted into each PCR tube. Primer (2 µl) and template DNA (2.0 µl) of 1:10 dilution were added. Heated lid was used instead of mineral oil. A control reaction containing all the components but no genomic DNA was run as a check for any contamination. Amplification reaction was carried out in a Master Cycler Personal (Eppendorf, Germany) where the PCR tubes were kept and the programme was run using the following cycles:

- 94ºC for 3 min (Initial denaturation)
- DNA denaturation at 92ºC for 1 min
- Annealing of primer to template DNA at 37ºC for 1 min } 40 cycles
- Primer extension at 72ºC for 2 min
- Final extension at 72ºC for 10 min

The details of decamer primers with their base sequence are given below:

Sl.No.	Primer code	Primer sequence	Sl.No.	Primer code	Primer sequence
1.	OPA-1	CAGGCCCTTC	17.	OPE-19	ACGGCGTATG
2.	OPA-2	TGCCGAGCTG	18.	OPE-20	AACGGTGACC
3.	OPA-3	AGTCAGCCAC	19.	OPF-2	GAGGATCCCT
4.	OPA-4	AATCGGGCTG	20.	OPF-5	CCGAATTCCC
5.	OPA-5	AGGGGTCTTG	21.	OPF-6	GGGAATTCGG
6.	OPE-3	CCAGATGCAC	22.	OPF-9	CCAAGCTTCC
7.	OPE-5	GCAGGGAGGT	23.	OPF-11	TTGGTACCCC
8.	OPE-6	AAGACCCCTC	24.	OPF-12	ACGGTACCAG
9.	OPE-8	TCACCACGGT	25.	OPF-13	GGCTGCAGAA
10.	OPE-9	CCTTCACCGA	26.	OPF-14	TGCTGCAGTA
11.	OPE-10	CACCAGGTGA	27.	OPF-15	CCATGCAGGT
12.	OPE-11	GAGTCTCAGG	28.	OPF-16	GGAGTACTGG
13.	OPE-13	CCCGATTCGG	29.	OPF-17	AACCCGGGAA
14.	OPE-14	TGCGGCTGAG	30.	OPF-18	TTCCCGGGTT
15.	OPE-16	GGTGACTGTG	31.	OPF-19	CCTCTAGACC
16.	OPE-17	CTACTGCCGT	32.	OPF-20	GGTCTAGAGG

The programme was completed within 4h. The amplified products were resolved on 1.2 per cent agarose gel with 1X TAE buffer system along with a standard molecular weight marker (λDNA digested with *Eco*RI / *Hind*III), stained with ethidium bromide, visualized under UV transilluminator and documented using Alpha Imager TM 1200 (Alpha Info Tech, USA).

3.10.2 Isolation of RNA

Total RNA was isolated from tender leaves of treated and control ginger plants by single step RNA isolation method (Chomczynski and Sacchi, 1987). Plants treated with RB-11 was selected for RNA isolation.

3.10.2.1 Precautions for RNA isolation

In order to avoid contamination with RNAase, a major constraint to RNA isolation, mortar, pestle, micro tips and micro centrifuge tubes used for isolation were treated with 0.1 per cent solution of diethyl pyrocarbonate (DEPC) overnight and autoclaved for inactivating DEPC. Water or any salt solution used in RNA isolation was treated with DEPC and then autoclaved. Hand gloves were worn to avoid contamination. Sterile pipettes were used and the working table was swabbed with 70 per cent ethanol before use.

3.10.2.2 Procedure for RNA isolation

Leaf tissue weighing 0.1 g was ground in liquid nitrogen using mortar and pestle. One ml Trizol reagent (Invitrogen, USA) was added to the homogenate and the sample was kept at room temperature for 5 min. The sample was then transferred to a 1.5 ml tube and centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatant was transferred to a fresh tube and 0.2 ml chloroform was added to it. The contents were mixed by inversion of the tube for 2 min and then it was centrifuged at 12,000 rpm for 15min at 4ºC. The upper aqueous phase containing RNA was carefully transferred again to a fresh tube and 0.5 ml isopropanol was added. The tube was kept at room temperature for 10 min and then centrifuged at 12,000 rpm for 15min at 4ºC. The pellet was washed with one ml 70 per cent ethanol by centrifugation at 7500rpm for 15min at 4ºC. The supernatant was discarded and the pellet was air dried for 10 min. Then the pellet was dissolved in 20 µl DEPC treated water. For checking purity of RNA, the samples were run on a 0.7 per cent (w/v) agarose gel as per 3.16.1.2. The RNA bands separated by electrophoresis were viewed and photographed using Alpha Imager-TM1200 (Alpha InfoTech, USA).

3.10.3 Preparation of cDNA

cDNA was prepared from good quality RNA isolated from treated and control ginger leaves using M-MuLV RT-PCR Kit (Bangalore, Genei). Procedure was followed according to manufacturer's guidelines.

To a sterile RNAse free 0.2ml tube, 9µl RNA was dispensed. 1µl oligo $(dT)_{18}$ primer was added to 9µl RNA in the tube. The vial with the contents was incubated at 65ºC for 10min and was kept at room tepmrature for 2 min to remove any secondary structure. The contents in the vial were given a brief spin. Then the given reagents were added in the following order.

RNAse inhibitor	-	1µl
DTT (0.1M)	-	1µl
Reverse Transcriptase buffer (5x)	-	4µl
dNTP mix (30Mm)	-	2µl
Reverse Transcriptase	-	0.5µl
Sterile water	-	1µl

The solution was mixed well and incubated at 42ºC for 1h. Then it was incubated at 94ºC for 2min to denature RNA-cDNA hybrids. A spin was given briefly and the vial was placed quickly on ice. The cDNA thus obtained was stored at – 20ºC.

3.10.3.1 Quantification of cDNA

Quantification of cDNA was carried out using UV spectrophotometer. 1µl of cDNA sample was used for analysis. The absorbance was measured at 260nm and 280nm and the cDNA concentration in pure sample was calculated using the following relationship.

OD_{260} = 1 is equivalent to 50 µg of double stranded DNA per ml.

Quantity of cDNA present in the sample = OD_{260} X 50 $\mu g ml^{-1}$.

3.10.3.2 RAPD assay with cDNA

The cDNA was diluted to 1:100, 1:20 and 1:10 and subjected to amplification with the selected 12 primers by PCR based characterization as in 3.10.1.5 of treated and control plants.

3.11 COMPATIBILITY OF PROMISING RHIZOBACTERIA TO PLANT PROTECTION CHEMICALS AND FERTILIZERS

The *in vitro* compatibility of the eight promising antagonists and the standard cultures of *P.fluorescens* to antibiotics, fungicides, insecticides and fertilizers commonly used in ginger plots were studied by Filter paper disc method.

3.11.1 Antibiotics

The following antibiotics at various concentrations used for *in vitro* evaluation are given below:

Sl. No.	Antibiotic	Concentration (ppm)
1.	Chloramphenicol	0.1, 0.25, 0.5
2.	Gentamicin	0.1, 0.3, 0.5
3.	Rifampicin	0.05, 0.15, 0.3
4.	Ampicillin	0.1, 0.25
5.	Streptomycin	0.1, 0.25
6.	Tetracycline	0.1, 0.3
7.	Kanamycin	0.5, 0.3
8.	Penicillin-G	0.1
9.	Cephalaxin	0.3
10.	Nalidixic acid	0.3
11.	Oxytetracycline	0.3

3.11.2 Fungicides

The following fungicides with different concentrations used for *in vitro* evaluation are given below:

Sl. No.	Chemical name	Trade name	Concentration (per cent)
1.	Mancozeb	Indofil M- 45	0.2, 0.3, 0.4
2.	Captan	Captaf	0.2, 0.3, 0.4
3.	Carbendazim	Bavistin	0.05, 0.1, 0.2
4.	Metalaxyl + Mancozeb	Master	0.2, 0.3, 0.4
5.	Potassium phosphonate	Akomin - 40	0.2, 0.3, 0.4
6.	Copper oxychloride	Fytolan	0.2, 0.3, 0.4
7.	Copper hydroxide	Kocide	0.1, 0.2, 0.3
8.	Copper sulphate	Shield	0.5, 1.0, 1.5

3.11.3 Insecticides

The insecticides with different concentrations used for *in vitro* evaluation are presented below:

Sl.No.	Chemical name	Trade name	Concentration (per cent)
1.	Carbaryl	Sevin 50 WP	0.2, 0.3 ,0.4
2.	Monocrotophos	Target 36 SL	0.1, 0.125, 0.15
3.	Chlorpyriphos	Durlax 20 EC	0.05, 0.1, 0.15
4.	Dimethoate	Rogor 30 EC	0.05, 0.1, 0.15
5.	Quinalphos	Ekalux 25 EC	0.05,0.1,0.2
6.	Imidachlorprid	Confidor 18 S	0.02, 0.03, 0.04
7.	Phorate	Phorate 10 G	0.05,0.1,0.2
8.	Carbofuran	Furadan 3 G	0.05,0.1,0.2
9.	Carbosulfan	Marshall 25 EC	0.1,0.15,0.2

3.11.4 Fertilizers

The following fertilizers and their concentrations used for *in vitro* evaluation are given below:

Sl.No.	Name	Concentration (per cent)
1.	Urea	1.0, 1.5, 2.0
2.	Rajphos	2.0, 2.5, 3.0
3.	Muriate of potash (MoP)	2.0, 2.5, 3.0
4.	Ammonium sulphate	2.0,2.5,3.0
5.	Factomphos	2.0, 2.5, 3.0

Antibiotic sensitivity with bacterial isolates was assessed with Hi Media antibiotic discs. In the case of fungicides, insecticides and fertilizers, in order to get a desired concentration, the required quantity was added to 100ml sterile water and autoclaved filter paper discs of 4mm diameter were soaked in these various concentrations of plant protection chemicals / fertilizers for a period of 30 min. Fertilizers were exposed to UV light for a period of 45 min to lessen the contamination. Three discs of antibiotic, fungicide, insecticide and fertilizer were placed on sterile King's B (KB) media seeded with the rhizobacterial isolate. Control consisted of KB medium alone inoculated with the antagonist. Three replications were maintained for each isolate. The inoculated Petri dishes were incubated at room temperature and the observations on inhibition zone around the discs were taken after 48h.

3.12 *In vitro* SENSITVITY OF PLANT PROTECTION CHEMICALS AND FERTILIZERS TO *Ralstonia solanacearum*

A similar method as mentioned in section 3.12 was followed for testing the effect of antibiotics, fungicides, insecticides and fertilizers on growth of the pathogen, *R.solanacearum*. All the antibiotics, fungicides, insecticides and fertilizers

used for *in vitro* testing against the antagonists were taken for this study also. Three replications were maintained in each case and observations were recorded after a period of 48h.

3.13 SENSITIVITY OF PROMISING RHIZOBACTERIAL ISOLATES AND *R. solanacearum* TO STRPETOCYCLINE

Streptocycline, a common agricultural preparation specific for bacterial wilt pathogen was used to test the sensitivity of the antibiotic to the eight promising rhizobacterial isolates as well as to *R.solanacearum.* A similar method as mentioned in section 3.12 was followed. Three concentrations *viz.,* 200, 300 and 400ppm of the antibiotic was used for the assay. Three replications were maintained and observations on the inhibition zone around the discs were taken after 48h.

3.14 MUTUAL COMPATIBILITY OF PROMISING RHIZOBACTERIA AND WITH REFERENCE CULTURES OF *Trichoderma* spp.

3.14.1 Mutual compatibility of PGPR

To assess the mutual compatibility with the eight selected rhizobacteria including the reference cultures of *P. fluorescens*, the organisms were streaked perpendicular to each other on the plates containing Nutrient agar medium (Manimala, 2003). Three replications were maintained. The plates were incubated for 48h at room temperature and observed for any lysis at the juncture between the antagonists.

3.14.2 Compatibility of PGPR isolates with *T. viride* and *T. harzianum*

The efficient eight promising PGPR isolates were tested individually with standard cultures of *T. viride* and *T. harzianum* for their compatibility. For this, five mm disc of the fungal culture were placed in the centre of the Petri plates and the rhizobacteria were streaked simultaneously on both sides leaving 2.25 cm away from

the edge of the Petri dish (Utkhede and Rahe, 1983). Plates with *T. viride and T. harzianum* alone served as control. Three replications were maintained for each bacterial isolate. Observations on growth of the fungus were taken at regular intervals upto five days. The per cent inhibition of mycelial growth of the fungus was calculated as mentioned in 3.5.1.

3.15 CHARACTERIZATION OF PROMISING ANTAGONISTS

Characterization of different promising bacterial antagonists *viz*., RB-11, RB-22, RB-82, RB-66, RB-11 and RB-77 was carried out following the methods as suggested in the Manual of Microbiological Methods, published by the Society of American Bacteriologists (1957) and also by the Bergy's Manual of Systematic Bacteriology, Vol I (Staley *et al.,* 1989). The cultural, morphological, physiological and biochemical characters of the isolates such as colony characters, Grams reaction, pigment production, oxidase and catalase reaction, arginine hydrolase, lecithinase, lipase and levan production, gelatin liquefaction, starch hydrolysis, citrate utilization, lysine decarboxylase, ornithine decarboxylase, urease test, phenylalanine deamination, nitrate reduction, H_2S production and mode of utilization of sugars *viz*., glucose, dextrose, sorbitol, dulcitol, inositol, mannose, maltose, mannitol, fructose, sucrose, lactose, cellobiose, adonitol, arabinose and glycerol were studied along with the reference cultures of *P. fluorescens* (P.f1 and P.f2). The Hi Assorted^{TM} Biochemical Test kit for Gram negative rods were also employed for characterization of the rhizobacterial isolates and compared with the Result Interpretation Chart given in the manual. Three replications were maintained for each isolate.

3.16 STATISTICAL ANALYSIS

Analysis of variance was performed on the data collected in various experiments using the statistical package MSTAT (Freed, 1986). Multiple comparison among treatment means was done using DMRT.

Results

4. RESULTS

Investigations on 'Plant growth promoting rhizobacteria (PGPR) mediated induced systemic resistance against bacterial wilt in ginger' were carried out at Department of Plant Pathology, College of Horticulture, Vellanikkara during 2003-2007 and the results are presented below:

4.1 ISOLATION AND CHARACTERISATION OF THE PATHOGEN

The bacterial wilt pathogen was isolated from infected ginger plants on Triphenyl Tetrazolium Chloride (TZC) agar medium. Isolation of the bacteria on TZC medium yielded circular, smooth, convex, creamish white, fluidal and slimy colonies with light pink centre after 24-48h of incubation. The pathogen was purified by dispensing a loopful of the culture in sterile water and by repeated streaking on TZC medium. The culture was preserved both in slants as well as in sterile water and stored under refrigerated conditions for further studies. Pathogenicity of the organism was established by inoculating fresh bacterial ooze obtained from infected ginger (Plate 4.1). Re-isolation from such infected plants yielded colonies resembling the original isolate of the bacterium.

Gram staining revealed that the isolate was Gram negative short rods (Table 4.1). The pathogen produced brown diffusible pigment in King's B medium supplemented with tyrosine but lacked fluorescence in King's B alone. The bacterium was found to be aerobic and positive to catalase and oxidase tests. The isolate produced levan from sucrose, utilized citrate as a carbon source and positive for urease test. The bacterium produced nitrite from nitrate and liberated hydrogen sulphide. It failed to produce ammonia from arginine and negative in lipase and lecithinase activity. It failed to liquefy gelatin and hydrolyze starch. The pathogen was weakly positive for lysine and ornithine decarboxylase. Among the 15 carbon compounds tested, all were utilized by the isolate with the exception of inositol,

Table 4.1. Cultural, morphological, and biochemical characteristics of bacterial wilt pathogen

Sl.No.	Cultural, morphological and biochemical characters	Reaction of pathogen	Sl.No.	Cultural, morphological and biochemical characters	Reaction of pathogen
1.	Grams reaction	-ve	21.	Ornithine decarboxylase	w
2.	Margin	entire	22.	Urease	+
3.	Elevation	convex	23.	Phenylalanine deamination	-
4.	Surface and Sheen	small, smooth and shining	24.	Nitrate reduction	+
5.	Density	translucent	25.	H_2S production	+
6.	Configuration	rod	i.	Utilization of sugars Glucose	+
7.	Colour in TZC	Cream colour with pink centre	ii.	Dextrose	+
8.	Pigments in KB with tyrosine	Brown, diffusable	iii.	Sorbitol	+
9.	Fluorescence (UV)	-	iv.	Dulcitol	+
10.	Pyocyanin production	-	v.	Inositol	-
11.	Oxidase test	+	vi.	Mannose	w
12.	Catalase test	+	vii.	Maltose	+
13.	Arginine dihydrolysis	-	viii.	Mannitol	+
14.	Lipase test	-	ix.	Fructose	+
15.	Levan production	+	x.	Sucrose	-
16.	Lecithinase test	-	xi.	Lactose	-
17.	Gelatin liquefaction	-	xii.	Cellobiose	-
18.	Starch hydrolysis	-	xiii.	Adonitol	-
19.	Citrate utilization	+	xiv.	Arabinose	-
20.	Lysine decarboxylase	w	xv.	Glycerol	w

+ - positive, - - negative, w - weak reaction

Plate 4.1 Symptoms of bacterial wilt in ginger on artificial inoculation

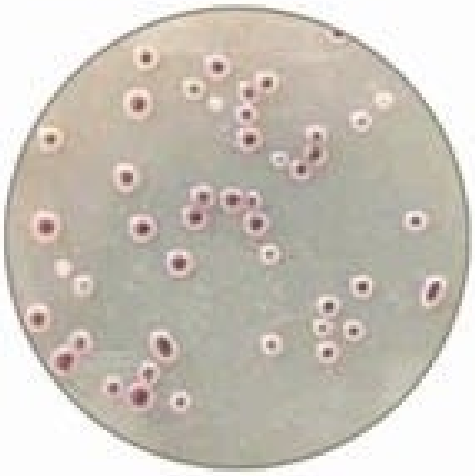


Plate 4.2 Typical colonies of *R. solanacearum* on TZC medium

lactose, cellobiose, adonitol and arabinose. The utilization of mannose and glycerol was weakly positive.

Based on morphological, cultural and biochemical characters coupled with pathogenicity, the pathogen was identified as *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (Yabuuchi *et al.*, 1995) (Plate 4.2). Since the pathogen could utilize all the sugars and alcohols *viz.,* maltose, lactose, cellobiose, mannitol, sorbitol and dulcitol, the pathogen was categorized as Biovar III.

4.2 ENUMERATION OF RHIZOSPHERE MICROFLORA OF GINGER AND ISOLATION OF RHIZOBACTERIA

Twenty four samples of rhizosphere soils of ginger were collected during the months of Nov-Dec 2003 from ginger growing areas of Wyanad, Thrissur and Palakkad districts. The total microflora *viz.,* fungi, bacteria and actinomycetes of soil samples were quantitatively estimated by serial dilution plating technique.

From the data, it is evident that the rhizosphere population of microbes varied with different locations (Table 4.2). The microbial population was high in samples from Silent Valley and Wyanad areas than those of other locations of Thrissur district and Alathur area. Among the microflora, bacteria were more predominant in all soils followed by fungi and actinomycetes. The population of fungi showed variations among the different rhizosphere soil samples. The highest count of fungi ranging from 29 x 10^2 to 35.45 x 10^2 cfu g^{-1}soil was observed from the soils of Silent valley area followed by ginger fields of Wyanad. The lowest count of 8.66 x 10^2 cfu g^{-1} soil was in Vellanikkara soils. The highest population of bacteria was recorded in Kalpetta soil of Wyanad district, followed by Silent valley area and the least in Vellanikkara soil. The population of actinomycetes varied with different locations with the maximum of 27.33 x 10^4 cfu g^{-1} soil in Silent valley area and the minimum of 3.50 x 10^4 cfu g^{-1} soil from Alathur area of Palakkad district.

Based on the cultural characters, representative colonies of bacteria obtained from the enumeration of total microflora of rhizosphere soils from different locations were selected. Further, isolation and selection of representative rhizobacterial isolates were carried out following serial dilution plate technique using Nutrient agar (NA), King's B, Soil extract agar (SEA), Methyl red (MR) and Crystal violet (CV) agar media. Altogether, 163 rhizobacterial isolates were selected and they were purified following standard protocols and maintained in NA slants as well as in sterile water and stored at 4°C for further studies.

4.3 PRELIMINARY SCREENING OF RHIZOBACTERIA AGAINST THE PATHOGEN

The antagonism of rhizobacterial isolates against bacterial wilt pathogen, *R. solanacearum* was tested on Nutrient agar plates by dual culture method. Out of the 163 bacteria screened, 45 were found to be antagonistic to the pathogen as evidenced by varying size of zone of inhibition.

These 45 bacterial isolates were further short listed based on the extent of zone of inhibition on the pathogen. The inhibition zone produced by the standard culture of *Pseudomonas fluorescens* from KAU and TNAU as well as an isolate of *Bacillus subtilis* was also studied. The inhibition zones produced by the isolates were scored on a scale as given in 3.4. All the isolates tested were antagonistic to *R. solanacearum* by producing inhibition zone varying from less than five to greater than 15mm.

From the results as depicted in Table 4.3, 20 isolates *viz.,* RB-4, RB-7, RB-11, RB-17, RB-18, RB-22, RB-31, RB-33, RB-36, RB-64, RB-66, RB-67, RB-69, RB-70, RB-71, RB-76, RB-77, RB-82, RB-144 and RB-157, showed prominent zone of inhibition above 15mm. Reference cultures of *P. fluorescens* of TNAU and KAU were also found to be equally effective against the pathogen producing a zone of inhibition above 15mm. However, *B. subtilis* did show only a slight zone of inhibition of less than five mm.

Table 4.2. Rhizosphere microflora of ginger from different locations

Sl.No.	Location	Fungi (x 10^2 cfu g^{-1} soil)	Bacteria (x 10^5 cfu g^{-1} soil)	Actinomycetes (x 10^4 cfu g^{-1} soil)
1.	Wyanad			
a.	Manathavady			
	M-1	23.33	45.33	7.33
	M-2	32.00	44.66	6.66
	M-3	34.66	40.00	9.33
	M-4	28.00	25.68	5.33
	Mean	29.50	38.92	7.16
b.	Kalpetta			
	K-1	25.66	59.33	14.33
	K-2	31.00	56.33	19.33
	K-3	35.00	60.00	8.32
	K-4	29.55	55.55	10.45
	Mean	30.30	57.80	13.11
2.	Thrissur			
a.	Vellanikkara			
	VK-1	8.66	34.33	13.00
	VK-2	8.66	32.00	12.66
	VK-3	10.00	28.32	15.36
	VK-4	9.45	27.56	8.00
	Mean	9.19	30.55	12.26
b.	Pattikkad			
	P-1	15.26	41.25	13.25
	P-2	21.35	33.52	8.56
	P-3	17.00	28.00	10.25
	P-4	11.12	36.54	7.45
	Mean	9.19	34.83	9.88
3.	Palakkad			
a.	Alathur			
	A-1	18.66	43.33	10.66
	A-2	23.00	56.33	12.00
	A-3	12.00	65.00	13.25
	A-4	17.80	54.32	3.50
	Mean	17.87	54.75	9.85
b.	Silent Valley			
	SV-1	31.33	52.00	27.33
	SV-2	29.00	45.33	22.33
	SV-3	28.33	45.00	20.66
	SV-4	35.45	58.50	18.00
	Mean	31.03	50.21	22.08

Table 4.3. *In vitro* antagonistic effect of different rhizobacteria against the pathogen

S. No.	Isolate No.	Inhibition	S. No.	Isolate No.	Inhibition
1.	RB-2 (SV-3)	+	25.	RB-67 (VK-2)	+++
2.	RB-4 (SV-1)	+++	26.	RB-69 (M-3)	+++
3.	RB-5 (SV-4)	+	27.	RB-70 (A-2)	+++
4.	RB-6 (SV-2)	+	28.	RB-71 (SV-3)	+++
5.	RB-7 (SV-2)	+++	29.	RB-73 (P-3)	+
6.	RB-9 (K-4)	++	30.	RB-74 (M-2)	+
7.	RB-11 (K-4)	+++	31.	RB-75 (P-4)	++
8.	RB-16 (K-2)	++	32.	RB-76 (M-3)	+++
9.	RB-17 (K-2)	+++	33.	RB-77 (M-1)	+++
10.	RB-18 (SV-2)	+++	34.	RB-78 (P-4)	+
11.	RB-22 (SV-4)	+++	35.	RB-81(M-4)	+
12.	RB-26 (A-1)	+	36.	RB-82 (K-2)	+++
13.	RB-29 (A-3)	+	37.	RB-85 (K-3)	+
14.	RB-30 (VK-3)	+	38.	RB-93 (M-1)	++
15.	RB-31 (SV-3)	+++	39.	RB-109 (VK-3)	++
16.	RB-33 (SV-3)	+++	40.	RB-113 (M-4)	+
17.	RB-36 (VK-1)	+++	41.	RB-121 (VK-4)	+
18.	RB-45 (VK-2)	+	42.	RB-133 (P-2)	++
19.	RB-49 (M-3)	+	43.	RB-141 (P-1)	+
20.	RB-52 (A-4)	+	44.	RB-144 (SV-1)	+++
21.	RB-55 (P-1)	++	45.	RB-157 (K-3)	+++
22.	RB-58 (K-4)	++	46.	Kerala *P.f*	+++
23.	RB-64 (K-1)	+++	47.	TNAU *P.f*	+++
24.	RB-66 (K-3)	+++	48.	*B.subtilis*	+

A-Alathur, SV- Silent Valley, K-Kalpetta, M-Mananthavady, VK-Vellanikkara,
P-Pattikkad, *P.f-Pseudomonas fluorescens*
Inhibition zone: Prominent: +++, Good: ++, Slight: +

4.4 *In vivo* EVALUATION OF SELECTED RHIZOBACTERIAL ISOLATES FOR GROWTH PROMOTION IN GINGER

A pot culture experiment (Plate 4.3) was laid out as described in Materials and Methods 3.4 to assess the growth promoting effect of the 20 selected antagonistic rhizobacterial isolates along with two standard cultures of *P. fluorescens* and one isolate of *B.subtilis.* Observations on germination percentage, pre and post emergence rot and other growth parameters *viz.,* number of tillers, number of leaves per tiller, as well as height of tillers were recorded at different intervals. The yield of ginger rhizomes was also recorded.

4.4.1 Per cent germination, pre-emergence and post emergence rot

Among the various treatments, maximum germination percentage of 90 was recorded with T_1 (RB-144) and T_{16} (RB-77) which was closely followed by 80 per cent germination in nine other rhizobacterial treatments (Table 4.4). The least germination percentage of 50 was recorded in T_8 (RB-67), T_{10} (RB-17), T_{12} (RB-4), T_{14} (RB-33) and T_{17} (*B.subtilis*). Rhizomes in absolute control (T_{25}) and PoP treatment (T_{24}) showed a per cent germination of 60. Observation on pre and post emergence rotting of ginger rhizomes was also recorded. A higher percentage of pre emergence rot of 80 per cent was noticed in T_{13} (RB-64), 50 per cent rot in T_8 (RB-67), T_{10} (RB-17), T_{12} (RB-4), T_{14} (RB-33) and T_{17} (*B.subtilis*). The lowest of 10 per cent rot was recorded with T_1 (RB-144) and T_{16} (RB-77). Observations on the pre emergence rot revealed that there was no rot in 10 treatments. Others showed a post emergence rot of 10 to 30 per cent with the maximum in T_{20} (RB-18).

4.4.2 Biometric observations

4.4.2.1 Number of tillers

The number of tillers after two, three, four and five months of planting showed a significant difference among the treatments (Table 4.5). After two months of planting, the maximum number of tillers was in treatment T_5 (RB-11), T_{16} (RB-77)

Plate 4.3 View of the pot culture experiment

Table 4.4. Effect of selected rhizobacterial isolates on germination, pre emergence and post emergence rot of ginger

Treatment	*Germination (%)	Pre-emergence rot (%)	Post-emergence rot (%)
T_1 (RB-144)	90	10	10
T_2 (RB-22)	80	20	20
T_3 (RB-82)	80	20	10
T_4 (RB-66)	80	20	10
T_5 (RB-11)	80	20	0
T_6 (RB-36)	70	30	10
T_7 (RB-151)	80	20	10
T_8 (RB-67)	50	50	10
T_9 (RB-31)	60	40	0
T_{10} (RB-17)	50	50	10
T_{11} (RB-70)	60	40	0
T_{12} (RB-4)	50	50	0
T_{13} (RB-64)	20	80	0
T_{14} (RB-33)	50	50	20
T_{15} (P.f1)	80	20	20
T_{16} (RB-77)	90	10	0
T_{17} (*B.subtilis*)	50	50	10
T_{18} (RB-7)	70	30	0
T_{19} (P. f2)	80	20	0
T_{20} (RB-18)	70	30	30
T_{21} (RB-69)	80	20	10
T_{22} (RB-71)	80	20	0
T_{23} (RB-76)	60	40	10
T_{24} (PoP)	60	40	0
T_{25} (Control)	60	40	10

* Mean of five replications. P.f-*Pseudomonas fluorescens*

Table 4.5. Effect of selected rhizobacterial isolates on number of tillers of ginger plants

Treatment	2 MAP		3 MAP		4 MAP		5 MAP	
	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control
T_1 (RB-144)	2.90^{ab}	+73.65	7.67^{bcdef}	+77.14	18.33^{a}	+129.13	18.67^{ab}	+93.1
T_2 (RB-22)	3.33^{ab}	+99.40	7.67^{bcdef}	+77.14	18.00^{ab}	+125.00	19.00^{a}	+96.5
T_3 (RB-82)	3.23^{ab}	+93.41	9.00^{abcde}	+107.85	14.00^{abcde}	+75.00	14.67^{abc}	+51.7
T_4 (RB-66)	3.00^{ab}	+79.64	8.67^{abcde}	+100.23	17.00^{abc}	+112.50	17.33^{abc}	+79.2
T_5 (RB-11)	4.33^{a}	+159.28	8.33^{abcdef}	+92.38	15.67^{abcd}	+95.88	16.33^{abc}	+68.9
T_6 (RB-36)	2.33^{ab}	+39.52	11.83^{ab}	+173.21	11.67^{abcde}	+45.88	13.33^{abc}	+37.8
T_7 (RB-151)	3.50^{ab}	+109.58	11.33^{abc}	+161.66	16.67^{abcd}	+108.38	17.33^{abc}	+79.2
T_8 (RB-67)	2.00^{b}	+19.76	6.67^{def}	+54.04	10.67^{bcde}	+33.38	12.00^{abc}	+24.1
T_9 (RB-31)	2.77^{ab}	+65.87	6.57^{def}	+51.73	11.33^{abcde}	+41.63	13.67^{abc}	+41.4
T_{10} (RB-17)	2.00^{b}	+19.76	6.67^{def}	+54.04	12.67^{abcde}	+58.38	12.33^{abc}	+27.5
T_{11} (RB-70)	2.67^{ab}	+59.88	7.33^{cdef}	+69.28	10.00^{cde}	+25.00	11.33^{abc}	+17.2
T_{12} (RB-4)	2.90^{ab}	+73.65	8.00^{abcdef}	+84.76	12.67^{abcde}	+58.38	13.33^{abc}	+37.8
T_{13} (RB-64)	2.67^{ab}	+59.88	5.33^{ef}	+23.09	10.67^{bcde}	+33.38	11.33^{abc}	+17.2
T_{14} (RB-33)	2.33^{ab}	+39.52	7.33^{cdef}	+69.28	12.33^{abcde}	+54.13	13.00^{abc}	+34.4
T_{15} (P.f1)	3.67^{ab}	+119.76	9.00^{abcde}	+107.85	9.33^{de}	+16.63	14.33^{abc}	+48.2
T_{16} (RB-77)	4.33^{a}	+159.28	12.33^{a}	+184.76	17.00^{abc}	+112.50	17.67^{ab}	+82.7
T_{17} (*B.subtilis*)	1.67^{b}	0.00	10.00^{abcd}	+130.95	11.33^{abcde}	+41.63	12.00^{abc}	+24.1
T_{18} (RB-7)	2.00^{b}	+19.76	10.00^{abcd}	+130.95	10.67^{bcde}	+33.38	11.00^{bc}	+13.8
T_{19} (P. f2)	4.33^{a}	+159.28	9.00^{abcd}	+107.85	12.33^{abcde}	+54.13	14.00^{abc}	+44.8
T_{20} (RB-18)	3.00^{ab}	+79.64	10.33^{abcd}	+138.57	12.00^{abcde}	+50.00	13.33^{abc}	+37.8
T_{21} (RB-69)	3.00^{ab}	+79.64	9.00^{abcde}	+107.85	13.33^{abcde}	+66.63	14.00^{abc}	+44.8
T_{22} (RB-71)	3.00^{ab}	+79.64	11.33^{abc}	+161.66	13.67^{abcde}	+70.88	16.67^{abc}	+72.4
T_{23} (RB-76)	3.17^{ab}	+89.82	10.00^{abcd}	+130.95	11.67^{abcde}	+45.88	11.67^{abc}	+20.7
T_{24} (PoP)	1.67^{b}	0.00	4.33^{f}	0.00	10.67^{bcde}	+33.38	11.67^{abc}	+20.7
T_{25} (Control)	1.67^{b}		4.33^{f}		8.00^{e}		9.67^{c}	

In each column figures followed by same letter donot differ significantly according to DMRT.
+-Increase, --Decrease. *P.f-Pseudomonas fluorescens*

and T_{19} (P.f2). The least number was with treatment T_{17} (*B.subtilis*), T_{24} (PoP) and T_{25} (Control) (1.67). Three months after planting, the highest number of tillers was in T_{16} (RB-77) and it was on par with 14 other treatments. Among these, 13 rhizobacterial treatments showed more than cent per cent efficiency over control.

Four months after planting, the number of tillers ranged from 8.0 in T_{25} (Control) to a maximum of 18.33 in T_1 (RB-144). The treatment T_1 was followed by T_2 (RB-22), T_4 (RB-66), T_7 (RB-151) and T_{16} (RB-77), which showed more than cent per cent efficiency over control. After five months of planting all the treatments had a positive effect in increasing the number of tillers compared to control. The maximum number of tillers was observed in T_2 (RB-22) followed by T_1, T_{16} and the minimum in T_{25} (Control).

4.4.2.2 Number of leaves per tiller

The data on the number of leaves revealed significant difference among the treatments (Table 4.6). The maximum number of leaves of 8.33 was observed in T_{22} (RB-71) at two months after planting and it was on par with 15 other treatments including control. Three months after planting, the maximum number of leaves was recorded in T_{16} (RB-77) followed by T_{22} (RB-71), T_5 (RB-11), T_1 (RB-144) and T_4 (RB-66) which showed a per cent increase over control of above 20 per cent. Four months after planting also there was significant difference among the treatments. The number of leaves ranged from 5.22 to 9.48 with the minimum in T_{18} (RB-7) and the maximum in T_5. After five months of planting, except the treatments T_{12} (RB-4), T_{13} (RB-64), T_{14} (RB-33) and T_{18} (RB-7), all others were on par with each other. The highest number of leaves was in plants in treatment T_2 (RB-22) followed by T_3 (RB-82) and T_5.

4.4.2.3 Height of tillers

Observations on height of tillers were recorded two, three, four and five months after planting. The results are presented in Table 4.7. Height of tillers, two

Table 4.6. Effect of selected rhizobacterial isolates on number of leaves of ginger plants

Treatment	2 MAP		3 MAP		4 MAP		5 MAP	
	Number of leaves / tiller	Per cent +/- over control	Number of leaves / tiller	Per cent +/- over control	Number of leaves / tiller	Per cent +/- over control	Number of leaves / tiller	Per cent +/- over control
T_1 (RB-144)	6.90^{ab}	+5.02	8.25^{abc}	+20.79	8.50^{ab}	+3.28	10.82^{ab}	+8.85
T_2 (RB-22)	7.17^{ab}	+9.13	7.72^{abcd}	+13.03	8.61^{ab}	+4.62	12.25^{a}	+23.24
T_3 (RB-82)	7.39^{ab}	+12.48	7.78^{abcd}	+13.91	8.56^{ab}	+4.01	11.40^{ab}	+14.69
T_4 (RB-66)	7.28^{ab}	+10.81	8.23^{abc}	+20.50	7.75^{abc}	-5.83	10.40^{abc}	+4.63
T_5 (RB-11)	6.83^{ab}	+3.96	8.4^{abc}	+22.99	9.48^{a}	+15.19	11.39^{ab}	+14.59
T_6 (RB-36)	6.22^{abc}	-5.33	6.68^{abcd}	-2.20	8.09^{ab}	-1.70	10.45^{ab}	+5.13
T_7 (RB-151)	6.81^{ab}	+3.65	6.83^{abcd}	0.00	7.53^{abc}	-8.51	10.59^{ab}	+6.54
T_8 (RB-67)	6.50^{ab}	-1.07	7.69^{abcd}	+12.59	8.09^{ab}	-1.70	9.31^{abcd}	-6.34
T_9 (RB-31)	6.17^{abc}	-6.09	6.95^{abcd}	+1.76	7.63^{abc}	-7.29	10.19^{abc}	+2.52
T_{10} (RB-17)	6.11^{bc}	-7.00	7.30^{abcd}	+6.88	7.56^{abc}	-8.14	10.37^{abc}	+4.33
T_{11} (RB-70)	6.55^{ab}	-0.30	7.07^{abcd}	+3.51	7.87bc^{a}	-4.37	8.86^{abcd}	-10.87
T_{12} (RB-4)	6.00^{bc}	-8.68	7.46^{abcd}	+9.22	7.67^{abc}	-6.80	8.68^{bcd}	-12.68
T_{13} (RB-64)	4.23^{cd}	-35.62	4.66^{e}	-31.77	5.32^{c}	-35.36	6.50^{d}	-34.61
T_{14} (RB-33)	5.63^{bcd}	-14.31	6.38^{bcde}	-6.59	6.92^{abc}	-15.92	8.04^{bcd}	-19.11
T_{15} (P.f1)	7.00^{ab}	+6.54	7.54^{abcd}	+10.40	9.08^{ba}	+10.33	11.13^{ab}	+11.97
T_{16} (RB-77)	6.75^{ab}	+2.74	8.67^{a}	+26.94	8.50^{ab}	+3.28	10.25^{abc}	+3.12
T_{17} (*B.subtilis*)	5.94^{bc}	-9.59	6.42^{bcde}	-6.00	6.69^{bc}	-18.71	8.88^{abcd}	-10.66
T_{18} (RB-7)	3.81^{d}	-42.01	4.58^{e}	-32.94	5.22^{c}	-36.57	6.94^{cd}	-30.18
T_{19} (P. f2)	6.70^{ab}	+1.98	8.48^{abc}	+24.16	9.12^{ab}	+10.81	10.17^{abc}	+2.31
T_{20} (RB-18)	5.92^{bc}	-9.89	6.47^{bcde}	-5.27	6.73^{abc}	-18.23	9.83^{abcd}	-1.11
T_{21} (RB-69)	7.00 ^{ab}	+6.54	7.44^{abcd}	+8.93	9.05^{ab}	+9.96	9.98^{abc}	+0.40
T_{22} (RB-71)	8.33^{a}	+26.79	8.58^{ab}	+25.62	9.08^{ab}	+10.33	10.25^{abc}	+3.12
T_{23} (RB-76)	5.64^{bcd}	-14.16	6.06^{cde}	-11.27	7.91^{abc}	-3.89	9.32^{abcd}	-6.24
T_{24} (PoP)	5.75^{bcd}	-12.48	7.09^{abcd}	+3.81	7.83^{abc}	-4.86	9.90^{abc}	-0.40
T_{25} (Control)	6.57^{ab}		6.83^{abcd}		8.23^{ab}		9.94^{abc}	

In each column figures followed by same letter donot differ significantly according to DMRT.
+-Increase, --Decrease. *P.f-Pseudomonas fluorescens*

months after planting showed significant difference among the treatments with the minimum of 13.74cm in T_{18} (RB-7) to the maximum of 24.79cm in T_4 (RB-66). Three months after planting, the height of treated plants ranged from 16.31 to 29.24 cm in T_{20} (RB-18) to T_2 (RB-22) with a per cent efficiency over control of -13.41 to 51.35.

After four months, plants in T_2 (RB-22) showed the maximum number of leaves with 31.16 per cent efficiency over control and the minimum in T_{23} (RB-76). The treatment T_2 was on par with 11 other ones. Significant difference among the treatments was also observed five months after planting with the maximum height in T_1 (RB-144), followed by T_6 (RB-36), T_2, T_4 (RB-66) and T_5 (RB-11).

4.4.2.4 Yield of rhizome

The data on the average yield of ginger rhizomes per plant are presented in Table 4.8. Analysis of the data revealed significant differences among the treatments. Plants in treatment T_{16} (RB-77) produced the maximum yield of 209.52 g which was on par with T_2 (RB-22), T_{15} (P.f1), T_{21} (RB-69) and T_4 (RB-66). The lowest yield was in T_{13} (RB-64) and it was on par with control and with eight other treatments.

4.5 SELECTION OF POTENTIAL RHIZOBACTERIAL ISOLATES

In the preliminary screening of 23 rhizobacterial isolates including the reference cultures of *P. fluorescens* (P.f1 and P.f2) and *B. subtilis* from the pot culture experiment, 11 potential rhizobacterial isolates which showed a promising effect in increasing the yield and yield attributing characters were selected and studied further for their growth promoting characteristics as given below. Based on the study, PGPR index of the isolates was calculated. The rhizobacterial isolates selected for the study are furnished in Table 3.5.

Table 4.8. Effect of selected rhizobacterial isolates on the yield of ginger plants

Sl.No.	Treatment	*Fresh weight (g)	Per cent +/- over control	Sl.No.	Treatment	*Fresh weight (g)	Per cent +/- over control
1.	T_1 (RB-144)	133.43^{bcde}	+ 121.46	14.	T_{14} (RB-33)	77.54^{def}	+ 28.70
2.	T_2 (RB-22)	178.63^{ab}	+ 196.48	15.	T_{15} (P.f1)	155.09^{abc}	+ 157.41
3.	T_3 (RB-82)	130.73^{bcde}	+ 116.98	16.	T_{16} (RB-77)	209.52^{a}	+ 247.75
4.	T_4 (RB-66)	146.48^{abcd}	+ 143.12	17.	T_{17} (*B.subtilis*)	94.75^{cdef}	+ 57.26
5.	T_5 (RB-11)	127.88^{bcde}	+ 112.25	18.	T_{18} (RB-7)	91.97^{cdef}	+ 52.65
6.	T_6 (RB-36)	102.75^{bcdef}	+ 70.54	19.	T_{19} (P. f2)	125.60^{bcde}	+ 108.46
7.	T_7 (RB-151)	122.96^{bcde}	+ 104.08	20.	T_{20} (RB-18)	122.33^{bcde}	+ 103.04
8.	T_8 (RB-67)	75.00^{def}	+ 24.48	21.	T_{21} (RB-69)	148.22^{abcd}	+ 146.01
9.	T_9 (RB-31)	101.14^{cdef}	+ 67.87	22.	T_{22} (RB-71)	135.77^{bcde}	+ 125.34
10.	T_{10} (RB-17)	60.97^{ef}	+ 1.20	23.	T_{23} (RB-76)	123.08^{bcde}	+ 104.28
11.	T_{11} (RB-70)	122.83^{bcde}	+ 103.87	24.	T_{24} (PoP)	107.88^{bcdef}	+ 79.05
12.	T_{12} (RB-4)	89.38^{cdef}	+ 48.35	25.	T_{25} (Control)	60.25^{ef}	
13.	T_{13} (RB-64)	45.17^{f}	- 25.03				

*Mean of five replications. In each column figures followed by same letter donot differ significantly according to DMRT. +-Increase, --Decrease. *P.f-Pseudomonas fluorescens*

4.5.1 Antagonistic index (AI) of potential rhizobacterial isolates

The selected isolates were screened for their inhibitory effect against *R. solanacearum* to find out the antagonistic index (AI) as per 3.5.1. It was observed that, after 24 h, the inhibition zone (IZ) ranged from 10.70 to 27.20 mm. The minimum value was shown by RB-71 and the maximum by P.f2 (Table 4.9). After 48 h of incubation, six isolates showed an inhibition zone above 20mm with the maximum in P.f2 followed by RB-144 and RB-11 (Plate 4.4A). These isolates showed a clear zone of inhibition demarcating the growth of pathogen and bacterium with more than 24 per cent inhibition of the pathogen. The least per cent inhibition of 15.3 and 16.20 was observed in RB-71 and RB-69 respectively. Data on inhibition zone score showed that except for RB-66, RB-151, RB-69, RB-71 and P.f1, all the isolates exhibited a score of three as shown in Table 4.9. The maximum antagonistic index (AI) of 888.75 was with P.f2 followed by RB-144 and RB-11. In addition, RB-22 and RB-82 also recorded comparatively high AI. The least AI of 260.10 and 291.60 was noticed with RB-69 and RB-71.

4.5.2 Vigour index

The 11 rhizobacterial isolates were bioassayed for the ability to promote seedling growth. The highest germination per cent of 96.67 was observed in sorghum seeds treated with RB-22, RB-82, RB-69 and P.f1 (Table 4.10). Maximum length of epicotyl was with seeds bacterized with RB-144 followed by RB-71 and RB-11. The highest length of hypocotyl was observed with RB-22, followed by RB-151 and RB-11(Plate 4.4B). Sorghum seeds bacterized with RB-22 were found to be a superior plant growth activator as it showed the highest vigour index (VI) of 7.26, closely followed by RB-151, P.f1, RB-144, RB-71 and RB-11. The least vigour index of 3.26 was observed in control followed by P.f2. It was noticed that except for plants in control and P.f2, all others showed a VI of more than four and therefore they belonged to the score of four.

Table 4.9. *In vitro* inhibitory effect of potential rhizobacterial isolates against *R. solanacearum*

Sl.No.	Isolates	*Inhibition zone (mm) (IZ) after		Score	*Per cent Inhibition (PI) (48 h)	Antagonistic Index (AI)
		24 h	48 h			
1.	RB-144	19.20	26.70	3	29.66	791.92
2.	RB-22	13.70	24.30	3	27.00	656.10
3.	RB-82	17.60	23.80	3	26.44	629.27
4.	RB-66	14.80	17.00	2	18.89	321.13
5.	RB-11	20.50	26.20	3	29.11	762.68
6.	RB-151	14.50	16.80	2	18.67	313.66
7.	RB-77	15.00	22.30	3	24.78	552.59
8.	RB-69	12.20	16.20	2	16.20	260.10
9.	RB-71	10.70	15.30	2	15.30	291.60
10.	P.f1	14.30	18.50	2	20.56	380.36
11.	P.f2	27.20	28.30	3	31.44	889.75

*Mean of three replications, P.f-*Pseudomonas fluorescens*
Scale for IZ zone : >1 <10 mm = 1; >10 <20 mm = 2; >20 <30 mm = 3 and > 30 mm = 4

Table 4.10. Vigour index of sorghum seeds due to treatment with potential rhizobacterial isolates

Sl.No.	Isolate	* Per cent Germination	*Shoot length (cm)	*Root length (cm)	Vigour Index (VI)	Score
1.	RB-144	86.67	5.62	2.27	6.84	4
2.	RB-22	96.67	3.91	3.60	7.26	4
3.	RB-82	96.67	3.46	2.17	5.44	4
4.	RB-66	86.67	3.37	2.34	4.95	4
5.	RB-11	90.00	4.13	3.38	6.76	4
6.	RB-151	93.33	3.87	3.56	6.93	4
7.	RB-77	93.33	2.88	2.04	4.59	4
8.	RB-69	96.67	2.73	1.76	4.34	4
9.	RB-71	93.33	4.65	2.64	6.80	4
10.	P.f1	96.67	3.85	3.25	6.86	4
11.	P.f2	86.67	2.23	1.87	3.55	3
12.	Control	86.67	2.51	1.25	3.26	3

*Mean of three replications, **P.f-Pseudomonas fluorescens*

Score : VI of >1 <2 = 1; VI of >2 <3 = 2; VI of >3 <4 = 3 and VI >4 = 4

4.5.3 Production of hydrogen cyanide

All the selected rhizobacterial isolates were tested for their ability to produce hydrogen cyanide (HCN). It was observed that none of the isolates were cyanogenic in nature and therefore scored as one.

4.5.4 Production of ammonia

Production of ammonia by the rhizobacterial isolates were detected by change in colour in the peptone broth media on addition of Nessler's reagent. Different isolates produced varying levels of ammonia (Table 4.11) (Plate 4.4C). The isolates RB-144, RB-22, RB-66, RB-11, RB-77, RB-71and P.f1, showed more production of ammonia and were thus scored as four. The least production of ammonia as evidenced by a faint yellow colour was noticed with RB-151 and P.f2.

4.5.5 Phosphorous (P) solubilization

The phosphorous solubilization capacity of the selected isolates was tested in Pikovaskya's TCP agar as well as in its broth. Of the nine rhizobacterial isolates and reference cultures of KAU and TNAU, only eight showed phosphate solubilization zones on Pikovaskya's TCP medium plates (Table 4.12) (Plate 4.4E). The remaining cultures *viz.,* RB-151, RB-77 and RB-71 did not show 'P' solubilization on agar medium but exhibited 'P' solubilization in liquid medium. The zone of 'P 'solubilization on agar plates ranged from 4.5 to 9.5 mm within one week. From the table, it is clear that RB-144 showed the maximum diameter of clear zone of 9.5mm followed by RB-82 and P.f2. The isolate RB-11, RB-22 and the P.f1 produced zone of above 7mm. Broth assay also proved the efficacy of the isolates in solubilization of phosphorous which ranged from 0.48 to 12.71 mg per 50 ml of the culture media, the maximum being with RB-144 itself.

Table 4.11. Production of ammonia by potential rhizobacterial isolates

Sl.No.	Isolate	Score
1.	RB-144	4
2.	RB-22	4
3.	RB-82	3
4.	RB-66	4
5.	RB-11	4
6.	RB-151	2
7.	RB-77	4
8.	RB-69	3
9.	RB-71	4
10.	P.f1	4
11.	P.f2	2
12.	Control	1

Score chart: Nil: 1, Low: 2, Medium: 3, High: 4

Table 4.12. Phosphorous solubilization by potential rhizobacterial isolates

Sl.No.	Isolate	*P solubilization zone (mm)	*P solubilization (mg / 50 ml)	Score
1.	RB-144	9.5	12.71	4
2.	RB-22	7.5	9.26	4
3.	RB-82	8.9	11.98	4
4.	RB-66	5.8	8.2	3
5.	RB-11	7.8	9.04	4
6.	RB-151	0	0.48	1
7.	RB-77	0	1.47	1
8.	RB-69	4.5	5.40	2
9.	RB-71	0	2.78	1
10.	P.f1	7.3	8.83	3
11.	P.f2	8.9	9.92	4
12.	Control	0	0	1

*Mean of three replications, *P.f-Pseudomonas fluorescens*

Score : >1<3 mg $50ml^{-1}$ = 1; >3<6 mg$50ml^{-1}$= 2; >6<9 mg $50ml^{-1}$ =3 and > 9 mg $50ml^{-1}$ =4

4.5.6 Assay of growth promoting hormones

4.5.6.1 Quantitative estimation of Indole Acetic Acid (IAA)

All the isolates produced varying levels of IAA ranging from 9.02 to 56.89 μgml^{-1} (Table 4.13). The maximum amount of IAA was produced by RB-22 (Plate 4.4D). The remaining isolates except RB-69 and RB-11 produced comparatively high quantity of IAA which ranged from 16.65 to 30.98 μgml^{-1} and were therefore scored as two.

4.5.6.2 Detection of auxins and gibberellins by thin layer chromatography

Observations of chromatograms to detect auxins and gibberellins by potential rhizobacterial isolates using alpha imager revealed that the isolates produced different metabolites with varying Rf values (Table 4.14). Among the various isolates, RB-144, RB-11 and P.f2 produced four metabolites with different Rf values and colours. The yellowish green colour by RB-144 and RB-11 indicated the presence of gibberellins, while, the red / violet colour showed the production of auxins (Plate 4.5).

Further, the chromatogram also showed the production of various other metabolites by different isolates. The blue / violet fluorescent bands noticed with most of the isolates except RB-66 and RB-77 indicated derivatives of auxin. It is also to be noted that except for RB-66 and RB-77, all the isolates produced more than one metabolite.

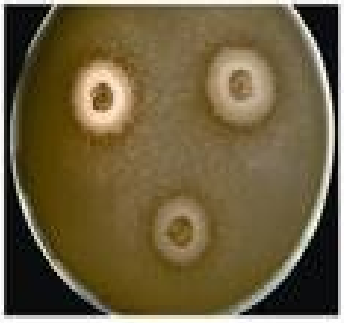
4.5.7 Determination of PGPR index

All the qualitative and quantitative data on plant growth promoting parameters *viz.,* vigour index (VI), phosphorous (P) solubilization, indole acetic acid (IAA), ammonia and hydrogen cyanide (HCN) production as well as antagonistic index (AI) were transformed into 1-4 scale and the PGPR index of the potential rhizobacterial isolates was calculated (Table 4.15).

Table 4.13. Production of indole acetic acid (IAA) by potential rhizobacterial isolates

Sl.No.	Isolate	*IAA (µg ml $^{-1}$)	Score
1.	RB-144	19.02	2
2.	RB-22	56.89	4
3.	RB-82	20.12	2
4.	RB-66	22.20	2
5.	RB-11	9.02	1
6.	RB-151	30.98	2
7.	RB-77	19.63	2
8.	RB-69	11.96	1
9.	RB-71	26.33	2
10.	P.f1	18.61	2
11.	P.f2	16.65	2
12.	Control	-	1

*Mean of three replications, *P.f-Pseudomonas fluorescens*
Score : >0<15 µgml^{-1} =1; >16<30 µgml^{-1}=2; >31<45 µgml^{-1} = 3 and > 46 µgml^{-1} = 4



A. Inhibition zone by RB-11 on the pathogen

B. Test for vigour index of PGPR isolates

C. Production of ammonia by the isolates

D. Production of IAA by the isolates

E. Zone of phosphorous solubilization produced by various isolates

Plate 4.4 Various attributes of plant growth promoting index (PGPI)

Among the 11 isolates, RB-22 showed higher PGPR index of 83.33 followed by RB-144, RB-82 and RB-11 with a value of above 70. Apart from RB-69, RB-151 and RB-71, remaining isolates *viz*., RB-66, RB-77 and P.f2 showed an index of above 60.

4.6 ASSAY OF PRODUCTION OF SECONDARY METABOLITES

Secondary metabolites like salicylic acid, antibiotics and siderophores produced by the potential rhizobacterial isolates were detected by various methods. Salicylic acid production was determined both by TLC and spectroscopy whereas antibiotics were assessed by chromatographic means alone. Siderophores were detected by U.V fluorescence, CAS assay as well as by spectrophotometry.

4.6.1 Spectroscopic and TLC analysis of salicylic acid

From the results furnished in Table 4.16, it is evident that, all the cell free culture filtrates (CFCF) of the rhizobacterial isolates produced salicylic acid in varying quantities. The highest quantity of salicylic acid was obtained with P.f2 followed by P.f1, RB-11, RB-22 and RB-66. The lowest quantity was noticed with RB-71 (Plate 4.6).

Qualitative analysis of salicylic acid from CFCF of six rhizobacterial isolates *viz*., RB-22, RB-82, RB-66, RB-11, P.f1 and P.f2 revealed its presence on chromatogram as indicated by brown spots with Rf value 1.0. However, it was not detected with the remaining isolates. Apart from salicylic acid, RB-22, RB-66 and P.f2 produced other metabolites on chromatograms with yellow colour and different Rf value.

4.6.2 TLC profile of antibiotics

Acetone extracts of culture supernatants from various isolates were subjected to thin layer chromatography (TLC) to detect production of antibiotics. The chromatograms developed were observed both under UV cabinet as well as in an

Table 4.14. TLC profile of growth regulators produced by potential rhizobacterial isolates

Sl.No.	Isolate	Rf value	Colour of Bands	
			Visible light	U.V.light
1.	RB-144	0.96 0.89 0.72 0.11	Red Dark Yellow Red Brown	- Yellowish green Red Brown
2.	RB-22	0.92 0.94 0.98	Light red Dark red Orange red	Violet Blue Violet
3.	RB-82	0.89 0.94 0.98	Brown Red Meroon Red Orange Red	Violet Blue Violet
4.	RB-66	0.91	Light Yellow	-
5.	RB-11	0.79 0.76 0.87 0.83	Blue Red Invisible Invisible	Blue Violet Yellowish green Violet
6.	RB-151	0.69 0.75 0.83	Pink Violet Red	Red Red Red
7.	RB-77	0.98	Red	-
8.	RB-69	0.80 0.67	Invisible Invisible	Blue Yellowish green
9.	RB-71	0.72 0.88	Yellow Red	- -
10.	P.f1	0.75 0.83 0.98	Violet Red Red	Red Red Red
11.	P.f2	0.61 0.68 0.77 0.96	Yellow Violet Red Orange	Red Red Red Red

*IAA-Indole acetic acid / Auxin (red), GA-Giberellic acid (yellowish green), Derivatives of IAA-Blue / violet fluorescence ,**P.f-Pseudomonas fluorescens*

Table 4.15. PGPR index of potential rhizobacterial isolates

Sl.No.	Isolate	Score for various parameters						*PGPR Index
		Seed germination	IAA production	NH_3 production	P solubilization	HCN production	Per cent inhibition	
1.	RB-144	4	2	4	4	1	3	75.00
2.	RB-22	4	4	4	4	1	3	83.33
3.	RB-82	4	2	3	4	1	3	70.83
4.	RB-66	4	2	4	3	1	2	66.67
5.	RB-11	4	1	4	4	1	3	70.83
6.	RB-151	4	2	2	1	1	2	50.0
7.	RB-77	4	2	4	1	1	3	62.50
8.	RB-69	4	1	3	2	1	2	54.17
9.	RB-71	4	2	4	1	1	2	58.33
10.	P.f1	4	2	4	3	1	2	66.67
11.	P.f2	3	2	2	4	1	3	62.50
12.	Control	3	1	1	1	1	1	33.33

Table 4.16. Spectronic assay and TLC profile of salicylic acid (SA) by potential rhizobacterial isolates

Sl.No.	Isolate	*SA ($\mu g\ ml^{-1}$)	Rf value in TLC plate	Colour of band in visible light
1.	RB-144	27.19	-	N.D
2.	RB-22	59.82	1.0, 0.88, 0.71	Brown, Yellow, Yellow
3.	RB-82	32.09	1.0	Brown
4.	RB-66	47.92	1.0, 0.90, 0.72	Brown, Yellow, Yellow
5.	RB-11	62.33	1.0	Brown
6.	RB-151	15.55	-	N.D
7.	RB-77	24.04	-	N.D
8.	RB-69	17.74	-	N.D
9.	RB-71	9.42	-	N.D
10.	P.f1	75.68	1.0	Brown
11.	P.f2.	101.11	1.0, 0.78, 0.56	Brown, Yellow, Yellow

*Mean of three replications. Standard of salicylic acid - brown colour with Rf value of 1.0
P.f – Pseudomonas fluorescens, N.D-Not detectable

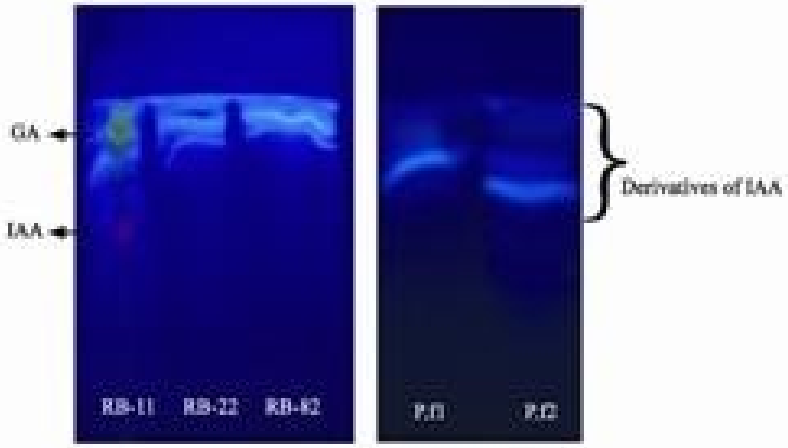


Plate 4.5 TLC profile of growth regulators
GA-Gibberilic acid, IAA-Indole acetic acid

Plate 4.6 Production of salicylic acid by PGPR isolates

Plate 4.7 TLC profile of antibiotics

iodine chamber and the characteristic colours developed with their Rf values are summarized in Table 4.17.

It was observed that all the isolates except RB-151, RB-69 and RB-71 produced various antibiotics. The antibiotic produced by the isolates were tentatively identified / detected as pyoluteorin, pyrrolnitrin, 2, 4 - diacetyl phloroglucinol (2, 4 DAPG) and pyocyanin. The isolates RB-144, RB-22, RB-82, RB-77 and the P.f2 produced blue spots with Rf value ranging from 0.33 to 0.37 comparable with pyoluteorin with standard Rf value of 0.36. With RB-22, RB-66, RB-11 and P.f1, a fluorescent spot of Rf value from 0.82 to 0.85 was noticed similar to pyrrolnitrin (Rf value 0.86) (Plate 4.7). The isolate RB-22 produced two other metabolites, one with Rf value of 0.11 which remained unidentified and the other blue spot with Rf value of 0.9 which may probably be pyocyanin (Rf value 0.88). Isolate RB-144 produced two other metabolites with Rf value 0.59 and 0.94. Apart from pyoluteorin and pyrrolnitrin, the culture supernatant of RB-82, RB-66, RB-11 and the P.f2 produced certain unidentified metabolites. Further, RB-66, RB-11 and P.f2 recorded a crimson yellow metabolite of Rf value ranging from 0.9 to 0.91with similarity to 2, 4-DAPG (Rf value 0.88).

4.6.3 Detection of siderophores

The ability of the 11 promising rhizobacterial isolates including the two reference ones were tested for their capacity to produce siderophores.

4.6.3.1 Detection of siderophores by UV fluorescence method

Among the bacterial cultures tested, the isolates *viz.,* RB-22, RB-82, RB-66 and RB-11 and the reference cultures only showed fluorescence under U.V. light (Plate 4.8).

Table 4.17. TLC profile of antibiotics produced by potential rhizobacterial isolates

Sl.No.	Isolate	Rf value	Iodine Chamber / Visible light	U.V.light	Probable Antibiotic
1.	RB-144	0.59 0.94 0.35	blue blue blue	- - -	unidentified unidentified Pyoluteorin
2.	RB-22	0.34 0.83 0.11 0.90	blue blue blue blue	blue blue - -	Pyoluteorin Pyrrolnitrin unidentified Pyocyanin
3.	RB-82	0.37 0.94	- blue	bluish green -	Pyoluteorin unidentified
4.	RB-66	0.82 0.61 0.91	- blue yellow	blue - -	Pyrrolnitrin unidentified 2,4 DAPG
5.	RB-11	0.83 0.60 0.90	- blue yellow	blue - -	Pyrrolnitrin unidentified 2,4 DAPG
6.	RB-151	-	-	-	*N.D
7.	RB-77	0.33	-	blue	Pyoluteorin
8.	RB-69	-	-	-	*N.D
9.	RB-71	-	-	-	*N.D
10.	P.f1	0.85 0.96	- blue	blue -	Pyrrolnitrin unidentified
11.	P.f2	0.34 0.61 0.91	blue blue yellow	- - -	Pyoluteorin unidentified 2,4 DAPG

Rf values of standards – Pyoluteorin (blue) (0.36), Pyrrolnitrin (Bluish green) (0.86)
Pyocyanin (blue) (0.88), 2, 4-diacetyl phloroglucinol (yellow) (0.88)
**P.f – Pseudomonas fluorescens*, *N.D-Not detectable

4.6.3.2 Chrome azurol (CAS) assay

The data presented in Table 4.18 indicated the production of siderophores by all bacterial strains except RB-151, RB-69 and RB-71. The isolates RB-22 and RB-11 produced a zone of colouration of 24 mm, while all others produced a zone of 12 to 23cm (Plate 4.9).

4.6.3.3 Iron dependent production of siderophores

The capacity of the rhizobacterial isolates to produce siderophores reduced as the concentration of $FeCl_3$ increased in the broth medium. Further, there was variation in the production of siderophores by the different isolates (Table 4.19 and Fig 4.1). Without the addition of $FeCl_3$, the maximum siderophores was produced by RB-22, followed by RB-66. The least production was by RB-77 and RB-144. At 200µm of $FeCl_3$, the isolates RB-144, RB-82, RB-66, RB-11 and P.f1 released only half the quantity of siderophore from the initial rate and the production further decreased at 300µm of $FeCl_3$, where the least production was noticed in RB-144 and RB-77. However, it was noticed that RB-22 produced the maximum quantity of siderophore and its capacity to produce siderophore was not much affected by $FeCl_3$ concentration.

4.7 POPULATION DYNAMICS OF THE POTENTIAL ANTAGONISTS

The rhizobacterial isolates were tested for their competence to colonize the rhizosphere soil. Rhizosphere population of the isolates was assessed at monthly intervals for three months. Population of isolates varied during different intervals of observation (Table 4.20). In general, as the time of sampling increased, there was reduction in the population of isolates from one month to three months. The bacterial population ranged from 11.67 to 32.0 $cfug^{-1}$ soil after one month where the minimum was in RB-144 and the maximum in RB-82. The population after two and three months slightly decreased when compared to the initial value. After two months, the

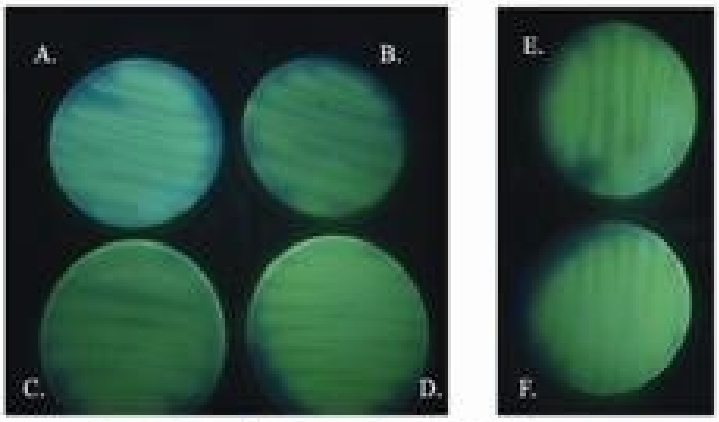


Plate 4.8 Detection of siderophores by U.V fluorescence

A. RB-22 B. RB-82 C. RB-66 D. RB-11 E. P.fl F. P.fl

Plate 4.9 Siderophore production by CAS assay

A. RB-22 B. RB-82 C. RB-66 D. RB-11 E. P.fl F. P.fl

Table 4.18. Siderophore production by promising rhizobacterial isolates by CAS assay

Sl.No.	Treatment	Zone of colouration on CAS plates (mm)
1.	RB-144	14
2.	RB-22	24
3.	RB-82	21
4.	RB-66	22
5.	RB-11	24
6.	P.f1	21
7.	RB-77	12
8.	P.f2	23
9.	RB-151	*N.D
10.	RB-71	*N.D
11.	RB-69	*N.D

*Mean of three replications, *N.D- not dectectable

Table 4.19. Production of siderophores by promising rhizobacterial isolates at different concentrations of $FeCl_3$

Sl.No.	Isolate	OD Value			
		Different concentrations of $FeCl_3$			
		0 μM	100 μM	200 μM	300 μM
1.	RB-144	0.152	0.137	0.092	0.046
2.	RB-22	1.720	1.580	1.400	1.330
3.	RB-82	0.965	0.476	0.392	0.280
4.	RB-66	1.660	0.865	0.676	0.616
5.	RB-11	1.100	0.528	0.504	0.464
6.	P.f1	0.452	0.374	0.293	0.213
7.	RB-77	0.148	0.144	0.105	0.100
8.	P.f2	1.130	0.802	0.742	0.596
9.	RB-151	0.125	0.114	0.108	0.101
10.	RB-71	0.134	0.122	0.114	0.109
11.	RB-69	0.144	0.135	0.119	0.103

P.f-Pseudomonas fluorescens, $FeCl_3$-Ferric chloride

highest population was detected for RB-77 and P.f1 and the lowest with RB-1. The population after three months ranged from 7.0 to 18.67 $cfug^{-1}$ soil.

4.8 *In vivo* EVALUATION OF POTENTIAL RHIZOBACTERIA FOR GROWTH PROMOTION AND DISEASE SUPPRESSION

An experiment was laid out to study the growth promoting effect of the nine potential antagonistic rhizobacterial isolates selected after the *in vitro* evaluation in comparison with the reference cultures of TNAU and KAU. In addition to the observations on germination percentage and biometric characters, changes in the phenol, protein and amino acid contents in the rhizobacteria inoculated plants due to infection were also studied. Moreover, observations on the natural incidence of pest and diseases and screening for disease resistance after challenge inoculation with the pathogen were also recorded.

4.8.1 Germination percentage

The data in Table 4.21 revealed that all the treatments except for T_{13} (Control) showed germination of 80 per cent and above. The maximum germination per cent of 100 was noticed in T_3 (RB-82), T_5 (RB-11), T_9 (P.f2) and T_{10} (RB-69) followed by T_1 (RB-144), T_2 (RB-22) and T_7 (P.f1) with 95 per cent. The least per cent germination was noticed in control (T_{13}). Pre emergence rot was maximum in T_{13} (Control) followed by T_3 (RB-82), T_{11} (RB-71) and T_{12} (PoP) in that order. The post emergence rot of 10 per cent was noticed in T_{11}, T_{12} and T_{13}.

4.8.2 Early sprouting of ginger plants

Observations were taken 20 and 25 days after planting (DAP) so as to record the effect of different bioagents in initiating early sprouting of ginger. After 20 days of planting, significant difference in the number of tillers was observed among the treatments with the maximum in T_5 (RB-11) and T_9 (P.f2) and these were on par with seven other treatments (Table 4.22) (Fig 4.2). Apart from plants in T_{12}

Table 4.20. Population dynamics of potential rhizobacterial isolates at different intervals

Sl.No.	Isolate	Bacteria (x 10^6 cfu g^{-1} soil)		
		*1 MAP	*2 MAP	*3 MAP
1.	RB-144	11.67	9.67	7.00
2.	RB-22	26.00	17.33	13.00
3.	RB-82	32.00	14.00	18.67
4.	RB-66	19.33	20.00	12.67
5.	RB-11	31.67	19.67	18.00
6.	RB-151	19.67	14.67	7.00
7.	RB-77	29.67	22.67	7.67
8.	RB-69	18.67	14.00	11.67
9.	RB-71	17.00	10.67	7.33
10.	P.f1	27.00	22.67	7.67
11.	P.f2	23.00	14.67	11.33

*Mean of three replications, **P.f-Pseudomonas fluorescens,* MAP: months after planting

Table 4.21. Effect of potential rhizobacterial isolates on the germination, pre emergence and post emergence rot of ginger

Sl.No.	Treatment	*Germination (%)	Pre-emergence rot (%)	Post emergence rot (%)
1.	T_1 (RB-144)	95	5	0
2.	T_2 (RB-22)	95	5	0
3.	T_3 (RB-82)	100	0	0
4.	T_4 (RB-66)	90	5	5
5.	T_5 (RB-11)	100	0	0
6.	T_6 (RB-151)	80	20	0
7.	T_7 (P.f1)	95	5	5
8.	T_8 (RB-77)	90	5	5
RB-71□9. 9.	T_9 (P.f2)	100	0	0
10.	T_{10} (RB-69)	80	10	10
11.	T_{11} (RB-71)	80	10	10
12.	T_{12} (PoP)	80	10	10
13.	T_{13} (Control)	70	30	10

*Mean of ten replications, *P.f-Pseudomonas fluorescens*

Table 4.22. Effect of potential rhizobacterial isolates on early sprouting of ginger

Sl.No.	Treatment	20 DAP			25 DAP		
		*Number of tillers	*Height of tillers	*Number of leaves per tiller	*Number of tillers	*Height of tillers	*Number of leaves per tiller
1.	T_1 (RB-144)	2.56 ^{bcd}	9.28 ^{a}	0.91 ^{abcd}	3.78 ^{ab}	14.83 ^{abc}	2.49 ^{a}
2.	T_2 (RB-22)	2.75 ^{ab}	8.54 ^{a}	1.33 ^{abc}	3.72 ^{ab}	18.71 ^{a}	2.48 ^{a}
3.	T_3 (RB-82)	2.53 ^{abc}	9.34 ^{a}	1.06 ^{abcd}	3.70 ^{ab}	16.53 ^{a}	2.11 ^{ab}
4.	T_4 (RB-66)	2.78 ^{ab}	10.88 ^{a}	1.68 ^{ab}	3.83 ^{ab}	15.68 ^{ab}	2.59 ^{a}
5.	T_5 (RB-11)	3.56 ^{a}	8.59 ^{a}	1.28 ^{abc}	4.11 ^{ab}	14.86 ^{abc}	1.96 ^{abc}
6.	T_6 (RB-151)	1.89 ^{abc}	8.44 ^{a}	1.08 ^{abcd}	3.50 ^{b}	15.54 ^{abc}	2.37 ^{a}
7.	T_7 (P.f1)	2.91 ^{ab}	11.49 ^{a}	1.87 ^{a}	5.72 ^{a}	10.43 ^{bc}	2.07 ^{abc}
8.	T_8 (RB-77)	2.42 ^{abc}	10.73 ^{a}	1.56 ^{abc}	3.50 ^{b}	15.21 ^{abc}	2.52 ^{a}
9.	T_9 (P.f2)	3.28 ^{a}	10.18 ^{a}	1.86 ^{a}	3.78 ^{ab}	18.11 ^{a}	2.50 ^{a}
10.	T_{10} (RB-69)	2.38 ^{abc}	7.82 ^{a}	0.88 ^{bcd}	3.44 ^{b}	15.14 ^{abc}	2.12 ^{ab}
11.	T_{11} (RB-71)	1.95 ^{bcd}	7.51 ^{a}	1.39 ^{abc}	3.33 ^{b}	13.76 ^{abc}	2.09 ^{ab}
12.	T_{12} (PoP)	1.33 ^{cd}	3.33 ^{b}	0.61 ^{cd}	2.47 ^{bc}	9.95 ^{c}	1.31 ^{c}
13.	T_{13} (Control)	0.97 ^{d}	3.36 ^{b}	0.31 ^{d}	1.39 ^{c}	10.56 ^{bc}	1.47 ^{bc}

*Mean of three replications, DAP-Days after planting. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Fig. 4.1 Production of siderophore by rhizobacterial isolates at various concentrations of FeCl3

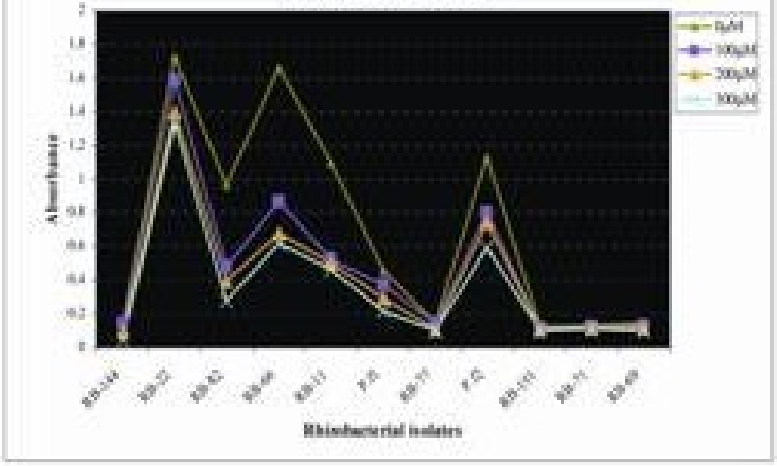
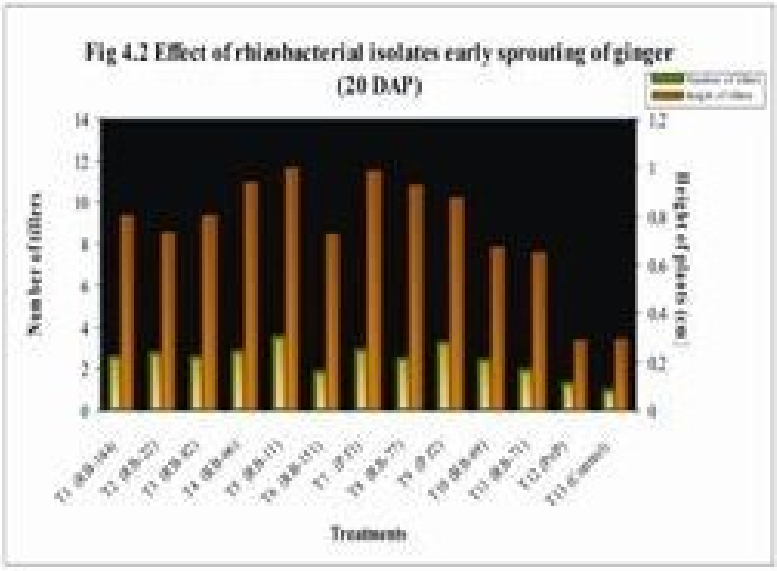


Fig 4.2 Effect of rhizobacterial isolates early sprouting of ginger (20 DAP)



(PoP) and T_{13} (Control), there was no significant difference among treatments with reference to height of tillers. On the other hand, the number of leaves per tiller varied among treatments where the maximum was noticed in T_7 (P.f1) and T_9 (P.f2) and these were on par with all other treatments except T_{10} (RB-69), T_{12} and T_{13}.

After 25 days of planting, there was significant difference among the treatments with regard to number and height of tillers and number of leaves as well. The maximum number of tillers of 5.72 was recorded in T_7 (P.f1) which was on par with T_1 (RB-144), T_2 (RB-22), T_3 (RB-82), T_4 (RB-66), T_5 (RB-11) and T_9 (P.f2). The maximum height was in T_2 (RB-22) and was on par with all other treatments except for T_7 (P.f1), T_{12} (PoP) and T_{13} (Control). Data on the number of leaves varied with different treatments. All the treatments except T_{12} and T_{13} were on par with each other.

4.8.3 Number of tillers

Observations (Table 4.23) on the number of tillers at different intervals revealed significant difference among the treatments. One month after planting, in general, the rhizobacterial treatments had a significant effect in increasing the number of tillers compared to control. During this period, plants treated with T_7 (P.f1) showed the maximum number of tillers followed by T_4 (RB-66). At two months after planting, the maximum tiller production was in plants in T_5 (RB-11) and the minimum in control (T_{13}). Three months after planting, T_5 closely followed by T_1 (RB-144) and T_4 produced the maximum tillers. At fourth and fifth month after planting, the maximum tiller production was in treatment T_5 followed by T_9 (P.f2) and the minimum in control.

4.8.4 Number of leaves per tiller

Observations on the number of leaves taken one month after planting showed significant difference among treatments (Table 4.24). Plants in various treatments recorded a per cent efficiency over control ranging from zero in T_{12} (PoP)

Table 4.23. Effect of potential rhizobacterial isolates on number of tillers of ginger plants

Sl.No.	Treatment	*1 MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Number of tillers	Per cent +/-over control	Number of tillers	Per cent +/-over control	Number of tillers	Per cent +/-over control	Number of tillers	Per cent +/-over control	Number of tillers	Per cent +/-over control
1.	T_1 (RB-144)	4.66^{abc}	+124.04	9.83^{ab}	+123.41	15.72^{a}	+135.68	20.00^{ab}	+56.49	27.50^{ab}	+61.76
2.	T_2 (RB-22)	4.50^{bc}	+116.35	7.92^{bcd}	+80.00	13.17^{ab}	+97.45	20.39^{ab}	+59.55	25.28^{ab}	+48.71
3.	T_3 (RB-82)	5.00^{abc}	+140.38	9.58^{abc}	+117.73	13.89^{ab}	+108.25	20.50^{ab}	+60.41	26.45^{ab}	+55.59
4.	T_4 (RB-66)	5.53^{ab}	+165.87	9.25^{abcd}	+110.23	15.78^{a}	+136.58	22.67^{ab}	+77.39	27.80^{ab}	+63.53
5.	T_5 (RB-11)	5.08^{abc}	+144.23	10.33^{a}	+134.77	16.28^{a}	+144.08	23.67^{a}	+85.21	28.44^{a}	+67.29
6.	T_6 (RB-151)	5.11^{abc}	+145.67	8.92^{abcd}	+102.73	13.78^{ab}	+106.60	20.72^{ab}	+62.13	22.39^{b}	+31.71
7.	T_7 (P.f1)	6.58^{a}	+216.35	8.30^{abcd}	+88.64	13.64^{ab}	+104.50	20.31^{ab}	+58.92	23.56^{ab}	+38.59
8.	T_8 (RB-77)	4.86^{abc}	+133.65	7.83^{bcd}	+77.95	11.42^{b}	+71.21	22.00^{ab}	+72.14	26.11^{ab}	+53.59
9.	T_9 (P.f2)	4.78^{abc}	+129.81	9.83^{ab}	+123.41	13.92^{ab}	+108.70	23.39^{a}	+83.02	28.28^{a}	+66.35
10.	T_{10} (RB-69)	4.50^{bc}	+116.35	7.20^{d}	+63.64	12.89^{ab}	+93.25	21.00^{ab}	+64.32	23.36^{ab}	+37.41
11.	T_{11} (RB-71)	4.14^{bc}	+99.04	7.57^{cd}	+72.05	12.44^{ab}	+86.51	20.55^{ab}	+60.80	22.56^{b}	+32.71
12.	T_{12} (PoP)	3.14^{cd}	+50.96	5.17^{e}	+17.50	7.78^{c}	+16.64	18.22^{b}	+42.57	22.22^{b}	+30.71
13.	T_{13} (Control)	2.08^{d}		4.40^{e}		6.67^{c}		12.78^{c}		17.00^{c}	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.24. Effect of potential rhizobacterial isolates on leaves of ginger plants

Sl.No.	Treatment	*1MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Number of leaves per tiller	Per cent +/- over control	Number of leaves per tiller	Per cent +/- over control	Number of leaves per tiller	Per cent +/- over control	Number of leaves per tiller	Per cent +/- over control	Number of leaves per tiller	Per cent +/- over control
1.	T_1 (RB-144)	3.67^{a}	+78.16	5.33^{ef}	+14.13	10.56^{abc}	+20.82	13.25^{ab}	+6.94	16.78^{abcd}	+11.05
2.	T_2 (RB-22)	3.47^{ab}	+68.45	7.25^{abcde}	+55.25	10.14^{bc}	+16.02	13.92^{ab}	+12.35	16.64^{abcd}	+10.13
3.	T_3 (RB-82)	3.25^{ab}	+57.77	5.62^{def}	+20.34	10.08^{bc}	+15.33	14.86^{a}	+19.94	17.97^{ab}	+18.93
4.	T_4 (RB-66)	3.28^{ab}	+59.22	7.17^{abcde}	+53.53	10.92^{ab}	+24.94	13.72^{ab}	+10.73	18.19^{a}	+20.38
5.	T_5 (RB-11)	3.11^{ab}	+50.97	7.33^{abcd}	+56.96	10.44^{abc}	+19.45	13.11^{ab}	+5.81	17.97^{abc}	+18.93
6.	T_6 (RB-151)	3.30^{ab}	+60.19	7.67^{abc}	+64.24	11.42^{ab}	+30.66	12.72^{ab}	+2.66	14.58^{d}	-3.51
7.	T_7 (P.f1)	2.94^{ab}	+42.72	8.50^{a}	+82.01	11.56^{ab}	+32.27	12.71^{b}	+2.58	16.61^{abcd}	+9.93
8.	T_8 (RB-77)	3.31^{ab}	+60.68	6.67^{bcde}	+42.83	9.63^{bc}	+10.18	13.55^{ab}	+9.36	16.44^{abcd}	+8.80
9.	T_9 (P.f2)	3.22^{ab}	+56.31	7.75^{abc}	+65.95	11.15^{ab}	+27.57	12.94^{ab}	+4.44	16.03^{abcd}	+6.09
10.	T_{10} (RB-69)	2.78^{b}	+34.95	8.67^{a}	+85.65	12.44^{a}	+42.33	13.85^{ab}	+11.78	14.85^{d}	-1.72
11.	T_{11} (RB-71)	2.83^{b}	+37.38	8.75^{a}	+87.37	11.5^{ab}	+31.58	12.61^{b}	+1.78	15.06^{cd}	-0.33
12.	T_{12} (PoP)	2.06^{c}	0.00	6.33^{cdef}	+35.55	8.79^{c}	+0.57	13.56^{ab}	+9.44	15.66^{bcd}	+3.64
13.	T_{13} (Control)	2.06^{c}		4.67^{f}		8.74^{c}		12.39^{b}		15.11^{cd}	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

to 78.16 T_1 (RB-144). Two months after planting, the maximum number of leaves was in T_{11} (RB-71) and it was on par with seven other treatments. The minimum number was in control (T_{13}). Likewise, after three and four months of planting the minimum number was in T_{13}. However, at five months after planting, the maximum number was in T_4 (RB-66) followed by T_3 (RB-82), T_5 (RB-11), T_1 (RB-144) and T_2 (RB-22) and the minimum in T_6 (RB-151), T_{10} (RB-69) and T_{11} (RB-71).

4.8.5 Height of tillers

The height of tillers in each treatment was also recorded at monthly intervals upto five months after planting. The results of the study shown in Table 4.25 revealed significant difference in the height of tillers among the treatments at different intervals. Maximum height of tillers after one and two months of planting was in T_5 (RB-11) which also showed maximum percentage efficiency over control. This was closely followed by T_3 (RB-82) and T_7 (P.f1). The least height was noticed in control (T_{13}). At three months, the maximum height was in T_9 and was on par with six others. However, at four and five months after planting, the maximum height was observed in T_4 (RB-66) which was on par with T_9 (P.f2), T_3 (RB-82), T_1 (RB-144), T_5 (RB-11) and T_7 (P.f1). The minimum height was observed in T_{10} (RB-69) on the fourth and fifth month.

4.8.6 Natural incidence of bacterial wilt, rhizome rot and pest

The effect of various treatments on the incidence of pest and diseases at different intervals showed significant difference. Ninety days after planting, shoot borer incidence was the highest in T_9 (P.f2) which was on par with T_{12} (PoP) (Table 4.26) (Fig 4.3). Further, the incidence of bacterial wilt was found in all treatments except T_3 (RB-82) and T_5 (RB-11). The maximum wilt incidence was in T_{10} (RB-69) and T_{13} (Control) closely followed by T_7 (P.f1), T_8 (RB-77) and T_9 (P.f2). Incidence of rhizome rot was noticed only in treatments T_3 (RB-82), T_6 (RB151), T7 (P.f1) and T_{13} (Control) where the highest incidence in control.

Table 4.25. Effect of potential rhizobacterial isolates on height of tillers of ginger plants

Sl.No.	Treatment	*1 MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control
1.	T_1 (RB-144)	16.89^{ab}	+49.73	29.60^{bcd}	+14.20	38.18^{de}	+7.04	46.33^{abc}	+10.31	52.00^{ab}	+14.29
2.	T_2 (RB-22)	18.71^{ab}	+65.87	28.08^{cd}	+8.33	40.28^{abcd}	+12.92	43.42^{cde}	+3.38	51.22^{abc}	+12.57
3.	T_3 (RB-82)	19.86^{a}	+76.06	30.91^{bc}	+19.25	41.78^{abc}	+17.13	47.44^{abc}	+12.95	56.46^{a}	+24.09
4.	T_4 (RB-66)	18.21^{ab}	+61.44	32.50^{ab}	+25.39	41.42^{abc}	+16.12	50.89^{a}	+21.17	56.50^{a}	+24.18
5.	T_5 (RB-11)	19.93^{a}	+76.68	35.25^{a}	+36.00	41.83^{abc}	+17.27	45.78^{abcd}	+9.00	51.33^{abc}	+12.81
6.	T_6 (RB-151)	18.19^{ab}	+61.26	26.83^{cd}	+3.51	38.15^{bcde}	+6.95	43.69^{cde}	+4.02	49.56^{bc}	+8.92
7.	T_7 (P.f1)	11.65^{c}	+3.28	30.22^{bcd}	+16.59	41.22^{abc}	+15.56	45.58^{abcd}	+8.52	50.00^{bc}	+9.89
8.	T_8 (RB-77)	16.75^{ab}	+48.49	25.97^{d}	+0.19	37.19^{cde}	+4.26	44.56^{bcd}	+6.10	49.00^{bcd}	+7.69
9.	T_9 (P.f2)	19.79^{a}	+75.44	32.50^{ab}	+25.39	44.66^{a}	+25.20	50.25^{ab}	+19.64	53.75^{ab}	+18.13
10.	T_{10} (RB-69)	16.22^{abc}	+43.79	27.13^{cd}	+4.67	33.70^{e}	-5.52	38.51^{e}	-8.31	43.46^{d}	-4.48
11.	T_{11} (RB-71)	15.11^{abc}	+33.95	28.75^{bcd}	+10.92	42.56^{ab}	+19.32	46.17^{abcd}	+9.93	48.19^{bcd}	+5.91
12.	T_{12} (PoP)	13.80^{bc}	+22.34	26.61^{cd}	+2.66	36.22^{de}	+1.54	40.39^{de}	-3.83	47.67^{bcd}	+4.77
13.	T_{13} (Control)	11.28^{c}		25.92^{d}		35.67^{de}		42.00^{cde}		45.50^{cd}	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.26. Effect of potential rhizobacterial isolates on incidence of pest and diseases

Sl.No	Treatment	*Shoot borer (%)	*Bacterial wilt (%)	*Rhizome rot (%)
		90 DAP	120 DAP	
1.	T_1 (RB-144)	4.00 (2.12) [bc]	10.0 (3.18) [a]	0.0 (0.71) [b]
2.	T_2 (RB-22)	4.70 (2.28) [b]	10.0 (3.18) [a]	0.0 (0.71) [b]
3.	T_3 (RB-82)	2.48 (1.72) [d]	0.0 (0.71) [b]	11.11(3.20) [a]
4.	T_4 (RB-66)	2.00 (1.58) [d]	10.0 (3.18) [a]	0.0 (0.71) [b]
5.	T_5 (RB-11)	2.00 (1.57) [d]	0.0 (0.71) [b]	0.0 (0.71) [b]
6.	T_6 (RB-151)	3.08 (1.89) [cd]	10.0 (3.18) [a]	20.0 (4.43) [a]
7.	T_7 (P.f1)	4.40 (2.21) [bc]	20.0 (4.43) [a]	10.0 (3.18) [a]
8.	T_8 (RB-77)	5.22 (2.38) [b]	20.0 (4.43) [a]	0.0 (0.71) [b]
9.	T_9 (P.f2)	7.04 (2.74) [a]	20.0 (4.43) [a]	0.0 (0.71) [b]
10.	T_{10} (RB-69)	4.31 (2.19) [bc]	22.22 (4.32) [a]	0.0 (0.71) [b]
11.	T_{11} (RB-71)	4.30 (2.18) [bc]	10.0 (3.18) [a]	0.0 (0.71) [b]
12.	T_{12} (PoP)	7.50 (2.83) [a]	10.0 (3.18) [a]	10.0 (3.18) [a]
13.	T_{13} (Control)	5.05 (2.35) [b]	22.22 (4.32) [a]	22.22 (4.32) [a]

*Mean of three replications, DAP-days after planting. In each column figures followed by same letter donot differ significantly according to DMRT. Figures in parenthesis are $\sqrt{x+0.5}$ transformed values. *P.f-Pseudomonas fluorescens*

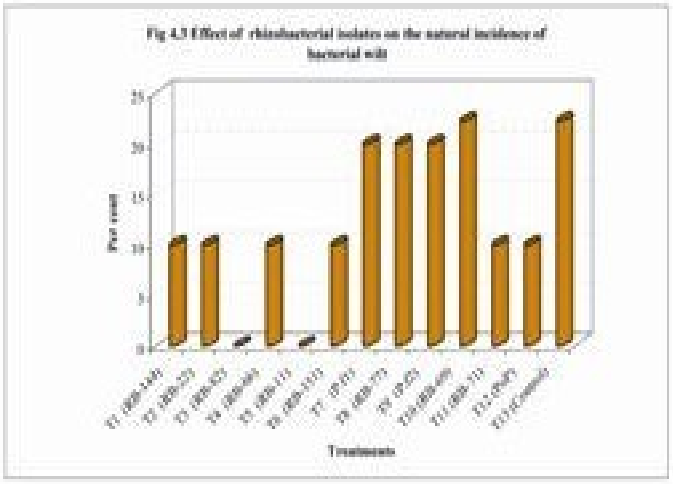


Fig 4.3 Effect of rhizobacterial isolates on the natural incidence of bacterial wilt

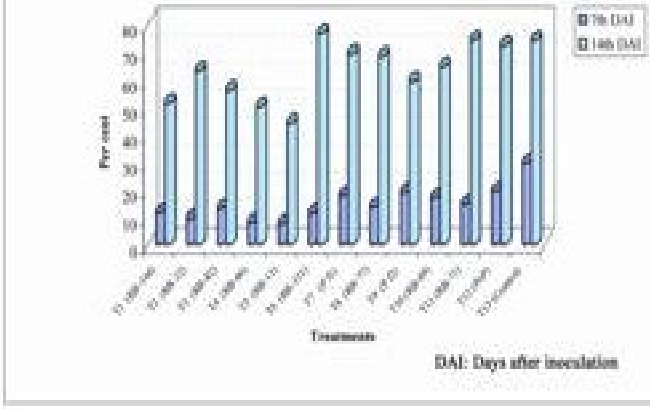


Fig 4.4 Effect of rhizobacterial isolates on the incidence of bacterial wilt after challenge inoculation with pathogen

4.8.7 Bacterial wilt incidence after challenge inoculation with the pathogen

The data presented in Table 4.27 revealed significant difference among the treatments. In general, all the plants in different treatments were infected to the bacterial wilt pathogen with varying percentage of incidence (Fig 4.4). The least per cent of infection was in T_5 (RB-11). It was on par with T_4 (RB-66), T_2 (RB-22), T_6 (RB-151) and T_{10} (RB-69) on the seventh day after inoculation. The highest incidence was in control (T_{13}). Fourteenth day of inoculation, the minimum disease incidence of 44.12 per cent was in T_5 (RB-144) which was closely followed by T_1 (RB-144), T_4 (RB-66), T_3 (RB-82) and T_9 (P.f2) and the maximum in T_6 (RB-151).

4.8.8 Yield of ginger, fresh root weight and top shoot dry weight

Statistical analysis of the data on yield of ginger revealed a significant difference among the treatments (Table 4.28). Fresh weight of rhizomes varied from 297.78g to 449.17g. The plants in T_5 (RB-11) recorded the maximum yield of followed by T_1 (RB-144), T_2 (RB-22), T_3 (RB-82 and T_4 (RB-66). Compared to plants in control, all the rhizobacterial treatments had an effect in increasing the rhizome yield. The least yield was recorded in T_{12} (PoP).

There was no significant difference among the treatments on the root weight of ginger six months after planting. However, the maximum root weight was noticed in T_1 (RB-144). Observations on dry weight of top shoot also showed no significant difference among treatments, the lowest weight in T_{13} (Control) and the highest in T_6 (RB-151). Analysis of data on number of roots and layers of rhizomes showed significant difference. The maximum number of roots was observed in T_9 (P.f2) which was on par with all other treatments except T_{13}, T_6 and T_1. Similarly, the number of layers in ginger rhizomes was the least in T_{12} ((PoP), T_7 (P.f1), T_8 (RB-77) and T_{11} (RB-71) and more in T_1 (RB-144), T_2 (RB-22) and T_6 (RB-151).

Table 4.27. Effect of potential rhizobacterial isolates on bacterial wilt after challenge inoculation

Sl.No	Treatment	*Per cent disease incidence			
		7th DAI		14th DAI	
		Per cent incidence	Per cent +/- over control	Per cent incidence	Per cent +/- over control
1.	T_1 (RB-144)	11.50^{bcd}	61.24	51.32^{cde}	30.81
2.	T_2 (RB-22)	9.56^{fg}	67.78	63.04^{abcd}	15.01
3.	T_3 (RB-82)	13.10^{ef}	55.85	56.15^{bcde}	24.3
4.	T_4 (RB-66)	8.01^{g}	73.0	50.00^{de}	32.59
5.	T_5 (RB-11)	7.70^{g}	74.05	44.12^{e}	40.52
6.	T_6 (RB-151)	11.44^{efg}	61.44	76.67^{a}	-3.37
7.	T_7 (P.f1)	17.86^{bc}	39.80	69.29^{abc}	6.58
8.	T_8 (RB-77)	13.67^{de}	53.93	68.34^{abcd}	12.79
9.	T_9 (P.f2)	19.06^{b}	35.76	59.36^{abcde}	19.97
10.	T_{10} (RB-69)	17.03^{efg}	42.60	64.68^{abcd}	7.86
11.	T_{11} (RB-71)	14.65^{cde}	50.62	74.18^{ab}	-0.01
12.	T_{12} (PoP)	19.45^{b}	34.44	72.36^{ab}	2.44
13.	T_{13} (Control)	29.67^{a}		74.17^{ab}	

*Mean of three replications, DAP-days after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.28. Effect of potential rhizobacterial isolates on yield, root weight, dry top shoot and number of roots layers

Sl.No.	Treatment	*Fresh weight of rhizomes		*Root weight	*Weight of dry top Shoot (g)	*No of roots	*Layers
		Yield (g)	Per cent +/- over control	Root weight (g)			
1.	T_1 (RB-144)	446.67[a]	+50.00	59.32[a]	36.58[a]	26.58[bc]	2.00[a]
2.	T_2 (RB-22)	435.00[a]	+46.08	57.83[a]	37.82[a]	27.67[abc]	2.00[a]
3.	T_3 (RB-82)	444.33[a]	+49.21	60.26[a]	42.79[a]	35.00[ab]	2.00[a]
4.	T_4 (RB-66)	435.83[a]	+46.36	62.45[a]	48.69[a]	37.00[ab]	1.83[ab]
5.	T_5 (RB-11)	449.17[a]	+50.84	56.08[a]	46.50[a]	29.25[abc]	1.83[ab]
6.	T_6 (RB-151)	356.67[ab]	+19.78	50.97[a]	50.28[a]	22.67[c]	2.00[a]
7.	T_7 (P.f1)	436.67[a]	+46.64	58.38[a]	48.38[a]	28.25[abc]	1.33[bcd]
8.	T_8 (RB-77)	385.00[ab]	+29.29	61.13[a]	45.27[a]	33.50[abc]	1.33[bcd]
9.	T_9 (P.f2)	440.83[a]	+48.04	67.31[a]	47.10[a]	38.33[a]	1.83[ab]
10.	T_{10} (RB-69)	351.67[ab]	+18.10	53.36[a]	38.40[a]	33.17[abc]	1.67[abc]
11.	T_{11} (RB-71)	350.00[ab]	+17.54	57.25[a]	39.19[a]	30.50[abc]	1.33[bcd]
12.	T_{12} (PoP)	371.10[ab]	+24.62	58.26[a]	47.52[a]	30.67[abc]	1.17[cd]
13.	T_{13} (Control)	297.78[c]		57.56[a]	36.10[a]	26.28[bc]	1.11[d]

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

4.8.9 Population dynamics of soil microflora

The effectof various treatments on the population of soil microflora *viz*., fungi, bacteria and actinomycetes was estimated one, three and five months after planting and the results are presented in Table 4.29.

4.8.9.1 Fungi

The data on the total fungal population showed significant fluctuation among the treatments during the first two intervals. The total fungal population one month after planting was minimum in treatment T_9 (P.f2) and the maximum in T_{12} (PoP). However, after three months, the highest population of 19 x 10^2 cfug $^{-1}$ soil was in T_{13} (Control) and the least of 10.67 in T_1 (RB-144). During the last observation, a slight built up of fungal population was noticed in certain treatments.

4.8.9.2 Bacteria

A considerable variation in the population of bacteria was noticed at different intervals of observation. One month after planting, the highest count was noticed in treatment T_1 (RB-144) and the lowest in T_5 (RB-11) and T_{10} (RB-69). After three months of planting, T_8 (RB-77) recorded the highest population which was on par with all treatments except for T_1 (RB-144). A gradual decrease in the population of bacteria was observed after five months of planting. During this period, T_{13} recorded the highest count and the lowest in T_3 (RB-82).

4.8.9.3 Actinomycetes

No significant difference in the population of actinomycetes was noticed among the treatments one and five months after planting. However, the highest population was observed in T_2 (RB-22) one month after planting and the lowest in T_3 (RB-82). After three months, a general increase in the population of actinomycetes

was recorded compared to the previous period of enumeration. The maximum count was noticed in T_4 (RB-66) and the minimum in T_7 (P.f1). After five months, the highest count was in T_{11} (RB-71) and the lowest count in control (T_{13}).

4.8.10 Changes in total phenols after challenge inoculation with pathogen

The qualitative and quantitative changes in phenol content before and after challenge inoculation of the pathogen was carried out.

4.8.10.1 Quantitative estimation of total phenols

Total phenol in the methanol extracts were estimated at different intervals and the results are presented in Table 4.30. In general, rhizobacterial treatments had a positive effect in increasing the phenol content of ginger compared to control (Fig 4.5). A significant difference in phenol content was noticed before inoculation of the pathogen. The highest phenol content was observed in T_1 (RB-144) and lowest in T_7 (P.f1), T_6 (RB-151) and T_2 (RB-22). There was constant rise of phenol in all treatments one day after inoculation. The highest content was in T_5 (RB-11) followed by T_4 (RB-66), T_2 (RB-22) and T_3 (RB-82) and these treatments showed high per cent increase in phenol content over control. The lowest phenol content was recorded in samples of T_{13} (Control). After three days of inoculation, the rate of increase in phenol over control ranged from 16.71to 77.06 per cent. The higher content was noticed in T_5 (RB-11) and lowest in control (T_{13}). In general, total phenol content after five days of inoculation also exhibited a high value in all the treatments with the lowest content in T_9 (P.f2) and the highest in T_7 (P.f1), closely followed by T_3 (RB-82). These treatments showed a per cent increase over control, ranging from 0.12 to 77.92.

4.8.10.2 TLC profile of phenols

Thin layer chromatography (TLC) of phenols was carried out as described in 3.8.2. The phenolic compounds appeared as dark blue spots when the TLC plates

Table 4.30. Effect of rhizobacterial isolates on changes in the content of total phenolics after challenge inoculation with pathogen

Sl.No.	Treatment	*Before inoculation		*1 DAI		*3 DAI		*5 DAI	
		Phenol µg g^{-1}	Per cent +/- over control	Phenol µg g^{-1}	Per cent +/- over control	Phenol µg g^{-1}	Per cent +/- over control	Phenol µg g^{-1}	Per cent +/- over control
1.	T_1 (RB-144)	771.00^{a}	+128.33	876.33^{abc}	+65.03	1037.00^{bc}	+41.60	1336.33^{abc}	+56.36
2.	T_2 (RB-22)	244.67^{def}	-72.46	1048.33^{ab}	+97.43	1073.00^{abc}	+46.52	1363.00^{ab}	+59.48
3.	T_3 (RB-82)	553.67^{abc}	+163.97	1004.33^{ab}	+89.14	1286.67^{a}	+75.70	1419.00^{a}	+66.03
4.	T_4 (RB-66)	516.67^{bc}	+153.01	1095.33^{ab}	+106.28	1037.00^{abc}	+41.60	1003.67^{bcd}	+17.43
5.	T_5 (RB-11)	413.67^{cde}	+122.51	1187.33^{a}	+123.60	1296.67^{a}	+77.06	1508.00^{a}	+76.44
6.	T_6 (RB-151)	232.67^{ef}	-68.90	760.37^{bc}	+43.20	1005.00^{c}	+37.23	1297.67^{abc}	+51.83
7.	T_7 (P.f1)	108.67^{f}	-32.18	756.33^{bc}	+42.44	1010.67^{bc}	+38.01	1520.67^{a}	+77.92
8.	T_8 (RB-77)	514.33^{bc}	+152.32	801.33^{abc}	+50.91	914.33^{cd}	+24.85	1234.87^{abcd}	+44.49
9.	T_9 (P.f2)	707.00^{ab}	+209.38	887.33^{abc}	+67.11	1045.33^{abc}	+42.74	855.67^{d}	+0.12
10.	T_{10} (RB-69)	478.67^{bcd}	+141.76	941.00^{ab}	+77.21	1103.80^{abc}	+50.72	1243.67^{abcd}	+45.51
11.	T_{11} (RB-71)	656.00^{ab}	+194.27	781.33^{bc}	+47.14	854.67^{cd}	+16.71	947.67^{cd}	+10.88
12.	T_{12} (PoP)	336.67^{cdef}	+99.70	910.00^{abc}	+71.37	917.67^{cd}	+25.31	992.67^{bcd}	+16.15
13.	T_{13} (Control)	337.67^{cdef}		531.00^{c}		732.33^{d}		854.67^{d}	

*Mean of three replications, DAI-Days after inoculation. +-Increse, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. Figures in parenthesis are √x+0.5 transformed values. *P.f-Pseudomonas fluorescens*

were sprayed with Folin-Ciocalteau reagent. The Rf values of the phenolic compounds were compared with the available standards which were also run in the same solvent.

The Rf values of the identified phenols and also of certain unidentified compounds studied are summarized in Table 4.31. From the table, it is clear that the number and type of phenols varied during the different intervals of observation. Only a single spot appeared on the chromatograms in all the treatments before inoculation of the pathogen, while, two to five spots was located from the extract of infected plants of different treatments after three days of challenge inoculation. Five phenols were observed in samples of treatment T_{10} (RB-69) while four in treatment T_1 (RB-144), T_7 (P.f1), T_8 (RB-77), T_9 (P.f2), T_{11} (RB-71) and T_{12} (PoP). After three days of inoculation, the presence of salicylic acid was noticed from the samples of all treatments. Others observed were pyrogallol, guaicol, hydroquinone and certain unidentified compounds. The number and type of phenolic compounds recorded after five days of inoculation varied from that of three days of inoculation. Here also, in seven samples presence of salicylic acid was noticed while catechol in seven other treatments. Five phenols were detected in samples of treatment T_2 (RB-22), T_5 (RB-11) and T_{11} (RB-71) and four in T_1 (RB-144), T_2 (RB-22), T_8 (RB-77), T_{10} (RB-69) and T_{12} (PoP). Only two phenols were detected in T_4 (RB-66), T_6 (RB-151), T_7 (P.f1) and T_{13} (Control).

4.8.11 Changes in total protein after challenge inoculation with pathogen

Total protein was estimated from ginger plants of different treatment and the results are furnished in Table 4.32 (Fig 4.6). Before inoculation, a significant difference was noticed among the plants in various rhizobacterial treatments, with the maximum content in 4.58 T_3 (RB-82) followed by T_1 (RB-144) and T_5 (RB-11). The least protein content was recorded in T_{10} (RB-69), T_8 (RB-77) and T_{12} (PoP). However, one day after inoculation there was fluctuation in the protein content except in T_1 (RB-144) and T_3 (RB-82). Here, all the treatments showed a gradual increase of protein content ranging from 1.9 to 5.51 mgg^{-1} compared to that of one day after inoculation. The lowest value was in T_{12} (PoP) and the highest in T_2 (RB -

Table 4.31. Profile of phenols in leaves of ginger on the day and after challenge inoculation of the pathogen (contd….)

Treatment	Before inoculation			3 Days after inoculation (3DAI)			5 Days after inoculation (5DAI)		
	Rf value	Standard	Probable phenol	Rf value	Standard	Probable phenol	Rf value	Standard	Probable phenol
T_7 (P.f1)	0.93	-	unidentified	0.19 0.43 0.74 0.97	- 0.46 0.77 0.99	unidentified Hydroquinone Guaicol Salicylic acid	0.48 0.92	0.46 -	Hydroquinone unidentified
T_8 (RB-77)	0.93	-	unidentified	0.17 0.43 0.76 0.97	- 0.46 0.77 0.99	unidentified Hydroquinone Guaicol Salicylic acid	0.18 0.44 0.69 1.0	- 0.46 0.69 0.99	unidentified Hydroquinone Catechol Salicylic acid
T_9 (P.f2)	0.92	-	unidentified	0.22 0.45 0.78 0.97	- 0.46 0.77 0.99	unidentified Hydroquinone Guaicol Salicylic acid	- 0.46 0.65 0.99	0.46 0.69 0.99	Hydroquinone Catechol Salicylic acid
T_{10} (RB-69)	0.92	-	unidentified	0.20 0.45 0.63 0.78 0.98	- 0.46 - 0.77 0.99	unidentified Hydroquinone - Guaicol Salicylic acid	0.18 0.43 - 0.62 0.97	- 0.46 0.69 0.99	unidentified Hydroquinone Catechol Salicylic acid
T_{11} (RB-71)	0.93	-	unidentified	0.19 0.45 0.97	- 0.46 0.99	unidentified Hydroquinone Salicylic acid	0.19 0.37 0.47 0.67 0.98	- - 0.46 0.69 0.99	unidentified unidentified Hydroquinone Catechol Salicylic acid
T_{12} (Chemical)	0.94	-	unidentified	0.21 0.36 0.79 0.97	- - 0.77 0.99	- - Guaicol Salicylic acid	0.17 0.39 0.65 0.97	- - 0.69 0.99	unidentified unidentified Hydroquinone Salicylic acid
T_{13} (Control)	0.93	-	unidentified	0.31 0.97	- 0.99	- Salicylic acid	0.44 0.98	0.46 0.99	Hydroquinone Salicylic acid

DAI-days after inoculation. *P.f-Pseudomonas fluorescens,* - standards not available

Table 4.31. Profile of phenols in leaves of ginger on the day and after challenge inoculation of the pathogen

Treatment	Before inoculation			3 Days after inoculation (3DAI)			5 Days after inoculation (5DAI)		
	Rf value	Standard	Probable phenol	Rf value	Standard	Probable phenol	Rf value	Standard	Probable phenol
T_1(RB-144)	0.93	-	unidentified	0.19 0.24 0.78 0.98	- 0.26 0.77 0.99	unidentified Pyrogallol Guaicol Salicylic acid	0.15 0.34 0.70 0.92	- - 0.69 -	unidentified unidentified Catechol unidentified
T_2 (RB-22)	0.92	-	unidentified	0.14 0.99	- 0.99	unidentified Salicylic acid	0.17 0.37 0.49 0.67 0.88	- - 0.46 0.69 -	unidentified unidentified Hydroquinone Catechol unidentified
T_3 (RB-82)	0.93	-	unidentified	0.14 0.99	- 0.99	unidentified Salicylic acid	0.38 0.48 0.74 0.89	- 0.46 0.77 -	unidentified Hydroquinone Guaicol unidentified
T_4 (RB-66)	0.92	-	unidentified	0.27 0.98	0.26 0.99	Pyrogallol Salicylic acid	0.44 0.90	0.46 -	Hydroquinone unidentified
T_5 (RB-11)	0.93	-	unidentified	0.20 0.44 0.98	- 0.46 0.99	unidentified Hydroquinone Salicylic acid	0.15 0.39 0.66 0.93 0.98	- - 0.69 - 0.99	unidentified unidentified Catechol - Salicylic acid
T_6 (RB-151)	0.93	-	unidentified	0.32 0.98	- 0.99	unidentified Salicylic acid	0.40 0.90	- -	unidentified unidentified

*Mean of three replications, DAI-days after inoculation. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

22). All the treatments except for T_{10} (RB-69), T_{11} (RB-71) and T_{12} (PoP) showed a per cent increase protein content over control rom 22.27 to 131.51 where the former was in T_8 (RB-77) and latter in T_2 (RB-22). Also, there was significant difference among treatments in protein at three days after inoculation. The increase was maximum in treatment T_9 (P.f2) which exhibited 137.4 per cent increase over control followed by T_3 (RB-82). Even though, T_{10} (RB-69) and T_{11} (RB-71) showed an increase in protein content compared to one day after inoculation, the content was less than that in control. At five days after inoculation, the protein content showed a steady decrease compared to previous observation in all the treatments except T_7 (P.f1), T_{10} (RB-69) and T_{11} (RB-71). However, compared to control, all the treatments showed a per cent increase ranging from 32.02 to 118.72 where the maximum was observed in T_5 (RB-11) which was on par with T_7 (P.f1), T_3 (RB-82) and T_6 (RB-151) in that order.

4.8.12 Changes in total free amino acids

4.8.12.1 Quantitative estimation of total free amino acids

The total free amino acid content in ginger leaves were estimated by means of ninhydrin reagent. The results are presented in Table 4.33. The total free amino acid content varied in treatments at different periods of observation (Fig 4.7). Before inoculation, the highest amino acid content was observed in plants in treatment T_3 (RB-82) with a per cent increase over control of 130.80 and it was on par with six other rhizobacterial treatments. The lowest content was in control (T_{13}).

One day after inoculation, in general, an increase in the amino acid content was observed in different treatments. The maximum amino acid content was noticed in treatment T_1 (RB-144) followed by T_4 (RB-66) and T_3 and these showed more than cent per cent increase over control. The least content was in control. Subsequently, there was a general increase in amino acid content compared to that of the previous observation. The maximum content was in T_2 (RB-22). Plants in treatment T_1 also showed a higher content while the lowest was in control. Five days

Table 4.33. Effect of rhizobacterial isolates on changes in total amino acid content after challenge inoculation with pathogen

Sl.No.	Treatment	*Before inoculation		*1 DAI		*3 DAI		*5 DAI	
		Amino acid mg g^{-1}	Per cent +/- over control	Amino acid mg g^{-1}	Per cent +/- over control	Amino acid mg g^{-1}	Per cent +/- over control	Amino acid mg g^{-1}	Per cent +/- over control
1.	T_1 (RB-144)	4.91^{ab}	+86.69	9.79^{a}	+250.90	7.17^{b}	+84.79	6.07^{abc}	+128.20
2.	T_2 (RB-22)	5.98^{a}	+127.38	4.69^{cd}	+68.10	10.54^{a}	+171.65	7.13^{ab}	+168.05
3.	T_3 (RB-82)	6.07^{a}	+130.80	7.32^{b}	+162.37	6.84^{bc}	+76.29	6.07^{abc}	+128.20
4.	T_4 (RB-66)	6.02^{a}	+128.90	8.26^{b}	+196.06	4.70^{bcd}	+21.13	7.38^{ab}	+177.44
5.	T_5 (RB-11)	3.71^{abc}	+41.06	5.36^{c}	+92.11	5.98^{bcd}	+54.12	7.76^{a}	+191.73
6.	T_6 (RB-151)	2.65^{bc}	+0.76	3.75^{de}	+34.41	4.21^{cd}	+8.51	4.39^{bcde}	+65.04
7.	T_7 (P.f1)	4.61^{ab}	+75.29	4.59^{cd}	+64.52	5.57^{bcd}	+43.56	6.07^{abc}	+128.20
8.	T_8 (RB-77)	3.77^{abc}	+43.35	3.32^{e}	+18.99	3.93^{d}	+1.29	3.31^{cde}	+24.44
9.	T_9 (P.f2)	3.42^{bc}	+30.04	3.88^{de}	+39.07	6.71^{bcd}	+72.94	1.87^{e}	+-29.70
10.	T_{10} (RB-69)	2.68^{bc}	+1.90	3.84^{de}	+37.63	5.44^{bcd}	+40.21	5.18^{abcd}	+94.74
11.	T_{11} (RB-71)	1.85^{c}	-29.66	2.80^{e}	+0.36	6.56^{bcd}	+69.07	1.85^{e}	-30.45
12.	T_{12} (PoP)	2.52^{bc}	-4.18	2.82^{e}	+1.08	4.75^{bcd}	+22.42	3.15^{cde}	+18.42
13.	T_{13} (Control)	2.02^{c}		1.52^{f}		3.88^{d}		2.66^{de}	

*Mean of three replications, DAI-days after inoculation. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Fig 4.5 Induction of phenol in ginger by potential rhizobacterial isolates

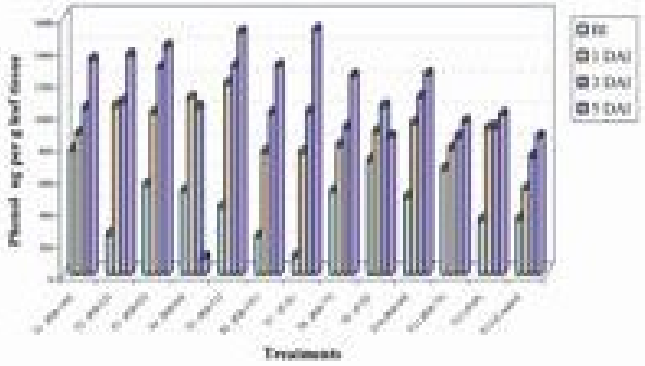


Fig 4.6 Induction of protein in ginger by potential rhizobacterial isolates

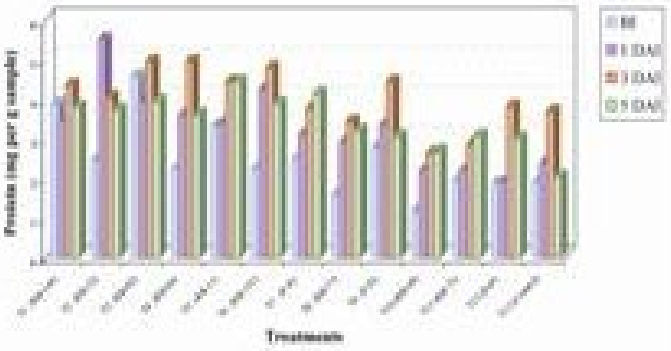
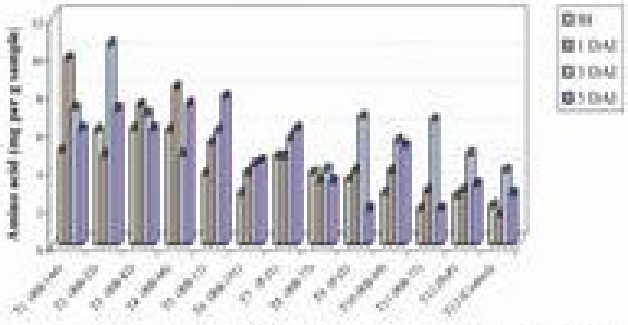


Fig 4.7 Induction of amino acids in ginger by potential rhizobacterial isolates



after challenge inoculation, significantly more content of amino acid was recorded in T_5 (RB-11) and less in T_{11} (RB-71).

4.8.12.2 TLC profile of amino acids

The amino acid profile in the leaves of ginger plants in rhizobacterial treatments on the day of inoculation, three and five days after challenge inoculation was studied by thin layer chromatography (TLC) in comparison with the standard ones (Table 4.34) (Plate 4.10).

It was noticed that apart from T_{13} (Control), plants in all rhizobacterial treatments recorded the presence of three amino acids with different Rf value. On the day of inoculation, valine was detected in eight treatments while hydroxyproline in all treatments. Amino acids like arginine, alanine, histidine were also detected in certain treatments. Three days after inoculation, the number of amino acids detected varied from three to five and the maximum number in T_1 (RB-144), T_2 (RB-22), T_3 (RB-82), T_8 (RB-77) and T_9 (P.f2). Probable amino acids recorded in plants in T_1, T_2 and T_3 were histidine, aspartic acid, glutamic acid and tyrosine in treatments T_1, T_2 and T_3 whereas alanine, valine and phenylalanine apart from histidine and glutamic acid were noticed in T_8 and T_9. The minimum number of amino acids was recorded in control (alanine, glutamic acid, phenyl alanine) and T_4 (RB-66) (alanine, valine, tyrosine). It is to be noted that the amino acid proline was detected only in sample T_5 (RB-11).

Five days after inoculation, there was not much change in the occurrence of number of amino acids but ther was a change in the type of amino acid in plants of treatments. Presence of phenylalanine, leucine / isoleucine was found common in all samples. During the sampling period, presence of methionine was detected in plants in T_8, T_9 and T_{12} in addition to the ones that were detected three days after inoculation.

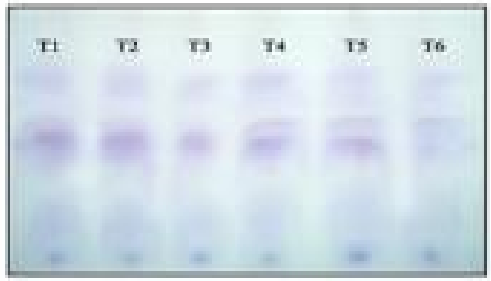
Table 4.34. Profile of amino acids in leaves of ginger on the day and after challenge inoculation of the pathogen

Treatment	Before inoculation				3 Days after inoculation (3DAI)				5 Days after inoculation (5DAI)			
	Rf value	Standard	Spot colour	Probable amino acid	Rf value	Standard	Spot colour	Probable amino acid	Rf value	Standard	Spot colour	Probable amino acid
T_1(RB-144)	0.54 0.71 0.91	0.55 0.69 -	Violet Yellow pink	Valine Hydroxy proline -	0.21 0.25 0.28 0.39 0.63	0.20 - 0.27 0.39 0.63	Pink Red orange Yellow Violet	Histidine, Lysine - Aspartic acid Glutamic acid Tyrosine	0.57 0.64 0.68 0.71	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenyl alanine Isoleucine/leucine
T_2 (RB-22)	0.55 0.70 0.91	0.55 0.69 -	Violet Yellow pink	Valine Hydroxy proline -	0.20 0.24 0.27 0.36 0.63	0.20 - 0.27 0.39 0.63	Pink Pink orange Yellow Violet	Histidine, Lysine - Aspartic acid Glutamic acid Tyrosine	0.56 0.64 0.69 0.72	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenyl alanine Isoleucine/leucine
T_3 (RB-82)	0.54 0.68 0.90	0.55 0.69 -	Violet Yellow Pink	Valine Hydroxy proline -	0.21 0.26 0.29 0.39 0.65	0.20 - 0.27 0.39 0.64	Pink Pink orange Yellow Violet	Histidine, Lysine - Aspartic acid Glutamic acid Tyrosine	0.54 0.64 0.69 0.71	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenyl alanine Isoleucine/leucine
T_4 (RB-66)	0.55 0.68 0.90	0.55 0.69 -	Violet Yellow pink	Valine Hydroxy proline	0.40 0.53 0.66	0.39 0.55 0.64	Violet Violet Violet	Alanine Valine Tyrosine	0.53 0.65 0.69	0.55 0.63 0.67	Violet Violet Violet	Valine Tyrosine Phenyl alanine
T_5 (RB-11)	0.54 0.68 0.91	0.55 0.69 -	Violet Yellow pink	Valine Hydroxy proline -	0.39 0.46 0.6 0.65	0.39 0.45 0.63 0.65	Violet Yellow violet Violet	Alanine Proline Tyrosine Phenyl alanine	0.54 0.64 0.69 0.72	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenyl alanine Isoleucine/leucine
T_6 (RB-151)	0.56 0.71 0.87	0.55 0.69 -	Violet Yellow pink	Valine Hydroxy proline -	0.24 0.27 0.37 0.66	0.25 0.27 0.36 0.64	Pink Orange Violet Violet	Arginine Aspartic acid Alanine Tyrosine	0.55 0.63 0.69 0.72	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenyl alanine Isoleucine/leucine

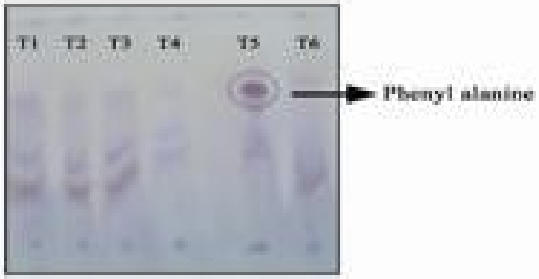
Table 4.34. Profile of amino acids in leaves of ginger on the day and after challenge inoculation of the pathogen (contd….)

Treatment	Before inoculation				3 Days after inoculation (3DAI)				5Days after inoculation (5DAI)			
	Rf value	Standard	Spot colour	Probable amino acid	Rf value	Standard	Spot colour	Probable amino acid	Rf value	Standard	Spot colour	Probable amino acid
T_7 (P.f1)	0.54 0.70 0.91	0.55 0.69 -	Violet Yellow Pink	Valine Hydroxy proline -	0.25 0.29 0.37 0.66	0.25 0.27 0.36 0.64	Pink Orange Violet Violet	Arginine Aspartic acid Alanine Tyrosine	0.53 0.64 0.69 0.71	0.55 0.63 0.67 0.72	Violet Violet Violet Red	Valine Tyrosine Phenylalanine Isoleucine/leucine
T_8 (RB-77)	0.22 0.42 0.72	0.2,0.21 - 0.69	Pink Violet Yellow	Histidine, lysine - Hydroxy proline	0.19 0.37 0.41 0.54 0.68	0.20 0.36 0.39 0.55 0.67	Pink Pink Orange red Pink Orange Violet	Histidine Alanine Glutamic acid Valine Phenyl alanine	0.37 0.56 0.65 0.67 0.72	0.36 0.55 0.65 0.67 0.72	Violet Violet Brick Red Violet Red	Alanine Valine Methionine Phenylalanine Isoleucine/leucine
T_9 (P.f2)	0.55 0.67 0.90	0.55 0.69 -	Violet Yellow Pink	Valine Hydroxy proline -	0.18 0.35 0.4 0.53 0.67	0.20 0.36 0.39 0.55 0.67	Pink Pink orange red Pink Orange Violet	Histidine Alanine Glutamic acid Valine Phenyl alanine	0.38 0.56 0.63 0.67 0.72	0.36 0.55 0.65 0.67 0.72	Violet Violet Brick Red Violet Red	Alanine Valine Methionine Phenylalanine Isoleucine/leucine
T_{10} (RB-69)	0.27 0.43 0.73	0.26 - 0.69	Pink Violet Yellow	Arginine - Hydroxy proline	0.18 0.37 0.41 0.67	0.20 0.36 0.39 0.67	Pink Pink Orange red Violet	Histidine Alanine Glutamic acid Phenyl alanine	0.39 0.57 0.67 0.72	0.36 0.55 0.67 0.72	Violet Violet Violet Red	Alanine Valine Phenylalanine Isoleucine/leucine
T_{11} (RB-71)	0.25 0.36 0.72	0.36 - 0.69	Pink Violet Yellow	Alanine - Hydroxy proline	0.20 0.35 0.41 0.69	0.20 0.36 0.39 0.67	Pink Pink Orange red Violet	Histidine Alanine Glutamic acid Phenyl alanine	0.37 0.54 0.66 0.72	0.36 0.55 0.67 0.72	Violet Violet Violet Red	Alanine Valine Phenylalanine Isoleucine/leucine
T_{12} (PoP)	0.25 0.32 0.71	0.26 0.31 0.69	Pink Violet Yellow	Arginine Serine Hydroxy proline	0.24 0.38 0.42 0.72	0.20 0.36 0.39 0.67	Pink Pink Orange red Violet	Histidine Alanine Glutamic acid Phenyl alanine	0.38 0.65 0.69 0.72	0.36 0.65 0.67 0.72	Violet Brick red Violet Red	Alanine Methionine Phenylalanine Isoleucine/leucine
T_{13} (Control)	0.30 0.67	0.31 0.69	Violet Yellow	Serine Hydroxy proline	0.37 0.42 0.69	0.36 0.39 0.67	Pink Orange red Violet	Alanine Glutamic acid Phenyl alanine	0.54 0.67 0.72	0.55 0.67 0.72	Violet Violet Red	Valine Phenylalanine Isoleucine/leucine

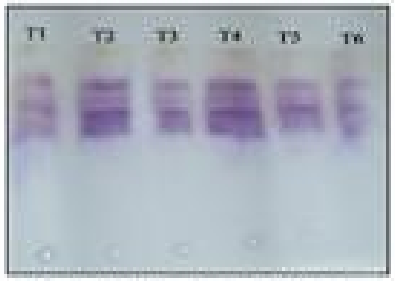
DAI-days after inoculation, *P.f-Pseudomonas fluorescens*



A. Before inoculation



B. Three days after inoculation



C. Five days after inoculation

Standards: 1. Glycine 2. Alanine 3. Serine 4. Cystein 5. Threonine 6. Valine 7. Leucine 8. Isoleucine 9. Methionine 10. Aspartic acid 11. Asparagine 12. Glutamic acid 13. Glutamine 14. Arginine 15. Lysine 16. Hydroxy Proline 17. Phenyl alanine 18. Tyrosine 19. Tryptophan 20. Histidine

Plate 4.10 TLC profile of amino acids before and after challenge inoculation

4.9 ASSAY OF DEFENSE RELATED ENZYMES

Based on the previous experiment on the role of antagonistic rhizobacterial isolates in promoting growth of ginger and also in imparting resistance against the bacterial wilt pathogen, further selection of most promising isolates were carried out and subjected to detailed investigation on their mechanism/s of induction of resistance. This was done by assessing the activity of defense related enzymes and their profile. The protein profile of plants in different treatments was assessed following native PAGE analysis. In addition to the observations taken as per 3.4, oil and oleoresin contents as well as NPK analysis of the rhizomes were also carried out. The following most promising isolates *viz*., RB-144, RB-22, RB-82, RB-66, RB-11 and RB-77 were used for the third pot culture experiment.

4.9.1 Germination percentage

It is evident from the Table 4.35 that all the rhizobacterial treated plants showed cent per cent germination except four treatments which also showed higher germination percentage. Comparatively less pre emergence rot was noticed in T_3 (RB-82), T_7 (RB-77), T_8 (P.f2) and T_{10} (Control) while post emergence rot was observed only in control.

4.9.2 Biometric observations

4.9.2.1 Number of tillers

There was no significant difference in the number of tillers among the treatments one month after planting (Table 4.36) (Fig 4.8). However, after two months, significantly more tiller production was noticed in T_4 (RB-66) and less in T_{10}. After three months of planting also, the number of tillers was the maximum in treatment T_4 (RB-66) but the minimum in T_8 (P.f2). Data after four and five months of planting revealed significant among treatments. The highest tiller production was

Table 4.35. Effect of promising rhizobacterial isolates on per cent germination, pre emergence and post emergence rot of ginger

Sl.No.	Treatment	Germination (%)	Pre-emergence rot (%)	Post emergence rot (%)
1.	T_1 (RB-144)	100	0	0
2.	T_2 (RB-22)	100	0	0
3.	T_3 (RB-82)	93.33	6.67	0
4.	T_4 (RB-66)	100	0	0
5.	T_5 (RB-11)	100	0	0
6.	T_6 (P.f1)	100	0	0
7.	T_7 (RB-77)	93.33	6.67	0
8.	T_8 (P.f2)	93.33	6.67	0
9.	T_9 (PoP)	100	0	0
10.	T_{10} (Control)	93.33	6.67	13.33

*Mean of 15 replications, *P.f-Pseudomonas fluorescens*

Table 4.36. Effect of promising rhizobacterial isolates on number of tillers of ginger plants

Sl.No.	Treatment	*1 MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control	Number of tillers	Per cent +/- over control
1.	T_1 (RB-144)	1.17^{a}	+3.54	4.90^{b}	+0.62	8.35^{b}	+11.78	13.17^{abc}	+41.31	17.67^{ab}	+25.50
2.	T_2 (RB-22)	1.17^{a}	+3.54	5.43^{ab}	+11.50	8.00^{b}	+7.10	16.56^{a}	+77.68	23.95^{a}	+70.10
3.	T_3 (RB-82)	1.13^{a}	0.00	5.32^{ab}	+9.24	7.95^{b}	+6.43	13.92^{abc}	+49.36	18.36^{ab}	+30.40
4.	T_4 (RB-66)	1.15^{a}	+1.77	6.15^{a}	+26.28	10.67^{a}	+42.84	14.90^{ab}	+59.87	19.27^{ab}	+36.86
5.	T_5 (RB-11)	1.17^{a}	+3.54	5.33^{ab}	+9.45	8.27^{b}	+10.71	13.65^{abc}	+46.46	20.17^{ab}	+43.25
6.	T_6 (P.f1)	1.10^{a}	-2.65	5.78^{ab}	+18.69	8.38^{b}	+12.18	14.39^{ab}	+54.40	18.64^{ab}	+32.39
7.	T_7 (RB-77)	1.13^{a}	0.00	5.12^{ab}	+5.13	8.18^{b}	+9.50	14.22^{abc}	+52.58	18.07^{ab}	+28.34
8.	T_8 (P.f2)	1.13^{a}	0.00	5.22^{ab}	+7.19	7.37^{b}	-1.34	13.40^{abc}	+43.78	18.53^{ab}	+31.61
9.	T_9 (PoP)	1.02^{a}	-9.73	5.13^{ab}	+5.34	7.72^{b}	+3.35	11.05^{bc}	+18.56	14.00^{c}	-0.57
10.	T_{10} (Control)	1.13^{a}		4.87^{b}		7.47^{b}		9.32^{c}		14.08^{c}	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

in T_2 (RB-22) and this one did not differ much with others except T_9 (PoP) and T_{10} (Control).

4.9.2.2 Number of leaves per tiller

The data on number of leaves per tiller one month after plating revealed that the maximum number was in T_4 (RB-66) and the minimum in T_8 (P.f2) (Table 4.37). After two months, plants in treatment T_2 (RB-22) recorded more number of leaves followed by T_5 (RB-11). During the last two observations, the maximum number of leaves was in T_5 but the minimum in T_{10} (Control). The percentage efficiency of treatments over control ranged from 28.17 to 49.70 five months after planting.

4.9.2.3 Height of tillers

The height of tillers one month after planting ranged from 14.97 to 27.92 cm where plants in treatment T_2 showed the highest value and the lowest by T_{10} (Control) (Table 4.38) (Fig 4.9). The per cent efficiency of the treatment over control two months after planting ranged from 16.29 to 45.53 where the maximum height was in T_3 (RB-82) followed by T_8 (P.f2). During three, four and five months observations, although there was no significant difference among the treatments, the maximum height was with plants in treatment T_6 (P.f1) at third and four months but in T_4 after five months. During these periods the least height was recorded in plants in T_{10}.

4.9.2.4 Pest and disease incidence

The data revealed comparatively more shoot borer incidence in T_3 and T_4 after 90 days after planting (Table 4.39). Data on rhizome rot incidence showed significant difference among treatments. The highest incidence was in T_{10} (Control) followed by T_8 (P.f2), T_9 (PoP) and T_4 (RB-66). It was noticed that there was no

Table 4.37. Effect of promising rhizobacterial isolates on number of leaves per tiller of ginger plants

Sl.No.	Treatment	*1 MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Number of leaves	Per cent +/- over control	Number of leaves	Per cent +/- over control	Number of leaves	Per cent +/- over control	Number of leaves	Per cent +/- over control	Number of leaves	Per cent +/- over control
1.	T_1 (RB-144)	3.55^{b}	+18.33	8.01^{ab}	+11.40	10.00^{ab}	-9.91	11.39^{bc}	+19.64	14.81^{a}	+46.92
2.	T_2 (RB-22)	4.00^{ab}	+33.33	9.13^{a}	+26.98	10.05^{ab}	-9.46	12.50^{ab}	+31.30	13.88^{a}	+37.70
3.	T_3 (RB-82)	4.00^{ab}	+33.33	7.85^{ab}	+9.18	9.78^{ab}	-11.89	11.99^{abc}	+25.95	14.50^{a}	+43.85
4.	T_4 (RB-66)	5.64^{a}	+88.00	7.67^{ab}	+6.68	10.26^{ab}	-7.57	12.68^{ab}	+33.19	14.20^{a}	+40.87
5.	T_5 (RB-11)	3.48^{ab}	+16.00	8.13^{ab}	+13.07	10.56^{a}	-4.86	14.53^{a}	+52.63	15.09^{a}	+49.70
6.	T_6 (P.f1)	4.28^{ab}	+42.67	8.12^{ab}	+12.93	9.60^{ab}	-13.51	12.41^{ab}	+30.36	13.10^{a}	+29.96
7.	T_7 (RB-77)	3.22^{b}	+7.33	7.88^{ab}	+9.60	10.00^{abc}	-9.91	12.00^{abc}	+26.05	12.92^{a}	+28.17
8.	T_8 (P.f2)	2.61^{b}	-13.00	7.73^{ab}	+7.51	9.17^{ab}	-17.39	10.92^{bc}	+14.71	13.22^{a}	+31.15
9.	T_9 (PoP)	3.05^{b}	+1.67	6.82^{b}	-5.15	8.16^{bc}	-26.49	11.43^{bc}	+20.06	12.92^{a}	+28.17
10.	T_{10} (Control)	3.00^{b}		7.19^{c}		11.10^{c}		9.52^{c}		10.08^{b}	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.38. Effect of promising rhizobacterial isolates on height of tillers of ginger plants

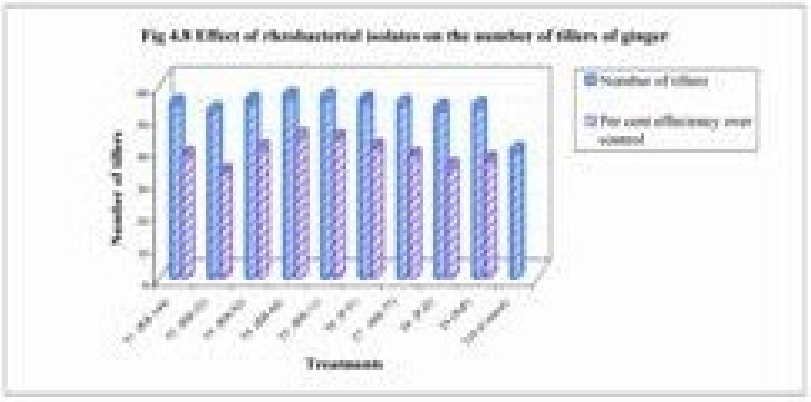
Sl.No.	Treatment	*1 MAP		*2 MAP		*3 MAP		*4 MAP		*5 MAP	
		Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control	Height of tillers (cm)	Per cent +/- over control
1.	T_1 (RB-144)	25.32^{a}	+69.14	38.8^{ab}	+35.38	44.21^{a}	+40.71	47.60^{a}	+31.78	53.48^{a}	+37.59
2.	T_2 (RB-22)	27.92^{a}	+86.51	37.3^{ab}	+30.15	43.83^{a}	+39.50	46.87^{a}	+29.76	51.39^{a}	+32.21
3.	T_3 (RB-82)	27.33^{a}	+82.57	41.71^{a}	+45.53	47.65^{a}	+51.65	51.39^{a}	+42.28	54.27^{a}	+39.62
4.	T_4 (RB-66)	27.18^{a}	+81.56	36.90^{ab}	+28.75	43.39^{a}	+38.10	51.49^{a}	+42.55	55.81^{a}	+43.58
5.	T_5 (RB-11)	26.29^{a}	+75.62	39.68^{ab}	+38.45	46.63^{a}	+48.41	49.14^{a}	+36.05	55.50^{a}	+42.78
6.	T_6 (P.f1)	23.08^{ab}	+54.18	39.33^{ab}	+37.23	50.23^{a}	+59.87	51.53^{a}	+42.66	54.38^{a}	+39.90
7.	T_7 (RB-77)	20.97^{abc}	+40.08	33.33^{bc}	+16.29	43.07^{a}	+37.08	49.33^{a}	+36.57	53.38^{a}	+37.33
8.	T_8 (P.f2)	16.30^{bc}	+8.88	41.53^{a}	+44.91	46.68^{a}	+48.57	48.63^{a}	+34.63	52.13^{a}	+34.11
9.	T_9 (PoP)	20.98^{abc}	+40.15	37.43^{ab}	+30.60	44.83^{a}	+42.68	48.66^{a}	+34.72	52.85^{a}	+35.97
10.	T_{10} (Control)	14.97^{c}		28.66^{c}		31.42^{b}		36.12^{b}		38.87^{b}	

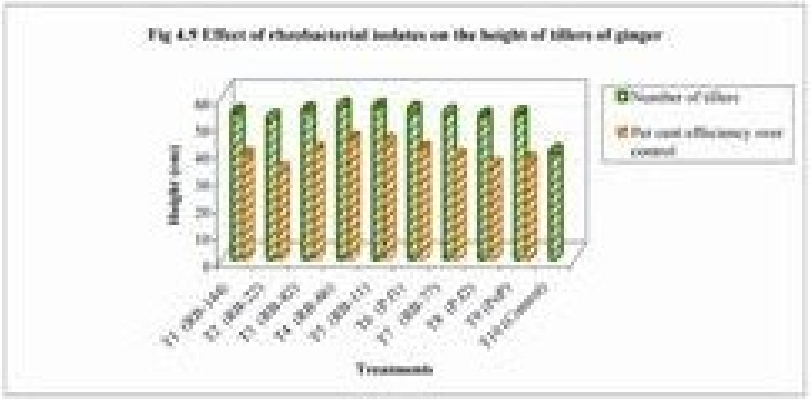
*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

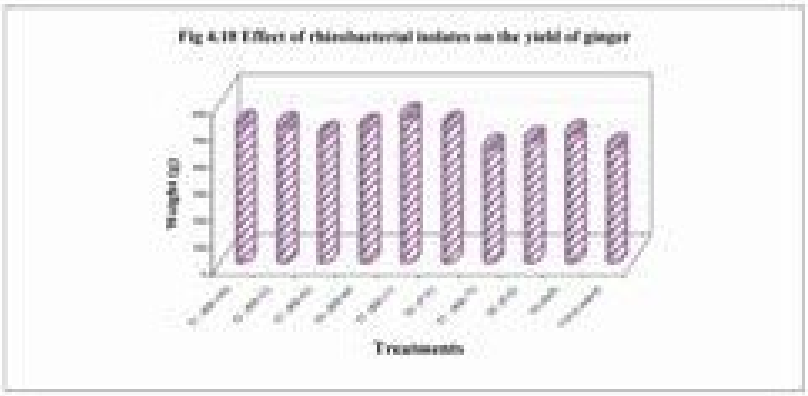
Table 4.39. Effect of promising rhizobacterial isolates on the incidence of pest and diseases

Sl.No.	Treatment	90 DAP	
		*Shoot borer incidence (%)	*Rhizome rot Incidence (%)
1.	T_1 (RB-144)	3.33 (1.93) [bc]	6.66 (2.68) [c]
2.	T_2 (RB-22)	2.22 (1.58) [c]	6.66 (2.68) [c]
3.	T_3 (RB-82)	6.66 (2.68) [a]	6.66 (2.68) [c]
4.	T_4 (RB-66)	6.66 (2.68) [a]	13.33 (3.72) [b]
5.	T_5 (RB-11)	5.00 (2.30) [ab]	0 (0.71) [d]
6.	T_6 (P.f1)	4.27 (2.18) [abc]	6.66 (2.68) [c]
7.	T_7 (RB-77)	3.33 (1.93) [bc]	6.66 (2.68) [c]
8.	T_8 (P.f2)	4.33 (2.19) [abc]	13.33 (3.72) [b]
9.	T_9 (PoP)	4.47 (2.23) [abc]	13.33 (3.72) [b]
10.	T_{10} (Control)	4.27 (2.18) [abc]	20.0 (4.51) [a]

*Mean of three replications, DAP-days after planting. . In each column figures followed by same letter donot differ significantly according to DMRT. Figures in parenthesis are √x+0.5 transformed values.
P.f-Pseudomonas fluorescens


Number of tillers
Per cent efficiency over control
Treatments


Number of tillers
Per cent efficiency over control
Treatments


Treatments

incidence of rhizome rot in plants treated with RB-11 (T_5). However, no natural incidence of bacterial wilt was noticed during this period.

4.9.2.5 Rhizome yield, fresh root weight and dry weight of top shoot

There was no significant difference among the treatments on the rhizome yield (Table 4.40) (Fig 4.10). However, the maximum yield was recorded in T_5 followed by T_1 (RB-144), T_6 (P.f1) and T_2 (B-22) in that order. The lowest yield was recorded in T_7 (RB-77) followed by T_{10}. Although, no significant difference on the fresh root weight and dry weight of top shoot was noticed, the maximum root weight was observed in T_5 (RB-11) while, the maximum dry weight of top shoot was with treatment T_7 followed by T_5. The least weight in both the cases was observed in T_8.

4.9.3 Induction of defense related enzymes in ginger

4.9.3.1 Assay of peroxidase (PO) activity

Studies on induction of peroxidase enzyme through spectral analysis revealed that the bacterized plants showed an increase in peroxidase activity over time (Fig 4.11). Compared to control, plants in various rhizobacterial treatments showed a higher peroxidase (PO) activity on the day of inoculation (Table 4.41). After three days, inoculation of *R. solanacearum* resulted in an increase in PO activity in all treatments except T_5 (RB-11). During this time, all the treatments except those in T_7 (RB-77) and T_8 (P.f2) expressed higher percentage increase of PO activity compared to control. The expression of peroxidase (PO) was elevated at five days after inoculation of the pathogen where the highest was noticed in T_2 (RB-22) and P.f1 (T_6) as well and the least in control (T_{10}). The percentage increase of PO activity over control was also more during this period.

4.9.3.2 Assay of polyphenol oxidase (PPO) activity

In general, the rhizobacterial treatments resulted in an increase in the polyphenol oxidase (PPO) activity of ginger (Fig 4.12). Apart from T_3 (RB-82), T_5

Table 4.40. Effect of promising rhizobacterial isolates on yield, root weight and dry shoot weight of ginger plants

Sl. No.	Treatments	*Yield of rhizomes		*Root weight		*Dry top shoot	
		Yield (g)	Per cent +/- over control	Root weight (g)	Per cent +/- over control	Weight (g)	Per cent +/- over control
1.	T_1 (RB-144)	508.75[a]	+20.72	68.89[a]	-3.42	45.67[a]	-42.31
2.	T_2 (RB-22)	502.50[a]	+19.23	66.39[a]	-6.93	68.61[a]	-13.34
3.	T_3 (RB-82)	467.17[a]	+10.85	82.89[a]	+16.21	79.17[a]	+0.00
4.	T_4 (RB-66)	496.67[a]	+17.85	79.44[a]	+11.37	62.22[a]	-21.41
5.	T_5 (RB-11)	536.11[a]	+27.21	89.17[a]	+25.01	92.5[a]	+16.84
6.	T_6 (P.f1)	505.00[a]	+19.83	87.92[a]	+23.26	76.25[a]	-3.69
7.	T_7 (RB-77)	419.28[a]	-0.51	67.39[a]	-5.52	116.67[a]	+47.37
8.	T_8 (P.f2)	448.33[a]	+6.38	57.17[a]	-19.85	41.67[a]	-47.37
9.	T_9 (PoP)	472.92[a]	+12.22	85.69[a]	+20.13	75.28[a]	-4.91
10.	T_{10} (Control)	421.44[a]		71.33[a]		79.17[a]	

*Mean of three replications, MAP-months after planting. +-Increase, --Decrease. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.41. Change in peroxidase (PO) activity in ginger plants by promising rhizobacterial isolates on challenge inoculation

Peroxidase Activity (Δ_{436} $mt^{-1}g^{-1}$ fresh tissue)							
Sl.No.	**Treatment**	**On the day of inoculation**		**3 DAI**		**5 DAI**	
		PO activity	**Per cent +/- over control**	**PO activity**	**Per cent +/- over control**	**PO activity**	**Per cent +/- over control**
1.	T_1 (RB-144)	1.07	+ 0.00	1.90	+ 7.95	2.27	+ 26.82
2.	T_2 (RB-22)	1.06	- 0.93	2.06	+ 17.05	2.91	+ 62.57
3.	T_3 (RB-82)	2.06	+ 92.52	2.14	+ 21.59	2.83	+ 58.10
4.	T_4 (RB-66)	1.99	+ 85.98	2.66	+ 51.14	2.24	+ 25.14
5.	T_5 (RB-11)	1.87	+ 74.77	1.87	+ 6.25	2.88	+ 60.89
6.	T_6 (P.f1)	1.44	+ 34.58	2.06	+ 17.05	2.91	+ 62.57
7.	T_7 (RB-77)	1.50	+ 40.19	1.74	- 1.14	1.84	+ 2.79
8.	T_8 (P.f2)	1.42	+ 32.71	1.63	- 7.39	2.32	+ 29.61
9.	T_9 (PoP)	1.74	+ 62.62	1.87	+ 6.25	1.98	+ 10.61
10.	T_{10} (Control)	1.07		1.76		1.79	

DAI – days after inoculation. +-Increase, --Decrease .*P.f-Pseudomonas fluorescens*

Table 4.42. Change in polyphenol oxidase (PPO) activity in ginger plants by promising rhizobacterial isolates on challenge inoculation

Polyphenol oxidase activity (Δ_{420} $mt^{-1}g^{-1}$ fresh tissue)							
Sl.No.	Treatment	Before inoculation		3 DAI		5 DAI	
		PPO activity	Per cent +/- over over control	PPO activity	Per cent +/- over control	PPO activity	Per cent +/- over control
1.	T_1 (RB-144)	0.51	+ 6.25	2.66	+ 112.80	3.64	+ 48.57
2.	T_2 (RB-22)	1.20	+ 150.00	2.14	+ 71.20	7.46	+ 204.49
3.	T_3 (RB-82)	0.26	- 45.83	1.03	- 17.60	3.81	+ 55.51
4.	T_4 (RB-66)	1.48	+ 208.33	1.46	+ 16.80	2.57	+ 4.90
5.	T_5 (RB-11)	0.34	- 29.17	2.06	+ 64.80	5.14	+ 109.80
6.	T_6 (P.f1)	1.29	+ 168.75	3.43	+ 174.40	3.86	+ 57.55
7.	T_7 (RB-77)	0.94	+ 95.83	4.97	+ 297.60	5.06	+ 106.53
8.	T_8 (P.f2)	1.29	+ 168.75	1.71	+ 36.80	3.30	+ 34.69
9.	T_9 (PoP)	0.13	- 72.92	0.81	- 35.20	4.54	+ 85.31
10.	T_{10} (Control)	0.48		1.25		2.45	

DAI – days after inoculation. +-Increase, --Decrease .*P.f-Pseudomonas fluorescens*

(RB-11) and T_9 (PoP), the polyphenol oxidase (PPO) activity was more in the bacterized plants than the control on the day of inoculation (Table 4.42). The minimum activity of PPO was observed in T_9 (PoP) while the maximum was in T_4 (RB-66). Further, PPO activity increased after three days of inoculation in almost all the treatments except for T_3 (RB-82) and T_9 (PoP). Similarly, after five days of inoculation, a profound rise in PPO activity was noticed in all the treatments with the maximum in T_2 (RB-22) followed by T_5 (RB-11) and T_7 (RB-77) and the least in T_{10} (Control).

4.9.3.3 Assay of phenylalanine ammonia lyase (PAL) activity

Results furnished in Table 4.43 revealed that in general all the rhizobacterial treatments induce the plants to synthesize more phenylalanine ammonia lyase (PAL) compared to control (Fig 4.13). In general, there was an additional increase in the enzymatic activity in plants in all treatments challenge inoculated with the pathogen. The activity of PAL on the day of inoculation ranged from 550.4 to 1002.2µmol transcinnamic acid g^{-1} fresh tissue where the minimum was in T_5 and the maximum in T_2. Three days after inoculation, a rapid and transient accumulation of PAL was induced in all the treatments except for T_2 which showed decrease compared to control. Likewise, after five days of inoculation, marked increase of PAL activity was noticed in all the treatments except T_2.

4.9.4 Isozyme analysis

4.9.4.1 Peroxidase enzyme (PO)

Native gel electrophoretic separation of enzyme extract of leaves from rhizobacterial treated plants after challenge inoculation, showed six isoforms designated as PO-1, PO-2, PO-3, PO-4, PO-5 and PO-6 in treatments T_1 (RB-144) to T_6 (P.f1). But in control plants only three isoforms *viz.,* PO-1, PO-2 and PO-3 were observed. The intensity of expression of PO-1 and PO-6 was more compared to other isoforms in all the treatments (Plate 4.11 and Fig 4.14). Further, the expression of

Table 4.43. Change in phenylalanine ammonia lyase (PAL) activity in ginger plants by promising rhizobacterial isolates on challenge inoculation

Phenylalanine ammonia lyase Activity (µmol transcinnamic acid g^{-1} fresh tissue)							
Sl.No.	Treatment	Before inoculation		3 DAI		5 DAI	
		PAL activity	Per cent +/- over control	PAL activity	Per cent +/- over control	PAL activity	Per cent +/- over control
1.	T_1 (RB-144)	599.5	+ 1.83	1108.8	+ 11.39	1687.7	+ 68.21
2.	T_2 (RB-22)	1002.2	+ 70.24	947.8	- 4.78	795.3	- 20.73
3.	T_3 (RB-82)	660.3	+ 12.16	1073.9	+ 7.89	1530.7	+ 52.57
4.	T_4 (RB-66)	731.5	+ 24.26	1103.9	+ 10.90	1609.2	+ 60.39
5.	T_5 (RB-11)	550.4	- 6.51	1104.8	+ 10.99	1604.3	+ 59.90
6.	T_6 (P.f1)	591.7	+ 0.51	1027.8	+ 3.25	1535.6	+ 53.05
7.	T_7 (RB-77)	923.3	+ 56.84	1212.8	+ 21.84	1599.4	+ 59.41
8.	T_8 (P.f2)	663.8	+ 12.76	1079.8	+ 8.48	1569.9	+ 56.47
9.	T_9 (PoP)	772.2	+ 31.17	1185.8	+ 19.13	1560.1	+ 55.50
10.	T_{10} (Control)	588.7		995.4		1003.3	

DAI – days after inoculation. +-Increase, --Decrease, *P.f-Pseudomonas fluorescens*

Fig 4.11 Peroxidase (PO) activity induced in response to challenge inoculation

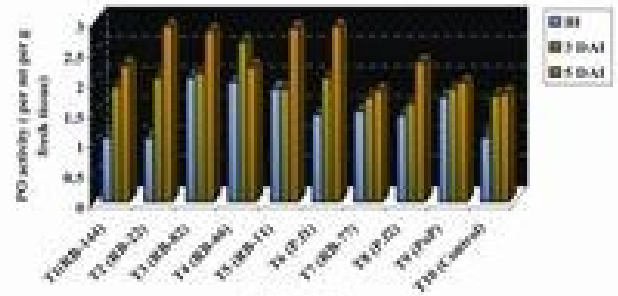


Fig 4.12 Polyphenol oxidase (PO) activity induced in responseto challeng inoculation

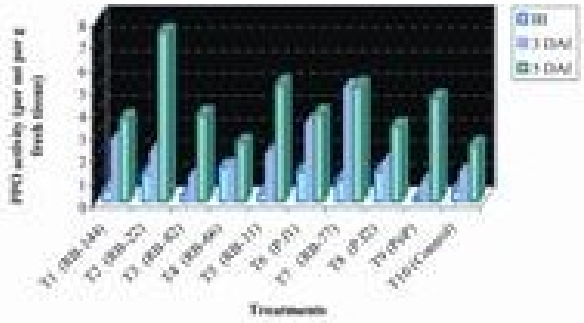
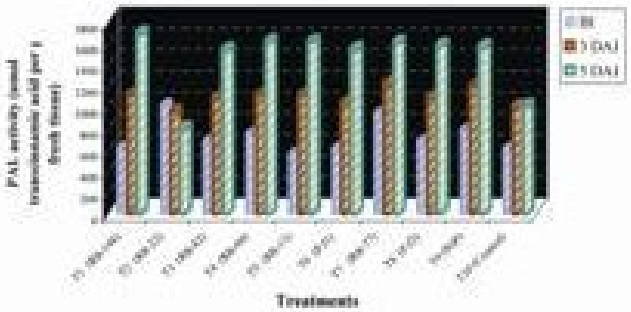


Fig 4.13 Phenyl alanine ammonia lyase (PAL) activity induced in response to challenge inoculation



PO-2 and PO-3 was more in rhizobacterial treated plants after challenge inoculation than control. Isoforms like PO-4 and PO-5 were absent in plants treated with T_7 (RB-77) and T_{10} (Control) as well as in plants treated with P.f2 (T_8). Four isoforms of peroxidase were noticed in plants in treatment T_7 (RB-77) and T_8 (P.f2). Moreover, plants in T_9 (PoP) lacked the expression of a single isoform, PO-4.

Relative mobility of the peroxizymes has been recorded and the values are presented in Table 4.44. Native PAGE analysis of the enzyme revealed three bands of Rm 0.32, Rm 0.49 and Rm 0.53 in all treated plant interactions. Three new bands of Rm 0.62, Rm 0.66 and Rm 0.88 were visualized in treatments T_1 (RB-144) to T_5 (RB-11) as well as in plants treated with P.f1. The relative mobility (Rm) value of 0.88 was noticed in T_7 (RB-77) as well as in T_8 (P.f2). An additional band of Rm 0.62 was also observed in plants in PoP treatment (T_9).

The banding pattern of peroxidase isoforms of 10 treatments including eight rhizobacterial isolates was also subjected to cluster analysis. It was observed that the isozyme pattern of peroxidase did not show much variation among the treatments (Fig 4.15). The figure indicated that all the treatments belonged to only one cluster. As a whole, the 10 treatments were classified into two sub cluster. In the first sub cluster, the treatments T_1, T_2 (RB-22), T_3 (RB-82), T_4 (RB-66), T_5 and T_6 had 100 per cent similarity index (SI) while T_9 showed 85 per cent SI with these six treatments. Further, the treatment T_7, and T_8 (P.f2) were found to have cent per cent SI whereas T_{10} showed about 85 per cent SI with T_7 and T_8.

4.9.4.2 Poylphenol oxidase enzyme (PPO)

Electrophoretic migration of PPO isoforms through Native PAGE analysis showed four isoforms designated as PPO-1, PPO-2, PPO-3 and PPO-4 in rhizobacterial treatments T_1 to T_8 while only three were noticed in T_9 and T_{10} (Plate 4.12 and Fig. 4.16). The expression of isoforms *viz.,* PPO-3 and PPO-4 was more in samples of all rhizobacterial treatments compared to isoforms PPO-1 and PPO-2. Even though, plants in T_9 and T_{10} showed only three isoforms, the expression of

Table 4.44. Relative mobility values of isoforms of peroxidase (PO) in ginger leaves of different treatments

	Relative mobility	Treatment									
		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
PO-1 **POX bands**	0.32	+	+	+	+	+	+	+	+	+	+
	0.49	+	+	+	+	+	+	+	+	+	+
PO-3	0.53	+	+	+	+	+	+	+	+	+	+
PO-4	0.62	+	+	+	+	+	+	-	-	+	-
PO-5	0.66	+	+	+	+	+	+	-	-	-	-
PO-6	0.88	+	+	+	+	+	+	+	+	-	-

Table 4.45. Relative mobility values of isoforms of polyphenol oxidase (PPO) in ginger leaves of different treatments

PPO bands	Relative mobility	Treatment									
		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
PPO-1	0.32	+	+	+	+	+	+	+	+	+	+
PPO-2	0.49	+	+	+	+	+	+	+	+	+	+
PPO-3	0.53	+	+	+	+	+	+	+	+	+	+
PPO-4	0.62	+	+	+	+	+	+	+	+	-	-

T_1: RB-144
T_2: RB-22
T_3: RB-82
T_4: RB-66
T_5: RB-11
T_6: P.f1
T_7: RB-77
T_8: P.f2
T_9: Chemical
T_{10}: Control

P.f-Pseudomonas fluorescens

+ - presence of band, - - absence of band

Plate 4.11 Native PAGE profile of peroxidase isoforms induced in response to challenge inoculation

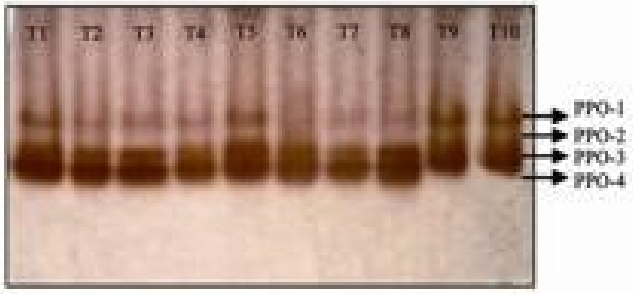


Plate 4.12 Native PAGE profile of polyphenol oxidase isoforms induced in response to challenge inoculation

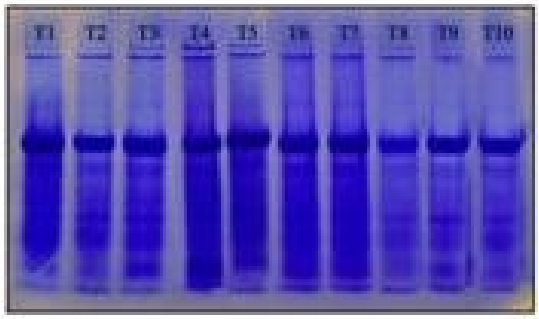


Plate 4.13 Native PAGE profile of protein induced in response to challenge inoculation

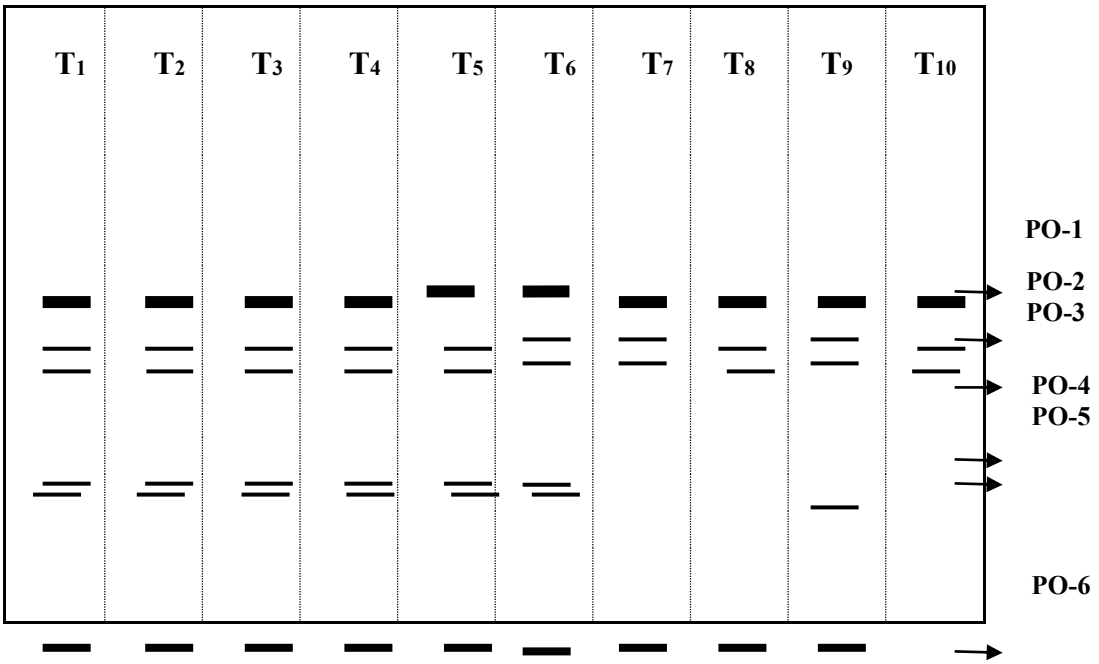


Fig.4.14 Native PAGE profile of isoforms of peroxidase induced in response to challenge inoculation

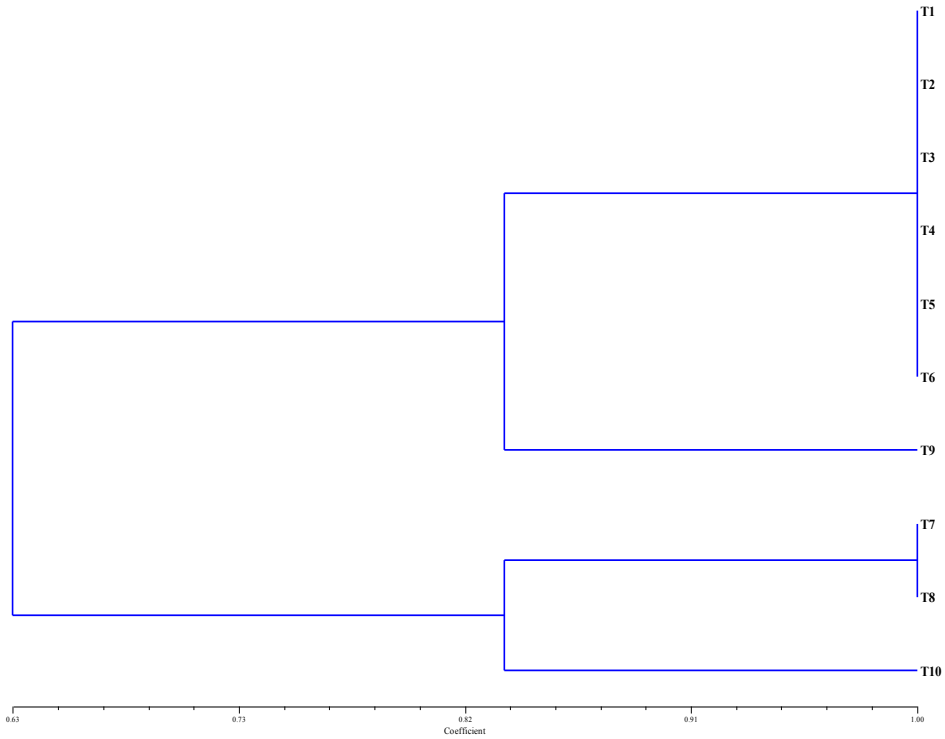


Fig.4.15 Dendrogram showing linkage between different treatments on isoforms of peroxidase of ginger after challenge inoculation

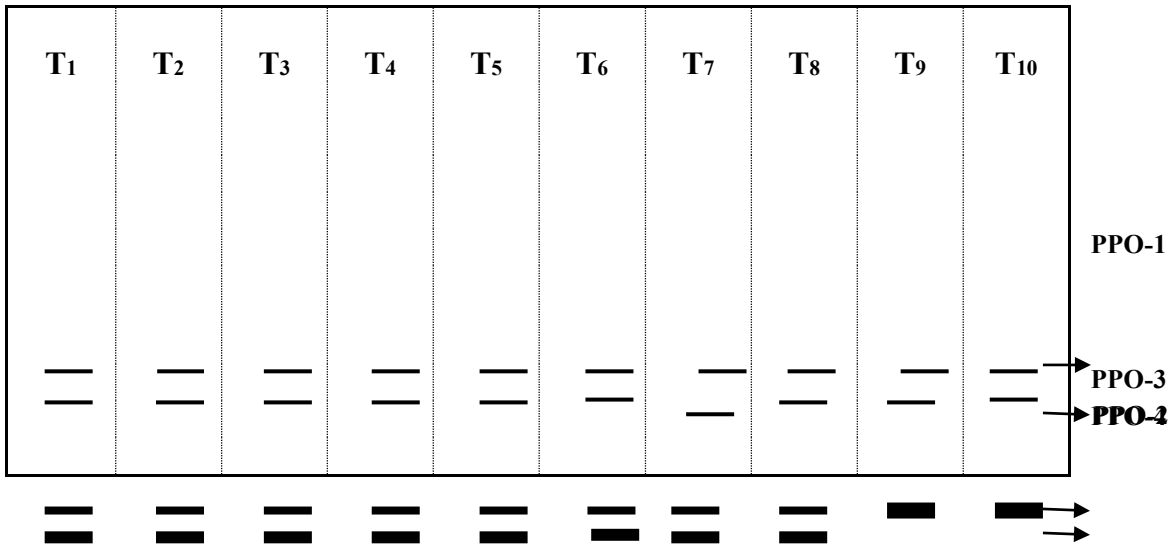


Fig.4.16 Native PAGE profile of isoforms of polyphenol oxidase (PPO) induced in response to challenge inoculation

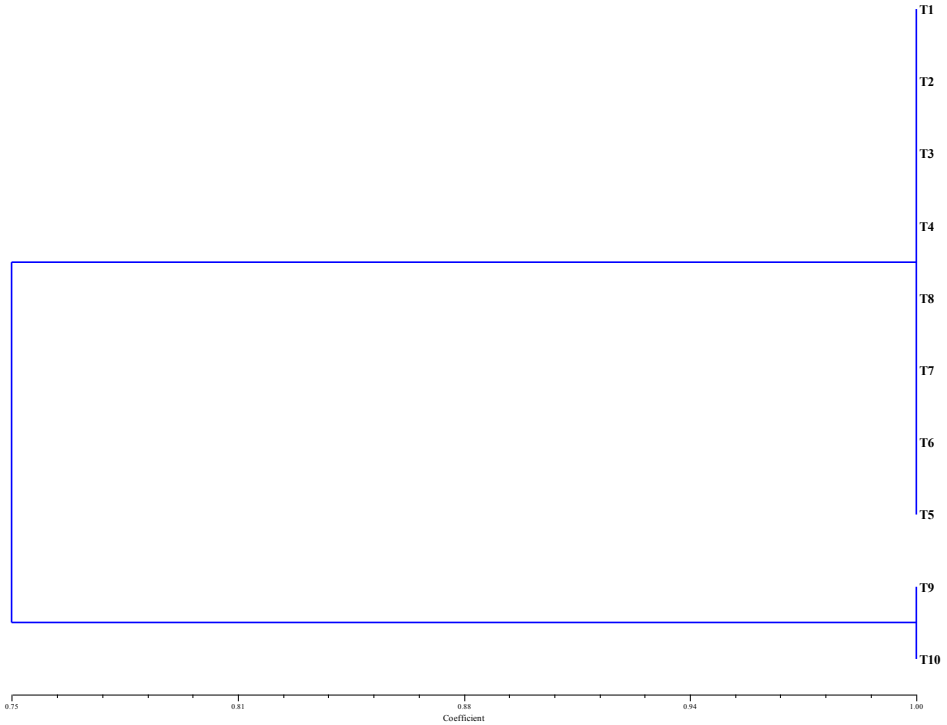


Fig. 4.17 Dendrogram showing linkage between different treatments on isoforms of polyphenol oxidase on challenge inoculation

PPO-3 in the same treatments was more pronounced than others. The relative mobility values of isoforms of PPO are presented in Table 4.47. Native PAGE analysis of the enzyme revealed bands of Rm value 0.32, 0.49 and 0.53 in all samples. An additional band of Rm 0.62 was observed in all the treatments with the exception of T_9 (PoP) and T_{10} (Control).

Cluster analysis of banding pattern of isoforms of PPO indicated presence of one cluster with no sub clusters (Fig 4.17). Except T_9 (PoP) and T_{10} (Control), the rest of the treatments showed 100 similarity index and these eight treatments possessed 75 per cent SI with T_9 and T_{10}.

4.9.4.3 Protein

The protein pattern of plants in various treatments inoculated with *R.solanacearum* was carried out to detect the variation in its profiles (Plate 4.13). The expression of protein profile was quite similar in number, relative intensity and position of bands in all treatments with few exceptions (Table 4.46 and Fig 4.18). A total of 10 bands were observed in the treatments from T_1 (RB-144) to T_5 (RB-11) while, the P.f1 treated plants as well as T_7 (RB-77), T_9 (PoP) and T_{10} (Control) were short of a band with relative mobility 0.1. Likewise, Rm of 0.6 was found absent in T_7 and the P.f2. Moreover, it was observed that the intensity of expression of bands with Rm 0.1, 0.13, 0.75, 0.85 and 0.98 was more compared to other bands.

The cluster analysis of banding pattern of protein is given in Fig 4.19. There was only one cluster with two sub clusters and treatments from T_1 to T_5 showed cent per cent similarity index (SI) and these five treatments were found to have 90 per cent SI with T_7 (RB-77). Similarly, T_8 (P.f2) showed 90 per cent SI with T_{10} (Control), T_6 (P.f1) and T_9 (PoP) while the latter three treatments had cent per cent SI with each other.

Table 4.46. Relative mobility values of protein bands in ginger leaves of different treatments by Native PAGE analysis

Protein bands	Relative mobility	Treatment									
		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}
P-1	0.10	+	+	+	+	+	-	-	+	-	-
P-2	0.13	+	+	+	+	+	+	+	+	+	+
P-3	0.22	+	+	+	+	+	+	+	+	+	+
P-4	0.32	+	+	+	+	+	+	+	+	+	+
P-5	0.45	+	+	+	+	+	+	+	+	+	+
P-6	0.53	+	+	+	+	+	+	+	+	+	+
P-7	0.60	+	+	+	+	+	+	-	-	+	
P-8	0.75	+	+	+	+	+	+	+	+	+	+
P9	0.85	+	+	+	+	+	+	+	+	+	+
P-10	0.98	+	+	+	+	+	+	+	+	+	

T_1: RB-144
T_2: RB-22
T_3: RB-82
T_4: RB-66
T_5: RB-11
T_6: P.f1
T_7: RB-77
T_8: P.f2
T_9: Chemical
T_{10}: Control

P.f-Pseudomonas fluorescens

+-presence of band, -- absence of band

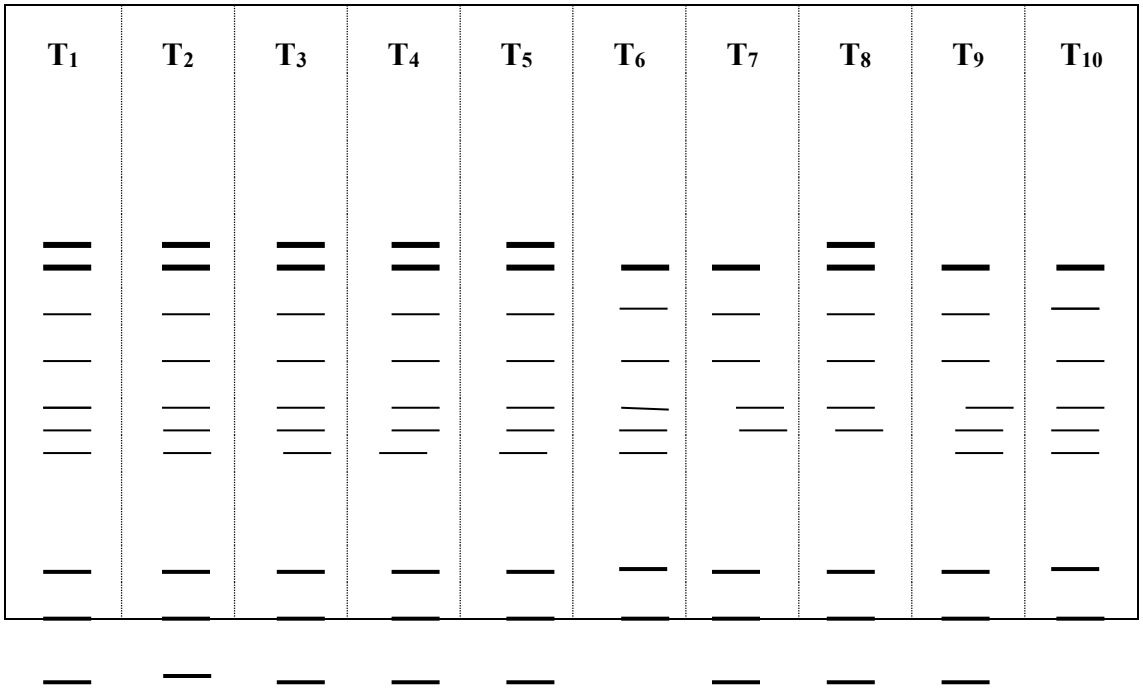


Fig. 4.18 Native PAGE profile of proteins from ginger leaves induced in response to challenge inoculation

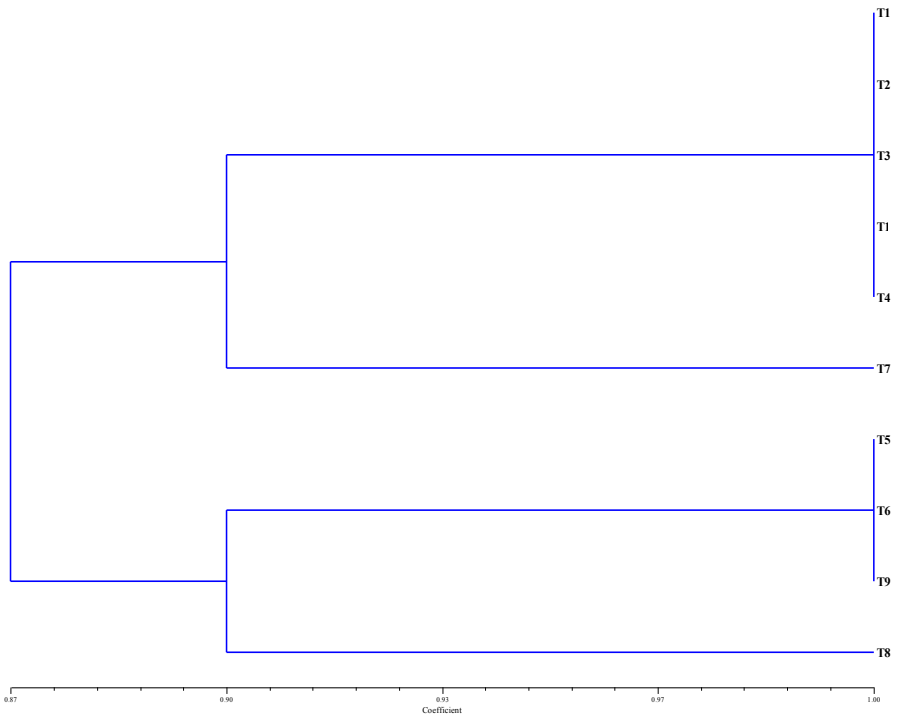


Fig.4.19 Dendrogram showing linkage between different treatments with respect to protein on challenge inoculation

4.9.5 Estimation of chlorophyll

The chlorophyll contents *viz*., a, b and total chlorophyll varied among plants in different treatments (Table 4.47). The chlorophyll content 'a' ranged from the minimum of 0.69 mgg^{-1} fresh tissue as observed in T_1 (RB-144) to the maximum of 1.21mgg^{-1} fresh tissue as witnessed in T_5 (RB-11). Chlorophyll 'b' content was less in treatment T_1 (RB-144) and more in T_3 (RB-82). Maximum total chlorophyll content of 2.21mg g^{-1} was recorded in samples of T_3 (RB-82) which was closely followed by that in T_5 (RB-11) and T_4 (RB-66). The least quantity of it was in samples of T_1 (RB-144), T_7 (RB-77), T_8 (P.f2) and T_9 (PoP).

4.9.6 Oil and oleoresin content of dry ginger rhizomes

From the Table 4.48 it is evident that there is no significant difference among the treatments on the oil content. However, the maximum oil content was recorded with rhizobacterial treatment T_3 (RB-82). On the other hand, the oleoresin content varied significantly among different treatments where the maximum content was with plants in T_1 (RB-144), T_3 RB-82) and T_2 (RB-22) and these were on par with all the other treatments except T_9 (PoP) and T_{10} (Control).

4.9.7 NPK content of ginger rhizomes

A significant difference was noticed among the treatments with respect to nitrogen. The highest nitrogen content was in T_7 (RB-77) and it was on par with all other treatments except for T_3 (RB-82) (Table 4.49). The highest phosphorous (P) content was in plants treated with T_5 (RB-11) and was on par with T_4 (RB-66) and the lowest was in T_6 (P.f1). The maximum potassium (K) content was in ginger rhizomes treated with T_5 (RB-11) and T_8 (P.f2) which was on par with four other rhizobacterial treatments. The least K content was observed in P.f1 and was on par with T_9 (PoP) and T_{10} (Control).

Table 4.47. Effect of rhizobacterial isolates in chlorophyll content in ginger leaves

Sl.No.	Treatment	*Chlorophyll a (mg g^{-1} fresh tissue)	Per cent +/- over control	*Chlorophyll b (mg g^{-1} fresh tissue)	Per cent +/- over control	* Total chlorophyll (mg g^{-1} fresh tissue)	Per cent +/- over control
1.	T_1 (RB-144)	0.69	- 0.34	0.23	- 0.91	0.92	- 0.49
2.	T_2 (RB-22)	0.95	+ 0.02	0.50	+ 0.12	1.46	+ 0.06
3.	T_3 (RB-82)	1.34	+ 0.31	0.87	+ 0.49	2.21	+ 0.38
4.	T_4 (RB-66)	1.14	+ 0.18	0.65	+ 0.32	1.80	+ 0.24
5.	T_5 (RB-11)	1.21	+ 0.23	0.71	+ 0.38	1.92	+ 0.29
6.	T_6 P.f1)	1.11	+ 0.16	0.58	+ 0.24	1.69	+ 0.19
7.	T_7 (RB-77)	0.73	- 0.27	0.29	- 0.52	1.02	- 0.34
8.	T_8 (P.f2)	0.86	- 0.08	0.37	- 0.19	1.22	- 0.12
9.	T_9 (PoP)	0.90	- 0.03	0.41	- 0.07	1.31	- 0.05
10.	T_{10} (Control)	0.93		0.44		1.37	

Table 4.48. Effect of promising rhizobacterial isolates on oil and oleoresin content of dry ginger rhizomes

Sl.No.	Treatment	Oil content (%)	Oleoresin content (%)
1.	T_1 (RB-144)	1.13[a]	5.74[a]
2.	T_2 (RB-22)	0.93[a]	5.01[abc]
3.	T_3 (RB-82)	1.20[a]	5.27[ab]
4.	T_4 (RB-66)	1.07[a]	4.72[abc]
5.	T_5 (RB-11)	0.97[a]	4.79[abc]
6.	T_6 (P.f1)	0.97[a]	4.72[abc]
7.	T_7 (RB-77)	0.73[a]	4.78[abc]
8.	T_8 (P.f2)	0.87[a]	4.75[abc]
9.	T_9 (PoP)	0.73[a]	4.47[bc]
10.	T_{10} (Control)	1.0[a]	4.01[c]

*Mean of three replications, MAP-months after planting. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

Table 4.49. Effect of promising rhizobacterial isolates on NPK contents of dry ginger rhizomes

Sl.No.	Treatment	N (%)	P (%)	K (%)
1.	T_1 (RB-144)	1.75 ^{ab}	0.30 ^{cd}	1.83^{abc}
2.	T_2 (RB-22)	1.63 ^{ab}	0.35 ^{bc}	1.92^{ab}
3.	T_3 (RB-82)	1.04 ^{b}	0.30 ^{cd}	1.73^{bc}
4.	T_4 (RB-66)	1.45 ^{ab}	0.43 ^{ab}	1.92^{ab}
5.	T_5 (RB-11)	1.40 ^{ab}	0.47 ^{a}	1.99^{a}
6.	T_6 (P.f1)	1.40 ^{ab}	0.21 ^{d}	1.50^{d}
7.	T_7 (RB-77)	1.99 ^{a}	0.29 ^{cd}	1.77^{abc}
8.	T_8 (P.f2)	1.54 ^{ab}	0.31 ^{cd}	1.99^{a}
9.	T_9 (PoP)	1.54 ^{ab}	0.29 ^{cd}	1.67^{cd}
10.	T_{10} (Control)	1.24 ^{ab}	0.37 ^{bc}	1.68^{cd}

*Mean of three replications, MAP-months after planting. In each column figures followed by same letter donot differ significantly according to DMRT. *P.f-Pseudomonas fluorescens*

4.10 MOLECULAR MECHANISM OF INDUCTION OF SYSTEMIC RESISTANCE

An effort was made to reveal the molecular mechanism of PGPR mediated induction of systemic resistance in ginger so as to locate whether there was any additional band unique to the induced plant. This was carried out through amplification of cDNA using random decamer primers.

4.10.1 Primer screening

For selecting random primers to amplify the cDNA from ginger variety, Himachal, a primer screening was carried out, for which, genomic DNA isolated from fresh ginger leaves were subjected to RAPD assay.

4.10.1.1 Isolation of DNA and its quantification

Genomic DNA was successfully isolated from ginger leaves using modified CTAB method as per the protocol given in 3.16.1.1. Upon electrophoresis on one per cent agarose gel, intact, clear and distinct DNA was observed and the quality and quantity of isolated DNA was assessed (Table 4.50) (Plate 4.14). The good quality DNA obtained was further used for screening of random primers for RAPD assay.

Table 4.50. Quality and quantity of DNA as revealed by spectrophotometry

Sl. No.	Parameter	DNA
1.	$OD_{260/280}$	2.09
2.	Quality	Good
3.	Quantity	5.75 $ngmg^{-1}$ leaf tissue

4.10.1.2 Screening of random primers

Initially RAPD profiles were generated using 32 random decamer primers of OPA, OPE and OPF series which were tested with the genomic DNA sample of ginger variety Himachal, so as to determine the suitability of each primer for further studies.

Out of the 32 primers screened, 12 primers of OPE and OPF series generated more number of fragments with clear and distinct bands using the template DNA of the sample were chosen for further studies (Table 4.51) (Plate 4.15). They were OPE 6, OPE 8, OPE 9, OPE 11, OPE 13, OPE 14, OPE 16, OPE 17, OPE 19, OPE 20, OPF 15, OPF 16, OPF 17, OPF 18 and OPF 19. Primers of OPA series and certain primers of OPE (OPE 5, OPE 6, OPE 9 , OPE 10) and OPF (OPF 2, OPF 5, OPF 6, OPF 9, OPF 11, OPF 12, OPF 13, OPF 14, OPF 15, OPF 16, OPF 20) series did not show any amplification. The details of each primer series are given below:

OPA series: Out of the 5 primers of OPA series screened, none of the primers gave amplification. So primers of OPA series were not selected.

OPE series: Out of the 13 primers of OPE series screened, 9 primers gave good amplification with 3-7 clear and distinct bands and hence selected.

OPF series: From among the 14 primers of OPF series, only 3 gave good amplification with 3-5 amplicons and were therefore chosen for further studies.

4.10.2 Isolation of RNA

Isolation of total RNA from PGPR treated (RB-11) and untreated control plants were carried out as per 3.10.2. The purity of RNA samples were detected by agarose gel (0.7%) electrophoresis and it was observed that five clear and distinct bands of treated ginger plants (28s, 18s, 5.8s, 5s and t-RNA) and three bands of control sample (28s, 18s and 5s) were obtained indicating good quality of RNA (Plate 4.16). Contamination with genomic DNA was absent in both the samples.

Table 4.51 Primer screening for DNA amplification in ginger

Sl.No.	Primer code	Number of bands	Quality of bands	Remarks
1.	OPA-1	0	No amplification	Not selected
2.	OPA-2	0	No amplification	Not selected
3.	OPA-3	0	No amplification	Not selected
4.	OPA-4	0	No amplification	Not selected
5.	OPA-5	0	No amplification	Not selected
6.	OPE-3	3	Distinct, clear bands	Selected
7.	OPE-5	0	No amplification	Not selected
8.	OPE-6	2	Less number of bands	Not selected
9.	OPE-8	3	Distinct, clear bands	Selected
10.	OPE-9	2	Less number of bands	Not selected
11.	OPE-10	0	No amplification	Not selected
12.	OPE-11	3	Distinct, clear bands	Selected
13.	OPE-13	6	Distinct, clear bands	Selected
14.	OPE-14	6	Distinct, clear bands	Selected
15.	OPE-16	5	Distinct, clear bands	Selected
16.	OPE-17	5	Distinct, clear bands	Selected
17.	OPE-19	7	Distinct, clear bands	Selected
18.	OPE-20	4	Distinct, clear bands	Selected
19.	OPF-2	1	Less number of bands	Not selected
20.	OPF-5	0	No amplification	Not selected
21.	OPF-6	1	Less number of bands	Not selected
22.	OPF-9	0	No amplification	Not selected
23.	OPF-11	0	No amplification	Not selected
24.	OPF-12	2	Less number of bands	Not selected
25.	OPF-13	1	Less number of bands	Not selected
26.	OPF-14	0	No amplification	Not selected
27.	OPF-15	1	Less number of bands	Not selected
28.	OPF-16	1	Less number of bands	Not selected
29.	OPF-17	4	Distinct, clear bands	Selected
30.	OPF-18	3	Distinct, clear bands	Selected
31.	OPF-19	5	Distinct, clear bands	Selected
32.	OPF-20	0	No amplification	Not selected

4.10.3 Synthesis of cDNA

cDNA of both treated and control samples were synthesized from RNA by RT PCR. The quantity of cDNA was assessed by spectrophotometry and is given in Table 4.52. The good quality cDNA thus obtained was then subjected to RAPD assay with the selected 12 primers.

Table 4.52. Quality and quantity of cDNA as revealed by spectrophotometry

Sl. No.	Parameter	cDNA sample	
		Treated	Control
1.	$OD_{260/280}$	1.82	1.80
2.	Quality	Fairly good	Fairly good
3.	Quantity	$4.57 \mu g ml^{-1}$	$3.75 \mu g ml^{-1}$

4.10.4 RAPD assay with cDNA

cDNA synthesized by RT PCR was subjected to RAPD assay. The 12 selected random primers as in 4.16.1.2 which produced clear distinct bands of 3-7 amplicons were used for the assay. A modified protocol of 3.16.1.4 was used for PCR based characterization of the selected treated and control plants with these primers. The cDNA was diluted to 1:100, 1:20 and 1:10 and subjected to amplification with these selected primers. However, no bands were visualized after subjecting the amplified products to agarose gel electrophoresis.

4.11 SENSITIVITY OF PROMSING RHIZOBACTERIAL ISOLATES TO PLANT PROTECTION CHEMICALS AND FERTILIZERS

The *in vitro* compatibility of the promising PGPR isolates and the reference cultures to antibiotics, fungicides, insecticides and fertilizers were studied.

Plate 4.14 Genomic DNA of ginger

S : Sample

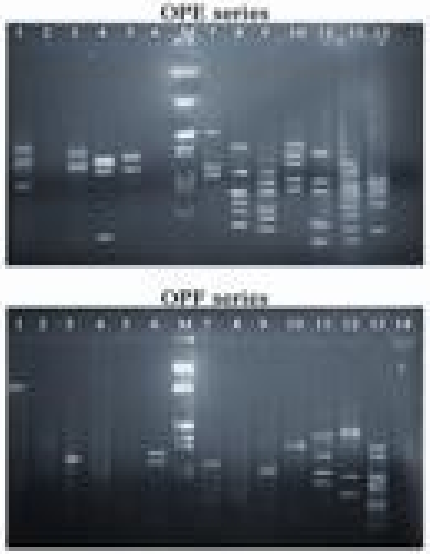


Plate 4.15 Primer screening for RAPD analysis

Plate 4.16 RNA of ginger

T : Treated

C : Control

4.11.1 Antibiotics

Antibiotics *viz*., Chloramphenicol, Gentamicin and Rifampicin at three concentrations; Ampicillin, Streptomycin, Tetracycline and Kanamycin at two concentrations and Penicillin-G, Cephalaxin, Nalidixic acid and Oxytetracycline at one concentration were used for this study. The results are presented in the Table 4.53.

Among the various isolates, Cephalaxin, Oxytetracycline and Nalidixic acid were compatible with all the isolates while, Penicillin-G were compatible with all the isolates except RB-77. Moreover, Ampicillin at both concentrations was compatible with all the isolates except RB-77. The lowest concentration of Tetracycline (0.05 ppm) was also compatible with the isolates except RB-144 and RB-77.

In general, Gentamicin at all concentrations was incompatible with the PGPR isolates with few exceptions. The isolates RB-1, RB-11, P.f1 and P.f2 was compatible to a certain extent with the lowest concentration of Chloramphenicol, lower two concentrations of Rifampicin and both the concentrations of Kanamycin (Plate 4.17). The rhizobacterial isolates *viz.,* RB-22, RB-82, RB-66 were also partially compatible with the lowest concentration of Chloramphenicol and Rifampicin and both the concentrations of Kanamycin, while, RB-77 was partially compatible with the lowest concentration of Chloramphenicol, Gentamicin and both concentrations of Kanamycin and Streptomycin whereas tetracycline at both concentrations were incompatible.

4.11.2 Fungicides

Eight fungicides *viz*., Indofil M-45, Captaf, Bavistin, Master, Akomin-40, Fytolan, Kocide and Shield each at three different concentrations were evaluated for their compatibility with the promising PGPR isolates and also with the standard culture of *P.fluorescens*. The results are furnished in Table 4.54.

Table 4.53. *In vitro* sensitivity of PGPR isolates to various concentrations of antibiotics

Sl.No.	Antibiotic	Concentration (ppm)	Inhibition zone (mm)			
			RB-144	RB-22	RB-82	RB-66
1.	Chloramphenicol	0.1	14.33^{ij} (3.85)	14.33^{h} (3.85)	10.33^{j} (3.29)	15.0^{g} (3.94)
		0.25	17.67^{g} (4.26)	18.83^{f} (4.40)	12.50^{i} (3.61)	18.67^{e} (4.38)
		0.50	19.33^{f} (4.46)	21.33^{d} (4.67)	15.83^{h} (4.04)	20.67^{d} (4.60)
2.	Gentamicin	0.1	24.33^{c} (4.98)	20.33^{e} (4.57)	22.67^{b} (5.31)	21.67^{c} (4.71)
		0.3	29.33^{b} (5.46)	22.67^{c} (4.81)	24.83^{d} (5.03)	23.83^{b} (4.93)
		0.5	31.50^{a} (5.66)	28.83^{a} (5.42)	29.67^{a} (5.49)	28.67^{a} (5.40)
3.	Rifampicin	0.05	13.83^{j} (3.79)	10.50^{i} (3.31)	7.83^{k} (2.89)	13.83^{h} (3.79)
		0.15	14.67^{i} (3.90)	20.83^{de} (4.62)	16.83^{g} (4.16)	23.67^{b} (4.92)
		0.3	16.83^{h} (4.16)	27.83^{b} (5.32)	20.83^{e} (4.62)	29.67^{a} (5.49)
4.	Kanamycin	0.05	12.17^{k} (3.56)	7.33^{k} (2.80)	7.83^{k} (2.89)	0^{k} (0.71)
		0.3	13.83^{j} (3.79)	8.67^{j} (3.03)	10.67^{j} (3.34)	9.83^{j} (3.21)
5.	Ampicillin	0.1	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)
		0.25	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)
6.	Streptomycin	0.1	20.33^{e} (4.57)	15.33^{g} (3.98)	18.83^{f} (4.40)	17.33^{f} (4.22)
		0.25	22.33^{d} (4.78)	18.83^{f} (4.40)	25.83^{c} (5.13)	21.33^{cd} (4.67)
7.	Tetracycline	0.05	14.67^{i} (3.90)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)
		0.3	20.50^{e} (4.58)	8.83^{j} (3.05)	0^{l} (0.71)	0^{k} (0.71)
8.	Penicillin G	0.1	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)
9.	Nalidixic acid	0.3	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	11.67^{i} (3.49)
10.	Oxytetracycline	0.3	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)
11.	Cephalaxin	0.3	0^{l} (0.71)	0^{l} (0.71)	0^{l} (0.71)	0^{k} (0.71)

Table 4.53. *In vitro* sensitivity of promising rhizobacterial isolates to antibiotics (contd…..)

Sl.No.	Antibiotic	Concentration (ppm)	Inhibiton zone (mm)			
			RB-11	P.f1	RB-77	P.f2
1.	Chloramphenicol	0.1	20.33^{e} (4.57)	12.33^{h} (3.58)	13.83^{h} (3.79)	14.33^{g} (3.85)
		0.25	25.50^{b} (5.10)	14.67^{g} (3.90)	17.0^{f} (4.18)	17.33^{e} (4.22)
		0.50	30.33^{a} (5.55)	18.50^{f} (4.36)	19.83^{d} (4.51)	21.33^{b} (4.67)
2.	Gentamicin	0.1	20.17^{e} (4.55)	30.33^{c} (5.55)	14.83^{g} (3.92)	17.0^{e} (4.18)
		0.3	24.83^{bc} (5.03)	31.33^{b} (5.64)	17.83^{e} (4.28)	20.67^{b} (4.60)
		0.5	29.83^{a} (5.51)	33.83^{a} (5.86)	20.33^{d} (4.57)	22.83^{a} (4.83)
3.	Rifampicin	0.05	0^{j} (0.71)	0^{j} (0.71)	18.33^{e} (4.34)	7.83^{j} (2.89)
		0.15	8.83^{h} (3.05)	12.83^{h} (3.65)	20.33^{d} (4.57)	15.83^{f} (4.04)
		0.3	10.83^{f} (3.37)	21.0^{e} (4.64)	23.33^{c} (4.88)	19.33^{c} (4.46)
4.	Kanamycin	0.05	7.33^{i} (2.80)	8.0^{i} (2.92)	12.50^{i} (3.61)	7.50^{j} (2.83)
		0.3	9.67^{g} (3.19)	17.83^{f} (4.28)	12.67^{i} (3.63)	9.67^{i} (3.19)
5.	Ampicillin	0.1	0^{j} (0.71)	0^{j} (0.71)	32.83^{b} (5.77)	0^{K} (0.71)
		0.25	0^{j} (0.71)	0^{j} (0.71)	40.83^{a} (6.43)	0^{K} (0.71)
6.	Streptomycin	0.1	21.83^{d} (4.72)	18.17^{f} (4.32)	12.83^{i} (3.65)	12.33^{d} (3.58)
		0.25	24.67^{c} (5.02)	23.33^{d} (4.88)	14.83^{g} (3.92)	18.33^{h} (4.34)
7.	Tetracycline	0.05	0^{j} (0.71)	0^{j} (0.71)	18.33^{e} (4.34)	0^{K} (0.71)
		0.3	0^{j} (0.71)	0^{j} (0.71)	20.67^{d} (4.60)	7.33^{j} (2.80)
8.	Penicillin G	0.1	0^{j} (0.71)	0^{j} (0.71)	41.83^{a} (6.51)	0^{K} (0.71)
9.	Nalidixic acid	0.3	0^{j} (0.71)	0^{j} (0.71)	0^{j} (0.71)	0^{K} (0.71)
10.	Oxytetracycline	0.3	0^{j} (0.71)	0^{j} (0.71)	0^{j} (0.71)	0^{K} (0.71)
11.	Cephalaxin	0.3	0^{j} (0.71)	0^{j} (0.71)	0^{j} (0.71)	0^{K} (0.71)

* Mean of three replications. In each column figures followed by same letters donot differ significantly according to DMRT. Figures in paranthesis are √x+0.5 transformed values

, *P.f-Pseudomonas fluorescens*

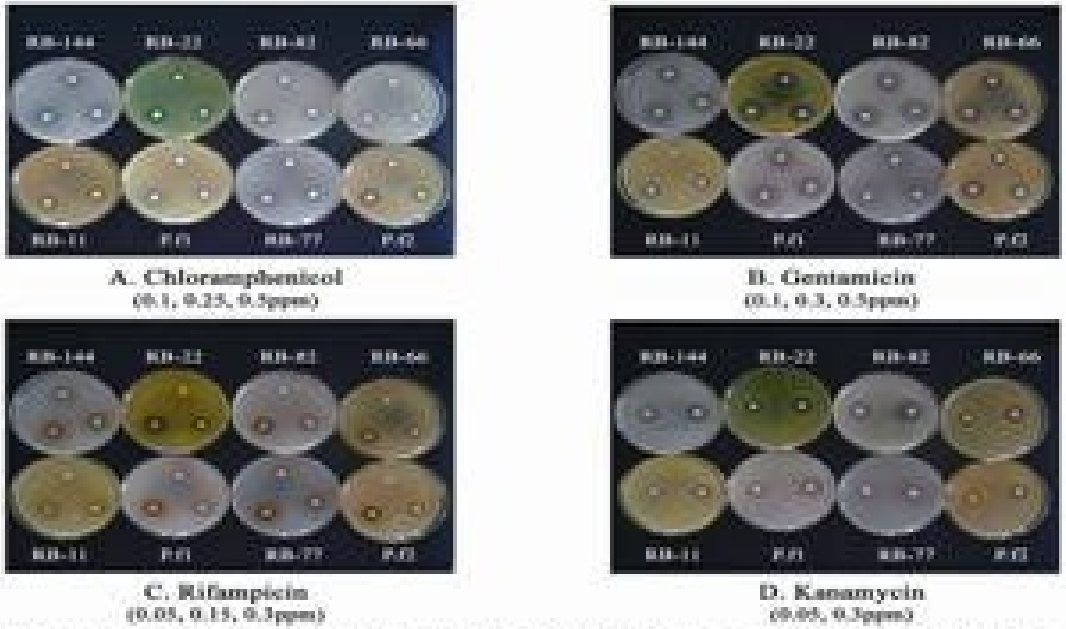


Plate 4.17 Sensitivity of promising PGPR isolates to various concentrations of antibiotics

*As concentration increases, inhibition zone increases

Among the different fungicides tested, with the exemption of copper fungicides, all others were found to be compatible with the PGPR isolates. Kocide at all concentrations showed inhibition zone ranging from 8.0 to 23.7 mm to all isolates except RB-144, RB-11 and P.f2. In general, Fytolan at all concentrations showed higher inhibition to all the isolates except RB-144 and RB-11. In the case of Shield, a copper sulphate fungicide, only the highest concentration showed an inhibitory effect against the PGPR isolates.

4.11.3 Insecticides

Insecticides *viz*., Sevin, Target, Duralax, Rogor, Ekalux, Confidor, Phorate, Furadan and Marshall at three different concentrations were used for the *in vitro* evaluation and it was observed that none of them had inhibitory effect against the PGPR isolates tested.

4.11.4 Fertilizers

Fertilizers *viz*., Urea, Rajphos, Muriate of Potash (MoP), Ammonium sulphate and Factomphos were also evaluated for their inhibitory effect and it was noticed that none of them were inhibitory to the rhizobacterial isolates tested.

4.12 *In vitro* SENSITIVITY OF PLANT PROTECTION CHEMICALS AND FERTILIZERS TO *Ralstonia solanacearum*

A similar method as mentioned in section 3.13 was followed for testing the effect of antibiotics, fungicides, insecticides and fertilizers on growth of *R.solanacearum*. All the antibiotics, fungicides, insecticides and fertilizers used for *in vitro* testing against the PGPR isolates were taken for this study also.

Table 4.54. *In vitro* sensitivity of promising PGPR isolates to various concentrations of fungicides

Sl.No.	Fungicide	Concentration (%)	Inhibition zone (mm)							
			RB-144	RB-22	RB-82	RB-66	RB-11	P.f1	RB-77	P.f2
1.	Shield	0.5	0^{c} (0.71)	0^{e} (0.71)	0^{c} (0.71)	0^{d} (0.71)	0^{d} (0.71)	0^{f} (0.71)	0^{e} (0.71)	0^{e} (0.71)
		1.0	0^{c} (0.71)	0^{e} (0.71)	0^{c} (0.71)	0^{d} (0.71)	0^{d} (0.71)	0^{f} (0.71)	0^{e} (0.71)	0^{e} (0.71)
		1.5	5.67^{b} (2.68)	7.33^{d} (2.80)	0^{c} (0.71)	7.33^{c} (2.80)	7.33^{bc} (2.80)	6.67^{e} (2.68)	8.0^{d} (2.90)	8.33^{d} (2.97)
2.	Fytolan	0.2	0^{c} (0.71)	25.67^{a} (5.12)	15.0^{b} (3.34)	20.33^{b} (4.57)	8.0^{ab} (2.91)	12.33^{b} (3.58)	23.33^{a} (4.88)	14.0^{b} (3.81)
		0.3	0^{c} (0.71)	26.0^{a} (5.15)	15.33^{a} (3.94)	22.33^{a} (4.77)	8.67^{a} (3.03)	20.0^{a} (4.53)	23.33^{a} (4.88)	18.0^{a} (4.30)
		0.4	7.67^{a} (2.85)	24.0^{b} (4.95)	10.33^{a} (3.98)	23.0^{a} (4.85)	7.17^{c} (2.77)	12.67^{b} (3.63)	24.67^{a} (5.02)	12.0^{c} (3.53)
3.	Kocide	0.1	0^{c} (0.71)	21.33^{c} (4.67)	16.33^{b} (3.27)	19.33^{b} (4.46)	0^{d} (0.71)	8.0^{d} (2.91)	17.0^{b} (4.18)	0^{e} (0.71)
		0.15	0^{c} (0.71)	23.33^{b} (4.88)	16.67^{a} (4.10)	22.67^{a} (4.81)	0^{d} (0.71)	10.67^{c} (3.34)	11.67^{c} (3.48)	0^{e} (0.71)
		0.2	0^{c} (0.71)	23.67^{b} (4.92)	16.67^{a} (4.14)	23.33^{a} (4.88)	0^{d} (0.71)	12.67^{b} (3.63)	22.33^{a} (4.78)	0^{e} (0.71)

* Mean of three replications. In each column figures followed by same letters donot differ significantly according to DMRT.
Figures in paranthesis are √x+0.5 transformed values

4.12.1 Antibiotics

Observations on the *in vitro* sensitivity of *R. solanacearum* to 11 different antibiotics at varied concentrations are presented in Table 4.55.

In general, it was observed that except Cephalaxin, all the antibiotics at different concentrations were inhibitory to the pathogen to a varying extent. The higher concentration of Gentamicin, Kanamycin and Tetracycline exhibited maximum inhibition with an inhibition zone of above 30mm (Plate 4.18). Similarly, the highest concentration of Chloramphenicol and Ampicillin and all concentrations of Rifampicin and Streptomycin sulphate inhibited the pathogen. Likewise, Penicillin G at 0.1 ppm and Nalidixic acid at 0.3 ppm also restricted the growth of the pathogen. Oxytetracycline at 0.3 ppm and the lowest concentration of Streptocycline (200 ppm) was comparatively compatible but the highest concentration of Streptocycline (400 ppm) recorded an inhibition zone of 22 mm.

4.12.2 Fungicides

Amongst the eight fungicides tested, only the copper fungicides *viz.,* Fytolan, Kocide and Shield exhibited inhibitory effect against the pathogen (Table 4.56). The higher two concentrations of Fytolan and the highest concentration of Kocide exhibited a per cent inhibition of more than 20. Though, the lowest concentration of Shield did not express any zone of inhibition, the higher two concentrations showed an inhibition zone of 10 and 13.5 mm respectively.

4.12.3 Insecticides

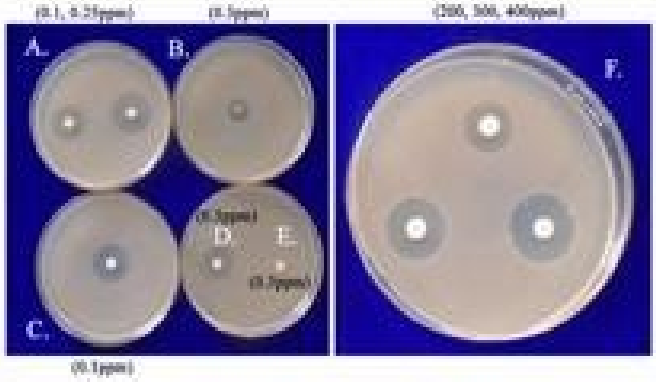
The insecticides as in 3.11.3 at three different concentrations were evaluated with the pathogen as well and it was observed that all the insecticides were not inhibitory to the pathogen

Table 4.55. *In vitro* sensitivity of *R.solanacearum* to antibiotics

Sl.No.	Antibiotic	Concentration (ppm)	*Inhibition zone (mm)
1.	Chloramphenicol	0.1	17.67^{h} (4.26)
		0.25	20.33^{f} (4.57)
		0.50	23.0^{e} (4.85)
2.	Gentamicin	0.1	24.0^{de} (4.95)
		0.3	27.67^{c} (5.31)
		0.5	30.33^{b} (5.55)
3.	Rifampicin	0.05	23.33^{e} (4.88)
		0.15	24.67^{d} (5.02)
		0.3	27.67^{c} (5.31)
4.	Kanamycin	0.01	12.33^{i} (3.58)
		0.05	24.67^{d} (5.02)
		0.3	30.33^{b} (5.55)
5.	Ampicillin	0.1	18.67^{g} (4.38)
		0.25	23.67^{de} (4.92)
6.	Streptomycin sulphate	0.1	23.67^{de} (4.92)
		0.25	26.67^{c} (5.21)
7.	Tetracycline	0.05	30.33^{d} (5.55)
		0.3	31.67^{b} (5.67)
8.	Penicillin G	0.1	24.67^{d} (5.02)
9.	Nalidixic acid	0.3	20.33^{f} (4.57)
10.	Oxytetracycline	0.3	12.33^{i} (3.58)
11.	Cephalaxin	0.3	0^{j} (0.71)

* Mean of three replications. In each column figures followed by same letters donot differ significantly according to DMRT. Figures in paranthesis are $\sqrt{x+0.5}$ transformed values

A. Rifampicin B. Kanamycin C. Chloramphenicol
D. Gentamicin E. Ampicillin F. Tetracycline



A. Streptomycin sulphate B. Oxytetracycline C. Penicillin
D. Nalidixic acid E. Cephalaxin F. Streptocycline

Plate 4.18 Sensitivity of *R.solanacearum* to various concentrations of antibiotics

*As concentration increases, inhibition zone increases

Table 4.56. *In vitro* sensitivity of *R.solanacearum* to fungicides

Sl.No.	Fungicide	Concentration (%)	*Inhibition zone (mm)
1.	Fytolan	0.2	15.5
		0.3	18.5
		0.4	21.0
2.	Kocide	0.1	8.5
		0.15	10.0
		0.2	18.5
3.	Shield	0.5	0.0
		1.0	10.0
		1.5	13.5

* Mean of three replications.

4.12.4 Fertilizers

In general, it was observed that all the fertilizers had an inhibitory effect to the pathogen (Table 4.57). The highest concentration of Factomphos showed the maximum inhibitory effect followed by that of Rajphos and Urea. This was followed by Factomphos (2.5 per cent) and the highest concentration of MoP also showed comparatively higher inhibitory effect.

4.13 SENSITIVITY OF PROMISING RHIZOBACTERIAL ISOLATES AND *R.solanacearum* TO STREPTOCYCLINE

Data on the *in vitro* sensitivity of *R. solanacearum* and PGPR isolates to the commonly used antibiotic, Streptocycline against bacterial wilt disease are presented in Table 4.58. The different concentrations of Stretocycline restricted the growth of the pathogen, exhibiting an inhibition zone (IZ) of above 25 mm with the maximum at 400 ppm concentration. However, all the rhizobacterial isolates were partially compatible as they expressed an IZ of less than 15 mm.

4.14 MUTUAL COMPATIBILITY OF PROMISING RHIZOBACTERIA AND WITH REFERENCE CULTURES OF *Trichoderma* spp.

4.14.1 Mutual compatibility of PGPR

To assess the mutual compatibility of the rhizobacterial isolates, the organisms were streaked perpendicular to each other as described in 3.14.1. It was observed that all the isolates were compatible with each other as none of the isolate showed a lysis at the juncture between the antagonists.

4.14.2 Compatibility of PGPR with *T. viride* and *T. harzianum*

The results of the compatibility studies with efficient promising PGPR isolates were tested individually with reference cultures of *Trichoderma viride* and

Table 4.57. *In vitro* sensitivity of *R.solanacearum* to fertilizers

Sl.No.	Fertilizer	Concentration (%)	*Inhibition zone (mm)
1.	Urea	1.0	12.00 ^{ef}
		1.5	14.67 ^{d}
		2.0	20.33 ^{bc}
2.	Factomphos	2.0	14.67 ^{d}
		2.5	19.00 ^{c}
		3.0	23.67 ^{a}
3.	MoP	2.0	11.33 ^{f}
		2.5	13.67 ^{de}
		3.0	18.67 ^{c}
4.	Rajphos	2.0	11.33 ^{f}
		2.5	14.83 ^{d}
		3.0	21.00 ^{b}
5.	DAP	2.0	5.67 ^{g}
		2.5	11.33 ^{f}
		3.0	13.67 ^{de}

*Mean of three replications. In each column figures followed by same letters donot differ significantly according to DMRT. Figures in paranthesis are $\sqrt{x+0.5}$ transformed values

MoP: Muriate of potash, DAP: diammonium phosphate

Table 4.58. *In vitro* sensitivity of streptocycline to the promising rhizobacterial isolates and *R. solanacearum*

Sl.No.	Isolate	Inhibition zone (mm) Concentration ppm		
		200	300	400
1.	RB-144	8.00	9.33	11.33
2.	RB-22	7.33	8.66	9.33
3.	RB-82	10.33	11.30	14.66
4.	RB-66	10.33	10.66	11.30
5.	RB-11	11.00	11.30	12.66
6.	P.f1	12.33	13.30	14.00
7.	RB-77	9.66	11.30	11.33
8.	P.f2	6.66	7.66	9.33
9.	*R .solanacearum*	21.66	28.0	33.33

* Mean of three replications. *P.f-Pseudomonas fluorescens*

Trichoderma harzianum as depicted in Table 4.59. Apart from RB-22, RB-66 and the P.f2, the remaining isolates were compatible with both the species of *Trichoderma* as they hardly inhibit each other in dual culture. It was also noticed that RB-22, RB-66 and P.f2 produced a zone of inhibition with both *T. viride* and *T. harzianum*. It was observed that a per cent inhibition (PI) of 34.59 was observed with RB-22 and *T. harzianum* and 36.0 per cent with *T. viride* (Plate 4.19). Similarly, a per cent inhibition of 14.28 was observed with RB-66 and *T. harzianum* and 21.89 per cent with *T. viride*. This was in line with TNAU culture (T_{19}) as it showed a PI of 31.03 with *T. harzianum* and 35.71 with *T. viride* respectively.

4.15 CHARACTERIZATION OF BACTERIAL ANTAGONISTS

The cultural, morphological and biochemical characters of the promising rhizobacterial isolates were studied along with the reference culture of *P. fluorescens* of KAU and TNAU and the results are presented in Table 4.60.

All the six rhizobacterial isolates including the P.f1 and P.f2 were Gram – ve short rods with round ends. The colonies of isolate RB-144 were spreading, transparent, flat, creamy with undulate margin, whereas those of RB-22 were rough, slimy, translucent, raised, greenish with undulate margin. RB-82 and RB-66 and P.f2 produced rough, translucent, slimy, raised, greenish yellow colonies with entire margin. RB-11 and P.f1 produced large, smooth, shining, translucent, fluidal, convex, yellowish green colonies with entire margin. Likewise, RB-77 produced the same characteristic features as that of RB-11 and P.f1, except the colonies are opaque and deep yellow in colour. Except RB-144 and RB-77, all the isolates produced water soluble pigments which showed fluorescence under U.V. RB-22 alone produced pyocyanin in King's A medium as evidenced by the blue black pigmentation (Plate 4.20).

All the isolates showed positive to catalase, oxidase and arginine dihydrolase tests and utilized citrate as carbon source. Apart from RB-1, all of them reduced nitrate and liquefied gelatin. Hydrogen sulphide was produced only by P.f1

Table 4.59. Mutual compatibility of promising rhizobacterial isolates with *T. viride* and *T.harzianum*

Sl.No.	Isolate	*T. harzianum*		*T. viride*	
		*CP / ICP	Per cent inhibition	*CP / ICP	Per cent inhibition
1.	RB-144	CP	-	CP	-
2.	RB-22	ICP	34.59	ICP	
3.	RB-82	CP	-	CP	36.0
4.	RB-66	ICP	14.28	ICP	
5.	RB-11	CP	-	CP	21.89
6.	RB-77	CP	-	CP	-
7.	P.f1	CP	-	ICP	
8.	P.f2	ICP	31.03	CP	35.71

*CP- Compatible, ICP- Incompatible, *P.f-Pseudomonas fluorescens*

Table 4.60. Cultural, morphological and biochemical characters of promising rhizobacterial isolates

Sl.No.	Cultural / morphological / biochemical test	RB-144	RB-22	RB-82	RB-66	RB-11	P.f1	RB-77	P.f2
1.	Grams Staining	-ve	-ve	-ve	-ve	-ve	-ve	-ve	
2.	Configuration	rod	rod	rod	rod	rod	rod	rod	rod
3.	Surface	spreading	large	large	large	large	large	large	-ve
4.	Sheen	dull	rough & shinning	rough & shinning	rough & shinning	smooth & shinning	smooth & shinning	Smooth & shinning	rough & shinning
5.	Fluidal / slimy	-	slimy	slimy	slimy	fluidal	fluidal	fluidal	slimy
6.	Elevation	flat	raised	raised	raised	convex	convex	convex	raised
7.	Margin	undulate	undulate	entire	entire	entire	entire	entire	entire
8.	Density	transparent	translucent	translucent	translucent	translucent	translucent	opaque	translucent
9.	Colour	cream	green	greenish yellow	greenish yellow	yellowish green	yellowish green	deep yellow	greenish yellow
10.	Pigments	-	WS	WS	WS	WS	WS	NWS	WS
11.	Fluorescence (UV)	-	+	+	+	+	+	-	+
12.	Pyocyanin production	-	+	-	-	-	-	-	-
13.	Oxidase test	+	+	+	+	+	+	+	+
14.	Catalase	+	+	+	+	+	+	+	+
15.	Arginine dihydrolysis	+	+	+	+	+	+	d	+
16.	Lipase	+	+	+	+	-	-	-	+
17.	Levan	-	-	-	-	-	-	-	-
18.	Lecithinase	-	-	+	+	+	+	-	+
19.	Gelatin liquefaction	-	+	+	+	+	+	d	+
20.	Starch hydrolysis	+	-	-	-	-	-	d	-
21.	Citrate utilization	+	+	+	+	+	+	+	+
22.	Lysine decarboxylase	-	d	+	+	-	-	-	d
23.	Ornithine decarboxylase	-	d	d	+	+	-	-	+
24.	Urease	-	-	d	d	+	+	-	-
25.	Phenylalanine deamination	-	-	-	-	-	-	-	-
26.	Nitrate reduction	-	+	+	+	+	+	+	+
27.	H_2S production	-	-	d	-	-	+	d	-

Table 4.60. Cultural, morphological and biochemical characters of promising rhizobacterial isolates (contd….)

Sl.No.	Cultural/ morphological/ biochemical test	RB-144	RB-22	RB-82	RB-66	RB-11	P.f1	RB-77	P.f2
	Utilization of sugars								
28.	Glucose	-	+	+	+	+	+	-	+
29.	Dextrose	+	+	+	+	+	+	-	+
30.	Sorbitol	-	-	-	-	-	-	-	-
31.	Dulcitol	-	-	-	-	-	-	-	-
32.	Inositol	-	-	-	-	-	-	-	-
33.	Mannose	-	+	+	+	+	+	-	+
34.	Maltose	-	-	-	-	-	-	+	-
35.	Mannitol	-	+	+	+	+	+	-	+
36.	Fructose	+	+	+	+	+	+	-	+
37.	Sucrose	-	-	-	-	-	-	-	-
38.	Lactose	-	-	-	-	-	-	-	-
39.	Cellobiose	-	-	-	-	-	-	-	-
40.	Adonitol	-	-	-	-	-	-	-	-
41.	Arabinose	-	-	-	-	-	-	-	-
42.	Glycerol	-	-	-	-	-	-	-	-

\+ - positive reaction
\- - negative reaction
d - doubtful / weak reaction
WS - water soluble
NWS - non-water soluble

Plate 4.19 Incompatibility of PGPR isolates with *T. viride*
A. RB-22 B. P/2 C. RB-66

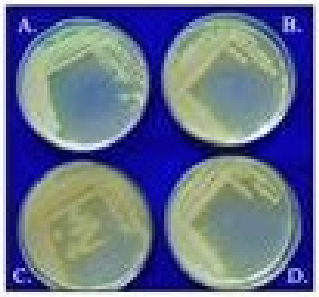


Plate 4.20 Identified cultures of PGPR isolates
A. *P. aeruginosa* (RB-22) B. *P. fluorescens* (RB-82) C. *P. fluorescens* (RB-66)
D. *P. fluorescens* (RB-11) E. Pyocyanin production by RB-22

isolate. RB-144 alone hydrolyzed starch. Except isolate RB-11, RB-77 and P.f1, the remaining ones showed lipase activity. Urease activity was positive only for RB-11 and P.f1, while positive lysine decarboxylase activity was recorded for RB-66 and RB-82 only. Only isolates, RB-82 and RB-11 were positive for ornithine decarboxylase, while RB-82, RB-66, RB-11, P.f1 and P.f2 expressed lecithinase activity. All isolates were incapable to produce levan from sucrose besides showing negative reaction for phenylalanine deamination.

Of the 15 carbon compounds tested, RB-22, RB-82, RB-66, RB-11, P.f1 and P.f2 utilized acid in glucose, dextrose, mannose, mannitol and fructose but was unable to utilize sorbitol, dulcitol, inositol, maltose, sucrose, lactose, cellobiose, adonitol, arabinose and glycerol. RB-144 was found positive for dextrose and fructose wheras RB-77 was positive only for maltose which has an affinity towards *P. pseudoalcaligenes*, however it warrants further invetsigation.

Based on the above cultural, morphological, and biochemical characters, isolate RB-22 was tentatively identified as ***Pseudomonas aeruginosa***; RB-82, RB-66 and RB-11 as ***Pseudomonas fluorescens*** biovar III (Plate 4.20) whereas RB-144 and RB-77 as could be barely identified by their genus and hence it is designated as a **'non-fluorescent Pseudomonad'**.

Discussion

5. DISCUSSION

Ginger, one among the major highly valued spice crops grown in the country, is renowned for its aroma, flavour and pungency. Though, Kerala contributes the largest share in total ginger production, bacterial wilt, incited by the ubiquitous soil borne pathogen, *Ralstonia solanacearum*, causes severe crop losses thus affecting the economic well being of the cultivators. The warm humid tropical climate of Kerala is conducive for the development of this wilt disease. Further, the management of the disease often did not give the desired effect owing to the nature of the pathogen, its survival and spread through soil and seed materials. None of the varieties of ginger are known to possess any resistant reaction against the disease. Even with this intricacy, the losses due to the disease can be restricted to a limited extent by the use of disease free planting material as well as by the application of antibiotics and copper based fungicides.

Nowadays, more emphasis is being given for the biological management of plant disease to alleviate the harmful effects of chemical pesticides in the ecosystem. Hence, efforts are being given for understanding of rhizosphere microbial population and how these could be utilized for sustainable agriculture (Lucy *et al*., 2004). Accordingly, a group of beneficial bacteria popularly termed as 'plant growth promoting rhizobacteria' (PGPR) are considered as potential agent for biological management of plant pathogens without causing any harm to Mother Nature. They are known to bring disease suppression by various modes of action in addition to induced systemic resistance (Wei *et al.,* 1996; Kumari and Srivastava, 1999; Zehnder *et al*., 2001; Mayak *et al*., 2004). Induced systemic resistance (ISR) is considered as an attractive alternative form of plant disease management, as it is based on the activation of extant resistance mechanisms in the plant and is effective against a broad spectrum of plant pathogens (van Loon *et al.,* 1998). Hence, it is pertinent to have detailed investigation of the use of PGPR as a possible biocontrol agent against this wilt disease of ginger. The purpose of this research is mainly three fold: (i) to explore the plant growth promoting rhizobacteria against bacterial wilt in ginger (ii) to study the role of antagonistic PGPR in bacterial wilt management and (iii) to

understand the mechanism of induction of systemic resistance in ginger due to PGPR.

5.1 ISOLATION AND CHARACTERISATION OF THE PATHOGEN

Beginning in 2003, the investigation was initiated with the isolation of the pathogen, *R. solanacearum* from infected ginger. The bacterium was isolated in TZC medium (Kelman, 1954) which yielded creamy white, pink centered virulent colonies and its pathogenicity established. Results of various cultural, morphological and biochemical characters of the pathogen studied as depicted in the previous chapter were in conformity with those reported by various workers (Kelman, 1954; Samuel, 1980; Jyothi, 1992; Prior and Steva, 1994; Mathew, 2001 and OEPP / EPPO, 2004). Hence, based on the characteristics studied and coupled with pathogenicity, the pathogen causing bacterial wilt of ginger was identified as *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* (Yabuuchi *et al.,* 1995). As the bacterium utilized all the sugars and alcohols *viz.,* maltose, lactose, cellobiose, mannitol, sorbitol and dulcitol, the pathogen was categorized as Biovar III which is in line with those reported by Rani (1994), OEPP / EPPO, (2004) and Kumar and Sarma (2004).

5.2 ENUMERATION OF RHIZOSPHERE MICROFLORA OF GINGER AND ISOLATION OF RHIZOBACTERIA

According to Cook and Baker (1983a) the starting point for biological control is often the isolation of potential antagonists from root environment where disease is lacking such as suppressive soil or from healthy plants in diseased fields. Further, selection and identification of growth promoting and disease suppressive biologically efficient microbes through *in vitro* and *in vivo* assays are crucial and is the first step towards the development of effective biological control before launching field evaluation (Weller, 1988). Hence, in the present study, attempts were made to select antagonistic bacteria prevalent in the ginger rhizosphere of different ginger growing areas of Wyanad, Thrissur and Palakkad districts. For this, a total of

24 soil samples were collected from the rhizosphere of healthy plants from diseased fields apart from the collection of rhizosphere soils from members belonging to Zingiberaceae family from Silent Valley biosphere and the quantitative estimation was carried out. Results of the study revealed more abundance of bacteria, fungi and actinomycetes in that order in different ginger fields in addition to the variation in their population dynamics. Jeyarajan *et al*. (1994) observed more abundance of bacteria in suppressive soils than that of conducive ones. Jubina and Girija (1998), while studying the microflora of rhizosphere soil of black pepper, also observed the abundance of soil bacteria. It is interesting to note that the microbial population was high in Silent Valley and Wyanad areas than those of other locations as these areas are known for unique treasure of biodiversity. Moreover, isolation of rhizobacteria in different media also helped in selecting appropriate colonies from a large population of bacteria.

5.3 PRELIMINARY SCREENING OF RHIZOBACTERIA AGAINST THE PATHOGEN

Since the present study is mainly intended to utilize the potential of antagonistic rhizobacteria, representative colonies of bacteria isolated from different locations and media were selected mainly based on colony characters. They were tested for their antagonistic action against the bacterial wilt pathogen. Out of the 163 bacteria screened, 45 were found to possess antagonistic property towards the pathogen. These 45 isolates were further short listed based on the extent of inhibition exerted by them on the pathogen, in comparison with that of reference cultures of *Pseudomonas fluorescens* from KAU (P.f1) and TNAU (P.f2) as well as an isolate of *Bacillus subtilis*. From among the 45 isolates, 20 isolates showed a zone of inhibition of above 15mm and hence they were selected. But *B.subtilis* did not show much inhibitory effect. Comparative studies with P.f1 and P.f2 also revealed prominent antagonistic action producing zone of inhibition of above 15mm. Such inhibitory property of rhizobacterial isolates especially those belonging to fluorescent pseudomonads towards *R. solanacearum* were reported by Opina and Valdez (1987), Ciampi-Panno *et al.* (1989); Piexoto *et al.* (1995); Furuya *et al.* (1997) and Akbar

(2002). However, Das *et al.* (2000) noticed less efficacy of *B. subtilis* than fluorescent pseudomonads against *R. solanacearum* of tomato. Further, it is also to be noted that that among the 20 rhizobacterial isolates which showed a prominent antagonistic action, eight were from Silent valley and six from Wyanad, places well known respectively for greater biodiversity and ginger cultivation, emphasizing the need for searching for antagonistic microbes from these places.

5.4 *In vivo* EVALUATION OF SELECTED RHIZOBACTERIAL ISOLATES FOR GROWTH PROMOTION IN GINGER

The selected 20 rhizobacteria were further tested in pot culture to assess their efficacy in promoting growth of ginger along with the reference cultures of *P. fluorescens* and *B. subtilis*. The experiment was laid out as delineated in the Materials and Methods. The rhizobacterial strains were inoculated by seed bacterization and also applied as foliar sprays and soil drench at 60 and 90 DAP. Ownley and Windham (2003) as well suggested seed bacterization as one of the most successful methods of introducing biocontrol agent into an agricultural system whereby the antagonists are delivered as close to the target as possible which will protect the planting material / seed from infection by soil borne pathogen. Further more, foliar sprays of antagonists and incorporation into soil are also common approach to biological control against target pathogen. Effectiveness of seed treatment and soil drenching of the rhizobacterial isolates were observed by many workers (Karuna *et al.,* 1997 and Akbar, 2002).

According to Kloepper *et al.* (1988) and Glick (1995), rhizosphere bacteria promote plant growth and yield either directly or indirectly. The indirect promotion of plant growth occurs when PGPR prevent the deleterious effects of plant pathogens on plants by production of inhibitory substances or by increasing the natural resistance of the host plants (Cartieaux *et al.,* 2003). Direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of uptake of nutrients from the environment which may include providing plants with fixed nitrogen, phytohormones, iron chelation by siderophore production,

soluble phosphate or the enzyme ACC deaminase that can lower the plant ethylene levels (Glick *et al*., 1999).

After planting of ginger rhizomes, observations on germination per cent and other biometric characters were recorded so as to find out the effect of various rhizobacterial treatments on growth promotion. Observations on germination percentage revealed that except for T_{10} (RB-17), T_{12} (RB-4), T_{14} (RB-33), T_{13} (RB-64) and T_{17} (*B.subtilis*), all the treatments were superior in increasing the germination per cent compared to absolute control (T_{25}) and the treatment (T_{24}) as per PoP. Though, all the selected rhizobacterial cultures showed inhibitory effect against the pathogen, under *in vitro* condition, some of them did show only a satisfactory effect in promoting the growth compared to control. But certain other treatments *viz.,* T_1 (RB-144), T_2 (RB-22), T_3 (RB-82), T_4 (RB-66), T_5 (RB-11), T_7 (RB-151), T_{16} (RB-77), T_{21} (RB-69), T_{22} (RB-71), T_{15} (P.f1) and T_{19} (P.f2) showed a profound effect in increasing the biometric characters especially with respect to the number and height of tillers. The yield of ginger rhizomes was also more than the control.

Although there are only few reports on the effectiveness of biocontrol agents in promoting growth of ginger, there exists a large body of literature describing the potential use of plant growth promoting rhizobacteria in other crops as agents for stimulating plant growth and managing soil and plant health. Rajan *et al.* (2002) noticed various endophytic bacterial strains isolated from different ginger tracts by enhanced tillering and overall growth of plants. van Peer and Schippers (1988) documented the increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Strains of fluorescent *Pseudomonas* antagonistic to *R. solanacearum*, isolated from rhizosphere of healthy egg plant increased plant height, total weight and root weight (Yungchun *et al*., 1997). According to Kumar (2002), Kumar *et al.* (2002) and Thomas and Vijayan (2003), seed coating with *P. fluorescens* resulted in increased seed germination and enhanced growth and vigour of various plants. Paul (2004) reported that the rhizobacterial strains significantly enhanced growth of black pepper.

5.5 SELECTION OF POTENTIAL RHIZOBACTERIAL ISOLATES

As already pointed out, direct mechanisms of plant growth promotion may involve the synthesis of substances by the bacterium or facilitation of uptake of nutrients from the environment, hence, from the preliminary screening in the pot culture experiment, 11 potential rhizobacterial isolates including the reference cultures which showed promising effect in increasing growth and yield of ginger were subjected to various analysis for studying the attributes which contribute to the growth promoting effect and a PGPR index was finally calculated for each isolate. It is also necessary to prove the antibacterial mechanism of each isolate because the production and especially the quantity of secondary metabolites are often specific for each isolate. Consequently, they were tested for the production of antibacterial antibiotics, growth regulators, hydrogen cyanide, ammonia, salicylic acid and also their effect on phosphorous solubilization in comparison with the reference strains of *P. fluorescens*. For calculation of PGPR index, these parameters were taken into consideration. Since the reference strain of *B. subtilis* did not show much effect on growth promotion as depicted in the above experiment, it was not included for further studies.

5.5.1 Antagonistic index (AI) of potential rhizobacterial isolates

As a first step to arrive at the PGPR index, the antagonistic index of the potential rhizobacterial isolates was calculated based on the zone of inhibition on the pathogen. Out of the 11 isolates including the two reference cultures of *P. fluorescens*, six isolates showed the maximum antagonistic index (AI) of above 500 with the maximum in P.f2 followed by RB-144 and RB-11. The least AI was noticed with RB-69 and RB-71. Similar results were reported for other pathosystems by various workers (Piexoto *et al.,* 1995; JianHua *et al.,* 1996; Silveira *et al.,* 1996; Yungchun *et al.,* 1997; Manimala, 2003) where they observed variation in the antagonistic reaction of different *Pseudomonas* spp. against *R. solanacearum.* According to Raupach and Kloepper (1998), the lytic activity by the rhizobacterial

antagonists against the pathogen is mainly due to their production of lytic enzymes or by inhibitory metabolites.

5.5.2 Vigour index

The 11 rhizobacteria including the reference cultures were further bioassayed for their ability to promote or inhibit the growth of sorghum seeds. In general, the seeds bacterized with rhizobacteria improved the vigour index. Among the isolates, RB-22 was the most effective growth activator followed by RB-151, P.f1, RB-144 and RB-71 thereby, indicating that these rhizobacteria had potentiality to enhance the growth of the seedlings. Such growth promoting effects of rhizospheric bacteria in various crops have been well documented (Kloepper and Schroth, 1978; Bhatia *et al.,* 2005; Dhoke and Kurundkar, 2005).

5.5.3 Production of hydrogen cyanide

With regard to the production of HCN, it was seen that all the rhizobacterial isolates tested were non-cyanogenic in nature when tested with King's B media. Bano and Mussarat (2003a) noticed low HCN production under iron limiting conditions. Lack of iron supplement in the media used in this study for HCN production may be the reason for failure in detection of HCN by the isolates. Such inability to produce HCN by rhizobacterial isolates have been noticed by other workers also (YongHoon *et al*., 2001 and Samanta and Dutta, 2004).

5.5.4 Production of ammonia

The ability of the rhizobacterial isolates for the production of ammonia, a volatile compound having direct bearing on biocontrol activity were tested and it was found that all the isolates produced ammonia. However, RB-151 and P.f2 produced less amount of ammonia while the others recorded higher production. Production of ammonia by rhizobacteria from mustard has been documented by Samanta and Dutta (2004) and concluded that ammonia production has a role in suppressing

S. sclerotiarum. Further, Ryu *et al*. (2003) opined that volatiles produced by PGPR strains trigger growth promotion and ISR in *Arabidopsis thaliana.*

5.5.5 Phosphorous (P) solubilization

It is well established that one of the important criteria for an efficient PGPR is their ability to transform unavailable 'P' to the available form. Thus, in the present study, the 'P' solubilizing capacity of 11 rhizobacterial isolates was assessed. Among the isolates, eight recorded 'P' solubilization in Pikovskya's solid media while the rest in liquid medium only. The maximum 'P' solubilization was with RB-144, RB-82 and P.f2. Such capacity of rhizobacterial isolates in solubilizing 'P' were documented by many workers (Katiyar and Goel, 2003; Dey *et al.,* 2004). Further, conferring resistance of plants to stress conditions by mobilizing 'P' for plant growth were also reported.

5.5.6 Assay of growth promoting hormones

It is very well established that many rhizobacteria including fluorescent pseudomonads produce plant growth promoting substances like gibberellins, cytokinins and IAA which can directly or indirectly modulate plant growth and development. Growth regulators like auxins and gibberellins are known to be produced by rhizobacteria in many phytosysytems which has a direct effect in growth and development of plants (Patten and Glick, 1996). Hence, in this study, the capacity of 11 rhizobacterial isolates in IAA production was assessed. All the isolates produced varying levels of IAA ranging from 9.02 to 56.89 μgml^{-1}. The maximum quantity was produced by RB-22 followed by RB-151 whereas RB-69 and RB-11 produced least quantity of IAA. After quantifying the IAA production by spectral analysis, further TLC analysis of growth regulators produced by the 11 rhizobacterial isolates were carried out. Chromatographic separation of the metabolites revealed that isolates RB-144, RB-11 and P.f2 produced four metabolites. Of the four metabolites, two spots were noticed with RB-144 and RB-11 which are suggestive of gibberellins and auxins. The red and the yellowish green colour suggests the

production of IAA and Gibberellins which were noticed with two isolates RB-144 and RB-11 while blue / violet coloured bands were developed with RB-144, RB-22, RB-82, RB-11 RB-151, P.f1 and P.f2 under U.V. which indicated production of derivatives of IAA with concomitant Rf values.

The role of plant growth regulators has been well demonstrated by many researchers throughout the globe in many different agricultural crops with different genera of beneficial bacteria (Suslow, 1982; Patten and Glick, 2002; Khalid *et al.,* 2004; Ebstam *et al.,* 2005). Production of IAA by different PGPR strains was quantified by many workers (Glick, 1995; Rubio *et al*., 2000; Bano and Mussarat, 2003a; Bhatia *et al.*, 2005). They also noticed variation among the rhizobacterial strains in the production of IAA.

5.5.7 Determination of PGPR index

Thus, based on the data on vigour index, 'P' solubilization, IAA, NH_3 and HCN production, the PGPR index of the 11 rhizobacterial isolates used in this experiment was determined, since PGPR index is a measure to detect the potentiality of the aforesaid bacteria. The rhizobacterial isolates showed different PGPR index (PGPI), among which, RB-22 recorded a higher PGPR index of 83.33 followed by RB-144, RB-11 and RB-82. The lowest index was with RB-151 (50). Similar line of work has been carried out by Samanta and Dutta (2004) and they observed differences in PGPI among the PGPR isolates tested. They concluded that among the PGPR parameters, 'P' solubilization and ammonia production had significant effect in attaining maximum PGPR index. Thus, the 11 isolates selected from the previous pot culture experiment possessed various mechanisms / attributes which might have played a role in growth promotion as well as their antagonistic activity against the pathogen. According to Chet (1990 and 1993), production of various compounds of microbial origin is involved in growth promotion as well suppression of pathogen.

5.6 ASSAY OF SECONDARY METABOLITES

After assessing the various parameters which are known to contribute to plant growth, an effort was carried out to detect the production of secondary metabolites like salicylic acid, antibiotics and siderophores by the rhizobacterial isolates as these secondary metabolites play a significant role in imparting resistance and disease suppression.

5.6.1 Spectroscopic and TLC analysis of salicylic acid

Quantitative estimation of the assay from cell free culture filtrates (CFCF) of the isolates revealed production of SA in varying quantities. The reference culture P.f2 produced the maximum quantity and the rest produced the same within a range of 9.42 to 75.68 μgml^{-1}. After the quantitative estimation, an attempt wasmade to separate SA and other related metabolites present in the CFCF by TLC. This chromatography study indicated the presence of SA as brown spot of Rf value 1.0 which was evident with six rhizobacterial isolates. It is interesting to note that in the TLC plate with RB-22, RB-66 and P.f2, two more spots were also visualized with yellow colour having different Rf values indicating the production of certain other metabolites in addition to SA by these isolates.

Salicylic acid is known to play a central role as a signaling molecule in plant defense against microbial attack (Reimann *et al.,* 2003). According to DeMayer and Hofte (1997), bacterially produced SA contributes to the induction of systemic resistance. However, van Loon *et al.* (1998) reported that the increase in SA in the bacterized plants was the result of induction by the bacteria in inducing SA synthesis in the plant or it is not clear that whether the plant takes up bacterially produced SA and translocate it to the leaves. Salicylic acid is a metabolite important in pathogen induced SAR and can induce systemic resistance to pathogens after root and soil treatment. Abeysinghe (2003) observed *P. fluorescens* strains WE16 and WE32 produced equal amount of SA *in vitro* unlike *Bacillus* strains and *P. putida* WE10 which didnot produce any detectable amount of SA, indicating that SA may

not play as a primary bacterial determinant for inducing systemic resistance. However, several workers reported the role of SA in induction of systemic resistance in various crops by different rhizobacterial isolates (DeMayer and Hofte, 1997; DeMeyer *et al.,* 1999; Audenaert *et al.,* 2002).

5.6.2 TLC profile of antibiotics

It is well established that antibiosis plays an active role in the biocontrol of plant disease and often acts in concert with competition and parasitism. According to Ownley and Windham (2003) antagonists produce effective growth inhibitory substances that are active against a wide group of micro organisms and such compounds are referred to as antibiotics with broad spectrum activity. Hence, an effort was made to find out whether the antagonistic rhizobacteria used in the present investigation were able to produce any antibiotic. For this, the culture supernatants of various isolates were subjected to TLC analysis. In general, most of the selected isolates produced antibiotics comparable with the Rf value of that of pyrrolnitrin, pyoluteorin, 2,4-diacetyl phloroglucinol (2, 4 DAPG) and pyocyanin. However, the isolates RB-151, RB-69 and RB-71 didnot produce any detectable antibiotic on the chromatogram. A blue coloured spot developed on the chromatogram of the isolates RB-144, RB-22, RB-82, RB-77 and P.f2 was identified as pyoluteorin while, the fluorescent one produced by RB-22, RB-66, RB-11 and P.f1 was recorded as pyrrolnitrin. The isolate RB-22 also produced another blue spot with different Rf value comparable with that of pyocyanin. RB-22 and RB-144 also produced two other unidentified metabolites. It is also interesting to note that the isolates RB-11, RB-66 and P.f2 showed a crimson yellow metabolite which was tentatively recorded as 2, 4-DAPG.

Here, it may be noted that detection of antibiotics in the chromatogram was made based on the Rf values of metabolites recorded and reported in the case of antagonistic fluorescent pseudomonads and other rhizobacterial strains reported by Gurusiddaiah *et al.* (1986) and Duffy and Defago 1997). Hence, further confirmation need to be carried out in comparison with the standard antibiotics. However, this

study indicated that the majority of the rhizobacterial isolates were able to produce many antibiotics which are capable of suppressing pathogenic organisms. Apart from these, isolates RB-144, RB-22, RB-82, RB-66, RB-11 and P.f2 are seen to produce certain other metabolites which did not match to the standard Rf values. It is to be noted that three isolates RB-66, RB-11 and P.f2 also produced 2, 4 DAPG, a phenolic metabolite known for its antibacterial activity (Rajendran *et al.,* 1998; Iavicoli *et al.*, 2003; Ahmadzadeh *et al*., 2004). Production of this compound in the suppression of bacterial leaf blight (BLB) of rice was documented (Velusamy *et al.*, (2003).

5.6.3 Detection of siderophores

Iron is a growth-limiting factor for the majority of organisms. In many cases unavailability of iron results in deleterious effects in the growth of organisms. In order to overcome this intricacy, bacteria have evolved mechanisms which under iron-limiting conditions selectively chelates iron for their own purpose and make it unavailable to others. This is true with the antagonistic bacteria which produce a metabolite, siderophore the production of which is correlated with antagonistic potential. Hence, the potential bacterial antagonists selected in this study were tested for their capacity to produce siderophores. The isolates *viz*., RB-22, RB-82, RB-66, RB-11 and the two reference cultures produced siderophores as evidenced either by their fluorescence or yellow halo zone. However, RB-144 and RB-77 produced only a small yellow zone indicating the production of low affinity siderophores and the remaining ones RB-64, RB-71 and RB-151 did not produce any siderophores. This implies their low competitiveness with the iron uptake mechanisms and may explain the weak antibiosis of these isolates. Similar findings of siderophore production by CAS assay were reported by Reddy *et al.* (2004) and Storey (2005).

The study also revealed that the capacity of the bacterial isolates to produce siderophores reduced as the concentration of $FeCl_3$ increased. Further, there was variation in the production of siderophores by the different isolates as evidenced by spectronic assay. Without the addition of $FeCl_3$, the maximum siderophores was

produced by RB-22, followed by RB-66. The least production of siderophores was by the culture RB-77, RB-144, RB-71, RB-69 and RB-151. When the concentration of $FeCl_3$ was increased, there was a concomitant reduction in the siderophore production. The iron dependent production of siderophores by these isolates was found to be in agreement with the findings of Kloepper *et al.* (1980b), Boruah and Kumar (2002), Villegas *et al.* (2002), Bano and Mussarat (2003a) and Storey (2005). They opined that in addition to production of siderophores, other mechanisms may also be involved in the suppression of the pathogens which needed critical study.

5.7 POPULATION DYNAMICS OF THE POTENTIAL ANTAGONISTS

For an efficient successful biocontrol agent, the primary criteria is its ability to establish and maintain the population under field conditions for a long period of time. Accordingly, in the present study, the 11 potential rhizobacterial isolates were tested for its ability to sustain their population in the rhizosphere under *in vivo* conditions for a period of three months. The population dynamics of the isolates were tested at monthly intervals. In general, it was found that as the time of sampling increased, there was a reduction in the population. However, it is pertinent to note that the population after two months was at a satisfactory level indicating that these rhizobacterial isolates are good colonizers which is in line with Bhatia *et al.* (2005) as they reported that as a result of aggressive root colonization, population of fluorescent *Pseudomonas* strains increased in rhizosphere upto 60 DAS but slightly decreased thereafter. All these findings clearly indicate successful establishment of the introduced bacteria in the rhizosphere. Evidence of rhizosphere microflora which serves as a defensive barrier for roots against the soil borne phytopathogens have been established by Weller (1988) Wei *et al.* (1996) and Nautiyal (1997).

5.8 *In vivo* EVALUATION OF POTENTIAL RHIZOBACTERIA FOR GROWTH PROMOTION AND DISEASE SUPPRESSION

After assessing the factors contributing to growth promotion of ginger and inhibiting activity against the pathogen by the 11 potential rhizobacterial

isolates, another pot culture experiment was carried out to assess the capacity of these isolates in inducing resistance reaction in ginger in terms of changes in phenol, proteins and amino acids on inoculation with the pathogen. Further, the effect of these rhizobacteria on the yield and yield attributing characters of ginger was studied. The response of the rhizobacterial treated plants on the incidence of bacterial wilt was also recorded after challenge inoculation.

5.8.1 Early sprouting, germination per cent and biometric characters of ginger plants

It was found that the rhizobacterial treatments had a positive effect in enhancing the sprouting of rhizomes, germination percentage and in reducing pre emergence rot compared to untreated plant. Several other studies also revealed the efficacy of rhizobacteria in augmenting plant growth and development and subsequent translation into higher yields. (Kloepper *et al.,* 1988; Polyanskaya *et al*., 2000; Bhatia *et al*., 2005). According to Kloepper and Schroth (1978), root colonization of introduced rhizobacteria had an effect in increasing the rate of plant growth. The capacity of the 11 rhizobacterial isolates to produce growth promoting substances and making available insoluble 'P' was established in the earlier *in vitro* studies in addition to the production of metabolites inhibitory to the growth of pathogens. These factors may be responsible for the better growth and development of the rhizobacterial treated plants as well as effect on the biotic stresses.

With regard to number of tillers, it is seen that, in general, the rhizobacterial treatments had a profound influence in increasing the number of tillers especially during the later stages of growth with the maximum being in plants treated with isolate T_5 (RB-11) followed by P.f2. The rhizobacterial treatments generally influenced production of more leaves compared to control. Similar to the observations on the number of leaves, there was variation among the treatments on the height of plants as well. Nevertheless, it is generally noticed that incorporation of bacterial isolates exerted some effect in increasing the height of plants. All these

results are in agreement with those reported by Yungchun *et al*. (1997), Thomas and Vijayan (2003) and Dhoke and Kurundkar, (2005).

5.8.2 Natural incidence of bacterial wilt, rhizome rot and pest

Diseases of fungal, bacterial and viral origin and in some instances even damage caused by insects and nematodes can be be reduced after application of PGPR (Ramamoorthy *et al.,* 2001; Tong *et al.,* 2002; Ryu *et al.,* 2003). Reduction of losses due to biotic stress like pest and disease attack due to application of rhizobacteria like *Pseudomonas* spp. has been noticed by Bong and Skiorowski (1991) and Ramanathan *et al.* (2002).

In this experiment, the natural incidence of rhizome rot and bacterial wilt of ginger as well as shoot borer incidence were recorded and a perusal of the data indicated that bacterization of the isolates RB-82 and RB-11 which was isolated from Kalpetta area of Waynad district had an effect in reducing the incidence as these plants were completely free from the disease. The results are in agreement with the findings of Anith *et al*., (2000), Devanath *et al.* (2002) and Sivaprasad (2002). However, only plants in four treatments exhibited some incidence of rhizome rot. The results were in agreement with those reported by Ram *et al*. (1997) and Rajan *et al.* (2000) as application of *P. fluorescens* in soil inhibited rhizome rot of ginger.

5.8.3 Bacterial wilt incidence after challenge inoculation with the pathogen

In view of the fact that, the data on the above aspects are based on the natural incidence of disease, confirmatory evidence of the beneficial effect of the bacterial isolates tested in this study need to be ascertained by artificial inoculation under green house conditions. Since the present study is mainly anticipated to harness the benefit of antagonistic rhizobacteria, another effort was made to assess the efficacy of the PGPR treatment against bacterial wilt pathogen upon challenge inoculation. Results of the experiment revealed significant difference among the treatments on the incidence of wilt disease. Observation on 7^{th} DAI revealed that, all

the rhizobacterial treatments had a positive effect in reducing wilt incidence compared to control pointing to the effect of rhizobacterial treatments in reducing the incidence to some extent. It is also to be noted that the isolate RB-66 and RB-11 from Kalpetta as well as RB-22 from Silent Valley recorded less than 10 per cent wilt incidence compared to others. After 14 DAI, the minimum disease incidence of 44.12 per cent was in T_5 (RB-144) which was closely followed by T_1 (RB-144), T_4 (RB-66), T_3 (RB-82) and T_9 (P.f2) and the maximum in T_6 (RB-151). The efficacy shown by RB-11 is from ginger rhizosphere from Kalpetta area of Waynad district. Observations on earlier studies revealed that ginger growing tracts of Waynad district are known for the epiphytotic occurrence of bacterial wilt of ginger. Oflate, incidence of this disease is comparatively less owing to the emergence and establishment of natural antagonistic ones and also probably due to the integrated disease management practices adopted by the farmers. Results obviously establish that PGPR are competent enough to offer protection against bacterial wilt disease in ginger to varying degrees compared to that of control. Related findings were accounted by other workers in this line. The effectiveness of *P. fluorescens* strain EM-85 in reducing bacterial wilt of ginger was noticed by Anith *et al.* (2000). According to Sivaprasad (2002) fluorescent *Pseudomonas* isolates P-1 was not only effective against bacterial wilt of ginger but also against other wilt of solanaceous plants. Further, Devanath *et al.* (2002) noticed the efficacy of *P. fluorescens* and *B. subtilis* in suppressing the growth of bacterial wilt pathogen of ginger.

5.8.4 Yield, number of roots, fresh root weight and dry top shoot weight

The rhizobacterial inoculants had a significant effect on the yield of ginger where the maximum yield was in plants bacterized with RB-11 followed by RB-144 and RB-82 respectively. Treatments with the two reference strains also effected an increase in yield. Thus, proving again the potentiality of use of PGPR in promoting the growth and yield of the crop. It is also to be noted that the isolates predominantly from ginger growing tracts as well as those from evergreen forest ecosystems like Silent Valley are found to be efficient enough compared to others. Widespread commercial use of PGPR inoculants has been reported on many crops

where PGPR are known as yield increasing bacteria (Kloepper, 1992). Seed bacterization with *Pseudomonas* strain RRLJ 008 enhanced yield in five test crops (aubergine, *Phaseolus vulgaris,* cabbage, Kohlrabi and tomato) under field conditions in Assam (Boruah and Kumar, 2002). JianHua *et al.,* (2004) enlighted that fluorescent *Pseudomonas* (J3) reduced the percent wilt incidence caused by *R. solanacearum* in tomato and increased yield from 180-237 per cent compared to *Bacillus* sp. and *Serratia* sp.

Observations on the fresh root and dry top shoot weight of ginger and number of roots and layers of ginger were also taken which in general are indicative of efficacy of rhizobacterial treatments in the maximum value of the above parameters. Strains of fluorescent *Pseudomonas*, isolated from rhizosphere of various crops increased plant height, shoot and root weight as reported by Yungchun *et al*. (1997); van Peer and Schippers (1988); Shishido and Chanway (2000) and Bhatia *et al.* (2005).

5.8.5 Population dynamics of soil microflora

Enumeration of the total count of microflora in the potting mixture of various treatments at different intervals was taken which revealed significant fluctuation among the various treatments. Though, a slight built up of fungal propagules was noticed in certain treatments 5 MAP, the bacterial count showed a gradual decrease. However, no significant difference in the population of actinomycetes among the treatments was noticed one and five MAP. The cause of this decrease in bacterial population may be due to the constant depletion of nutrient availability in the rhizosphere especially from the plant roots as root exudates. There are several reports of the plants supporting the enhanced proliferation of rhizosphere microflora. The exudates contain mainly low molecular organic compounds such as sugars, amino acids and organic acids (Bachmannen and Kinsel, 1992). Additonally, organic substances are made available to soil microbes through constant root and root hairs.

5.8.6 Changes in total phenol after challenge inoculation with pathogen

5.8.6.1 Spectroscopic analysis of total phenol in ginger

Phenols are known to occur in all plants investigated so far. Some of them occur constitutively while others are formed as a part of an active defense response in the host (Nicholson and Hammerschmidt, 1992, Kuc, 1995). These compounds are known to enhance mechanical strength of cell wall and also inhibit the invading pathogens, thereby conferring resistance either directly or indirectly through activation of post-infection responses in the hosts (Harborne, 1988). According to Wei *et al.* (1991) and Ramamoorthy *et al.* (2002), seed bacterization with PGPR resulted in accumulation of phenolic compounds in host plants thus offering a practical way of immunizing plants against the pathogen. The present investigation was therefore taken up to assess the effect of the rhizobacterial treatments on the quantitative and qualitative changes in phenol content in ginger, challenge inoculated with the wilt pathogen.

Higher accumulation of total phenolics was noticed in ginger plants treated with rhizobacterial isolates upon challenge inoculation compared to control. In general, observations taken before inoculation showed higher phenol content in rhizobacterial treated plants. There was a constant rise in phenol content 1 DAI, the highest quantity was recorded with RB-11. Almost similar trend of increase in the content was observed in plants in other treatments also at other periods. Thus, indicating the role of rhizobacterial treatments in activating defense related compounds. Further, it may be noted that there was comparatively less incidence of bacterial wilt in rhizobacterial treated plants upon challenge inoculation. This clearly indicates the positive response of PGPR in reducing the disease by production of phenols. Higher accumulation of phenolics in other plants pretreated with *P. fluorescens* challenged with the pathogen was reported by Singh (2003), and Vivekananthan *et al.* (2004).

5.8.6.2 TLC profile of phenols

However, a mere accumulation of total phenols will not give a clear picture on the role played by individual phenolic compounds in imparting resistance. Bennett and Wallsgrove (1994) opined that several phenolics with different modes of action are associated with plant defense mechanism. Consequently, an attempt was made to elucidate the profile of phenolic compounds formed due to infection by TLC. Extracts of plant samples of all treatments taken before inoculation showed only a single spot on the chromatogram with Rf value of 0.92 to 0.93. However, it is interesting to note that due to inoculation, there was a change in number and type of phenolics with the accumulation of new ones at different intervals. Three DAI, formation of salicylic acid was noticed from the samples of all treatments which are in line with the observations of Singh *et al.* (2003). Others noticed were pyrogallol, hydroquinone, guiacol and certain unidentified compounds. More number of phenolic compounds was noticed 5 DAI with a difference in the type of phenol detected from that of 3 DAI. During this period, presence of catechol and certain unidentified compounds were detected in addition to the earlier mentioned ones. Presence of five phenols were noticed in plants treated with T_2 (RB-22), T_5 (RB-11), T_{11} (RB-71), four in T_1 (RB-144), T_2 (RB-22), T_8 (RB-77) and T_{10} (RB-69) whereas two in T_4 (RB-66), T_6 (RB-151), T_7 (P.f1) and T_{13} (Control). Thus, it is pertinent to note that production of more number of phenols in PGPR treated plants might have helped in reducing the incidence of bacterial wilt upon artificial inoculation. Though, the exact mechanism of alteration of phenolic profile in plants by PGPR is not very clear, phenols at high concentration are known to inhibit the microorganism. The presence of phenolic compounds in plants and their synthesis in response to infection have been associated with resistance (Ingham, 1972). Initial accumulation of phenolics in response to infection and a general increase in host metabolism leading to formation of relatively non-toxic secondary metabolites which ultimately serve as precursors of compounds for resistance reaction have been documented (Nicholson and Hammerschmidt, 1992). Hence, it may be presumed that application of rhizobacterial strains in ginger helps to modify the composition of phenolics upon infection through synthesis of new ones thereby exerting resistance reaction.

5.8.7 Changes in total protein after challenge inoculation with pathogen

The total protein content of ginger in different treatments was also assessed. In general, there was more protein content in rhizobacterial treated ginger plants than that of control. One and 3 DAI, there was a gradual increase in the protein content in majority of the treatments and later on, the content showed a gradual decline. Here too, 5 DAI, all the rhizobacterial treated plants showed an increase in total protein content compared to control with the maximum in plants treated with RB-11 followed by those treated with P.f1. Enhanced protein synthesis appears to be a universal phenomenon in host pathogen (resistant) interaction especially in incompatible ones. *De novo* synthesis of new proteins has also been reported by Yamamoto and Tani (1986). Such synthesized protein may not be inhibitory but may activate the production of defense-related chemicals such as phenolics, lignins, phytoalexins etc. The increase in protein content following infection may be due to the formation of newer proteins. Staples and Stahmann (1964) observed a new protein in bean leaves inoculated with *U. phaseoli* as well as increase in the number of isozyme of acid and alkaline phosphatase. Many inhibitory proteins have been detected in plants treated with PGPR strains (Vigers *et al.,* 1991 and Swegle *et al.,* 1992) which might have been involved in enhancing the disease resistance against plant pathogens.

5.8.8 Changes in total free amino acids

5.8.8.1 *Spectroscopic analysis of amino acids in ginger*

Since there was an increase in protein content in rhizobacterial treated challenge inoculated plants, efforts were made to elucidate if there was any change in the number, type and content of amino acids in ginger plants as amino acids are the building blocks of proteins, the chief structural component of plant cells and always exhibit a change during infection. On spectroscopic analysis, it was observed that in general the rhizobacterial treatments had an effect in the amino acid content of ginger. A significant increase one DAI was observed with the maximum amino acid content in T_1 (RB-144) followed by T_4 (RB-66) and T_3. Subsequently, there was a

general increase in amino acid content on three and five DAI compared to that of the previous observation. The increase in amino acid content following infection may be due to the activated synthesis of amino acids by the pathogen or due to degradation of proteins in the cell by proteolytic activity (van Andel, 1966).

5.8.8.2 ***TLC profile of amino acids***

In order to find out the various types of amino acid formed in treated plants on challenge inoculation, TLC analysis was performed and the individual amino acids were identified in comparison with the standards. Apart from T_{13} (Control), plants in all rhizobacterial treatments showed the presence of three amino acids with different Rf value. Before inoculation, presence of hydroxy proline was detected in plants of all treatments. Three DAI, the number of amino acids detected varied from three to five and the maximum number in T_1 (RB-144), T_2 (RB-22), T_3 (RB-82), T_8 (RB-77) and T_9 (P.f2). Presence of histidine, aspartic acid, lysine, glutamic acid and tyrosine were detected in treatments T_1, T_2 and T_3 whereas alanine, and phenylalanine from T_8 (RB-77) and T_{13} (Control). The minimum number of amino acids was recorded in control and T_4 (RB-66). Presence of proline was detectedd only in sample RB-11 (T_5) and also the concentration of phenylalanine was found very high in the same treatment as indicated by a dark red spot. Five DAI, there was not much change in the number of amino acids in plants of different treatments with few exceptions. Presence of phenylalanine, leucine / isoleucine was found common in all samples. During the sampling period, presence of methionine was also detected in certain treatments. Such occurrence of more number of amino acids especially those which play a role in defense reaction like PAL has been established. Tyrosine was detected in most of the rhizobacterial treatments in general, which was absent in control at all intervals of observation.

Accumulation of aromatic amino acids *viz*., phenylalanine, tyrosine and tryptophan in tobacco tissues inoculated with the pathogen *P. tabaci* was observed by Pegg and Sequira (1968) due to the enhanced aromatic biosynthesis. The brilliant work of Kuc and his co workers (Kuc *et al*., 1959) indicated role of phenyl alalnine

in imparting resistance in apple against *V. inequalis.* Sadasivan (1968) also indicated the role of aromatic metabolites phenylalanine and tyrosine in disease resistant rice varieties to *D. oryzae* / *P. oryzae*. It is also to be noted that phenylalanine and proline plays a major role in plants under stress conditions especially when subjected to pathological stress. Much work has not been done on this line and information is scanty on the induction of amino acids in rhizobacterial treated plants upon challenge inoculation with pathogen.

5.9 ASSAY OF DEFENSE RELATED ENZYMES

The utilization of plant's own defense mechanism is a fascinating arena of research recently practiced all over the world to manage plant disease. Plants have latent defense mechanism against pathogens, which can be systemically activated upon exposure of plants to PGPR strains or infection by pathogen (Baker *et al.,* 1997). This phenomenon is called induced systemic resistance (ISR) / systemic acquired resistance (SAR) (Tuzun and Kuc, 1991). The mechanism facilitated by PGPR organisms operates through the activation of multiple defense compounds at sites distant from the point of pathogen attack (Dean and Kuc, 1985). The inducers include pathogens (Hammerschimdt, 1999), PGPR (Leeman *et al.,* 1995), Ramamoorthy *et al.,* 2002), chemicals and botanicals (Singh *et al.,* 1990). In some cases, the level of induced protection can be enhanced further when both ISR and SAR are activated simultaneously (van Wees *et al.,* 1997), indicating that the effects of both types of resistance are additive. Peroxidase, lipoxygenase and phenylalanine ammonia lyase (PAL) are linked to the ISR pathway regulated by jasmontes and ethylene, which in turn is activated by rhizobacteria (van Loon *et al.,* 1998). Of late, investigations on mechanisms of biological control by plant growth promoting rhizobacteria revealed that several strains protect the plants from various pathogens in several crops by activating defense genes encoding PO, PPO, PAL, catalase and other enzymes (Chen *et al*., 2000; Thangavelu *et al.,* 2003; Bharathi *et al.,* 2004) and induction of systemic resistance is thought to confer resistance against a broad spectrum of plant pathogens (Sticher *et al*., 1997).

In this study, the effect of eight most promising rhizobacterial isolates *viz*., RB-144, RB-22, RB-82, RB-66, RB-11, RB-77, P.f1 and P.f2 in conferring resistance in ginger against the pathogen was studied by assessing the defense related enzymes and their profile. Isolates viz., RB-151, RB-69 and RB-71 were discarded based on the previous *in vitro* and pot culture experiment as they did not show a good response in comparison with that of other isolates. The protein profile of treated plants was assessed by native PAGE analysis. In addition to the growth promoting effect of the bacterial isolates, chlorophyll, oil and oleoresin and NPK contents of the rhizomes were also estimated.

5.9.1 Germination per cent, biometric observations, pest and disease incidence

As in the case of previous experiment, all the promising isolates had a profound influence in the germination percentage as well as other biometric characters studied when compared to that of control and the observations are in conformity with those discussed in 5.4. The percentage of shoot borer and rhizome rot incidence was recorded and it was observed that comparatively more incidence was in T_3 (RB-82) and T_4 at 90 DAP. However, at 120 DAP, shoot borer attack was not observed in T_1 (RB-144), T_4 (RB-66) and T_5 (RB-11) but the highest incidence was observed in T_9 (PoP). Likewise, the highest rhizome rot incidence was in T_{10} (Control) followed by T_8 (P.f2), T_9 (PoP) and T_4 (RB-66). However, no natural incidence of bacterial wilt was noticed during the study owing to the particular weather conditions prevailing during the period.

5.9.2 Induction of defense related enzymes in ginger

When pathogens invade plants, major physiological changes are induced and plant defense enzymes are generally activated. Inspite of major advances in our understanding of the defense responses in various plants, information on PGPR mediated resistance in ginger against bacterial wilt is scanty. Hence, we made an

attempt to identify the useful rhizobacterial isolates with the capacity to induce systemic resistance in ginger against bacterial wilt disease.

5.9.2.1 Spectronic and electrophoretic assay of peroxidase (PO)

Appreciable increase in peroxidase activity over time was observed in all PGPR treated ginger plants after challenge inoculation. Compared to control, plants in various rhizobacterial treatments showed a higher PO activity during the first observation. Three DAI, the per cent increase in PO activity was more in all treatments except that of T_7 and the P.f2 (T_8).The expression of PO activity was at an elevated stage at five DAI of the pathogen in plants in all treatments where the highest was noticed in T_2 (RB-22) and P.f1 (T_6). Native gel electrophoresis revealed six isoforms of PO in plants treated with rhizobacteria *viz.,* RB-144, RB-22, RB-82, RB-66, RB-11 and P.f1 after challenge inoculation and their expressions were prominent when compared to control which showed the presence of only three isoforms. Further, it was noticed that in rhizobacterial treatments more number of isoforms of peroxidase was noticed compared to control.

Peroxidase is the key enzyme in the biosynthesis of lignin (Bruce and West, 1989 and Brisson *et al.* 1994) and is also reported to be involved in disease resistance and wound healing (Gasper *et al.,* 1982). Induced systemic resistance (ISR) once expressed, activates multiple potential defense mechanisms that induce increased activity of peroxidases which showed resistance to various plant pathogens. Colonization of PGPR has been reported to induce metabolic changes in the host especially the increased production of peroxidases. The increase in PO activity is thought to be one of the key components in local and systemic disease resistance which has been elicited in different plants (Chen *et al.*, 2000) due to pathogen infection. Ramammoorthy *et al.* (2002) observed that foliar application of *P. fluorescens* induced PO activity in tomato. Groundnut plants treated with *P. fluorescens* and challenge inoculated with *A. alternata* recorded significant increase of PO isozyme (Chitra *et al.,* 2006).

5.9.2.2 Spectronic and electrophoretic assay of poly phenol oxidase (PPO)

Polyphenol oxidases (PPO) are enzymes which use molecular oxygen to catalyze the oxidation of mono phenolic and ortho diphenolic compounds to highly toxic quinones and hence it has been assigned a role of disease resistance. In the present investigation, in general, the rhizobacterial treatments had a profound influence in increasing the polyphenol oxidase (PPO) activity of ginger. Apart from plants in T_3, T_5 and T_9, the PPO activity was more in the bacterized plants than the control on the day of inoculation. However, PPO activity increased at three DAI in almost all the treatments except in T_3 (RB-692) and T_9. Likewise, at five DAI, a profound rise in PPO activity was noticed in all the treatments with the maximum in T_2 (RB-22). The activity of PPO increased several fold upto three days after challenge inoculation and declined thereafter. This might be due to the alteration of redox potential of the host leading to an abrupt rise in the activity of PPO (Vidhyasekaran, 1988). Chen *et al.* (2000) reported that various rhizobacteria induced the PPO activity in cucumber root tissues. Native PAGE analysis of PPO revealed four isoforms in plants treated with rhizobacteria, while only three were noticed in plants treated as per PoP and control. Expression of PPO1 and PPO2 isoforms were found in all the groundnut plants treated with Pf1 while additional PPO3, PPO4 and PPO5 were noticed in Pf1 treated plants followed by challenge inoculation with the pathogen (Chitra *et al.,* 2006).

5.9.2.3 Spectronic assay of phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase (PAL) is an enzyme associated with the production of specific phenolic compounds including some isoflavanoids, phytoalexins and lignins (Vidhyasekaran, 1997). It plays an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.,* 1997) which could be induced during plant-pathogen interaction. In the present study, in general, it was observed that rhizobacterial treatments and challenge inoculation with pathogen triggered the activity of PAL compared to control over time. Three and five DAI of the pathogen, there was a rapid and transient

accumulation of PAL in all the treatments except for T_2. The rate of increase was more in rhizobacterial treated plants compared to control. Similar findings were also observed due to PAL in different crops (Nayar, 1996; Ramanathan *et al*., 2002; Paul, 2004). Plants in treatment T_2 initially had higher levels of PAL but when these plants were inoculated with the pathogen, the levels were reduced compared to other treatments or control. It may be due to early induction of PAL by PGPR with subsequent non activation of enzyme activity on inoculation as suggested by Chen *et al.* (2000). Contrary to this, in all treatments upon five days of challenge inoculation, the activity of PAL increased several fold except for T_2. The increased PAL in the PGPR applied plants might be conferring resistance against *R. solanacearum* by making a physical barrier stronger or chemically impervious to the hydrolytic enzymes produced by the pathogen.

5.9.2.4 Protein

Protein profile of various treatments inoculated with *R.solanacearum* revealed a total of 10 bands in treatments from T_1 (RB-144) to T_5 (RB-11), whilst the P.f2 treated plants as well as T_7 (RB-77), T_9 and T_{10} was short of a band. In the rhizobacterial treated plants, there is a possibility of expression of newly induced proteins which may play a role in the defense mechanism. The induction of protein has been reported in several crop plants after treatment with biotic and abiotic elicitors (Reiss and Bryngelsson, 1996; Silue *et al*., 2002 and Kagale *et al.,* 2004).

5.9.3 Estimation of chlorophyll

Since one of the factors which favour better growth of plants is the quality and quantity of photosynthetic pigments, in the present investigation, the effect of rhizobacterial treatments on the chlorophyll content was estimated. Chlorophyll content *viz*., a, b and total chlorophyll varied among different treatments. It was noticed that the least chlorophyll 'a' content was in T_1 (RB-144) and maximum witnessed in T_5 (RB-11). In the same way, chlorophyll 'b' content was found to be the least in treatment T_1 and maximum in T_3 (RB-82). The highest total chlorophyll

content was observed in T_3 which was closely followed by T_5 and T_4 (RB-66) and the least in T_1, T_7, T_8 and T_9. According to Govindasamy *et al.* (2003), enhanced chlorophyll content was correlated with higher percentages of 'N' and 'P' in sunflower treated with PGPR.

5.9.4 Yield, oil and oleoresin and NPK contents

During the pot culture study, the maximum yield was recorded in T_5 followed by T_1 (RB-144), T_6 (P.f2) and T_2 (RB-22). The least yield was recorded in T_7 (RB-77) closely followed by T_{10} (Control). This is in conformity with the earlier studies which once again emphasize the beneficial effect of rhizobacterial isolates in increasing yield and yield attributes. Though, not significant, the treatments had an effect in increasing the fresh root weight and weight of dry top shoot of ginger plants. The least weight in both the cases was observed in T_8. van Peer and Schippers (1988) documented the increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains.

The oil and oleoresin content determines the quality of ginger rhizome. It was observed that rhizobacterial treatments had no significant effect on the oil content. However, there was significant effect in oleoresin content in rhizobacterial treated plants indicating the possibility of the role of these isolates in oleoresin content. However, the exact role played by the isolates in increasing the quality parameters of ginger need to be further ascertained. Similarly, PGPR are known to promote growth of plants by making available required nutrients either in available or non-available form. The NPK analysis of rhizobacterial treated plants showed a significant difference in various treatments. All the isolates used in the study were found to solubilize complex forms of 'P' to the plant available form as observed in the *in vitro* studies. The strains also mobilized higher uptake of 'N' and 'K' in the treated plants which was in agreement with that reported by Paul *et al*. (2004). The content of 'N' and 'P' in groundnut kernels were significantly higher in treatments inoculated with PGPR cultures according to Pal *et al.* (2003)

5.10 MOLECULAR MECHANISM OF INDUCTION OF SYSTEMIC RESISTANCE

Molecular tools are increasingly important in modern plant research. Hence, an approach to analyze the molecular basis on induction of systemic resistance was made as it may perhaps be possible to understand whether an additional band has occurred on addition of the promising rhizobacteria. For this, the initial step was to isolate DNA from the ginger variety, Himachal, in order to select the random primers which would amplify the cDNA.

5.10.1 Isolation of DNA

The genomic DNA was extracted from the leaves of ginger by modified CTAB method as described b Doyle and Doyle (1987). Leaf tissue of ginger being fibrous, homogenizing in presence of liquid nitrogen was carried out, which, according to He *et al*. (2003), could reduce DNase activity. Moreover, tender leaves were used for DNA isolation which yielded good quality and quantity of DNA which is in agreement with Babu (2000) who observed that young leaves usually contain actively dividing cells with lesser concentration of extranuclear materials like protein, oil, carbohydrates and other metabolites that interfere with nucleic acid extraction. The extraction buffer used contained EDTA, which effectively chelates Mg^{2+} ions and mediate aggregation of nucleic acid and ß-mercaptoethanol, an important component in DNA isolation was used for disrupting protein disulfide bonds, thus capable of initiating protein degradation.

The detergent used in lysis buffer was SDS which could act as a nuclease inhibitor and is often used to dissolve membranes. Lipids and denaturing proteins were removed by chloroform: isoamyl alcohol treatment. Isopropanol (0.6 volume) was used only for initial precipitation of DNA at low temperature (-20°C) and two volumes of ethanol was used for final precipitation. The pellet was dissolved in TE buffer for long-term storage. The EDTA present in TE buffer chelates and removes Mg^{2+} ions, which are required for nuclease activity. Finally, it was noticed that

quality of DNA obtained was good with an intact band, as revealed by agarose gel electrophoresis. Since no RNA contamination was noticed along with the bands, treatment with RNase was not given. Similar method of extraction of DNA from different ginger varieties / cultivars of ginger has been described by various workers (Rout *et al.,* 1998; Wahyuni, 2003 and Therakulpisut *et al.,* 2005).

The optical density values of diluted preparation of DNA were found out using UV spectrophotometer. The DNA preparation recorded an OD_{260}/OD_{280} of 2.09, indicating good quality DNA without much RNA or protein contamination and the quantity of DNA was recorded as 5.75 $ngmg^{-1}$ fresh tissue. Since one PCR reaction requires only 25-50 ng template, the DNA recovered was found to be sufficient for further PCR reactions.

5.10.2 Screening of random primers for amplification of DNA by RAPD aasay

RAPD assay was carried out as per the protocol of Demeke *et al.* (1992). For RAPD analysis, 32 random primers of OPA, OPE and OPF series were screened using the genomic DNA. It was noticed that 12 primers of OPE and OPF series gave good amplification and the number of bands obtained for each primer ranged from 3 to 7. Amplification by primers of the same series *viz*., OPE and OPF with various varieties / cultivars of ginger has been reported by Suja (2006) and Srinivasan (2006). It was also observed from the study that primers of OPA series (OPA 01 to OPA 5) did not produce any amplification. However, contrary to the above findings, Therakulpisut *et al*. (2005) observed that the primers of OPA 01 to OPA 20 produced strong reproducible bands in *Zingiber* spp. The genetic variation through RAPD markers has been highlighted in a number of *Zingiber* spp and ginger varieties by various workers with a different set of primers. Rout *et al.* (1998) observed that out of 15 decamer primers screened for DNA amplification, three primers *viz*., OPC 04, OPC 19, OPC 17 produced amplification. Similarly, Theerkulpisut *et al.,* (2005) noticed OPW 01 to 10 produced strong bands with *Zingiber* spp. According to Srinivasan (2006), OPB 2 and OPC 5 were used to detect the genetic uniformity in three ginger varieties by RAPD assay. Palai and Rout (2007) observed that with

ginger variety, Surabhi, only A and N series primers produced relatively more amplification fragments compared to C and D series of primers. Likewise, RAPD primer of OPC 02, OPA 02, OPD 020 and OPN 06 in *Z. officinale* was documented by Nayak *et al*. (2005).

5. 10.3 Isolation of RNA

Total RNA was isolated from rhizobacterial treated and control plants of ginger using Trizol reagent. Trizol reagent combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate immediate and most effective inhibition of RNse activity. Tender leaves were ground in liquid nitrogen for RNA isolation and the sample was homogenized or lysed in Trizol reagent. Similar work was carried out by Chen *et al.* (2005) where they isolated RNA from rhizomes of *Zingiber officinale* in liquid nitrogen and total RNA was extracted using Trizole Reagent.

The homogenate was later separated into aqueous and organic phase by chloroform addition and centrifugation whereby, RNA remains exclusively in the aqueous phase, DNA in the interphase and proteins in the organic phase. RNA was precipitated later from the aqueous phase by addition of isopropanol, washed with ethanol and solubilized. The major problem during RNA isolation is the degradation of RNA due to contamination by ribonuclease / RNases which are very stable enzymes and generally require no cofactors to function. To overcome this problem, all glasswares, tips, tubes mortar and pestle were dipped in 0.1 per cent DEPC solution overnight and were washed thoroughly with double distilled water, autoclaved and finally baked in oven. This chemical inactivates RNase by covalent modification and RNA was eluted in a final volume of 20µl of DEPC treated water. Agarose gel electrophoresis analysis of RNA revealed three intact bands of control sample corresponding to 28s, 18s and 5s r RNA, whereas five bands were observed in the treated sample (28s, 18s, 5.8s, 5s and t-RNA) indicating good quality RNA of both the samples without any degradation. No genomic DNA contamination as indicated by the agarose gel electrophoresis was observed. These RNAs were stored

at -70ºC. The entire procedure was completed within 1h and later subjected to RT-PCR for preparation of cDNA.

5. 10.4. Preparation of cDNA

Complementary DNA was synthesized from RNAs of both the treated and control samples. The first step involved synthesis of first strand of cDNA with RNA as the starting template using the enzyme Reverse transcriptase. The efficiency of the reaction is highly dependent on the quality and quantity of the starting RNA template. Hence, it is important to have intact RNA as starting template. Total RNA extract (9µl) and 1µl of oligo dT primer were incubated at 65ºC for 10min. After 10min incubation, sample was kept at room temperature for 2min to remove any secondary structure. To the denatured RNA, 10µl of reverse transcription reaction mixture was added to provide a final volume of 20µl. The quality and quantity of cDNA thus obtained was subjected to spectral assay and it was noticed that fairly good quality cDNA with an OD value of 1.82 and 1.80 were obtained in treated and control samples. These are in agreement with the findings of Kiefer *et al*. (2000) in cDNA prepared from various crop plants. RNse, which works in the absence of cofactors and divalent cations can be inhibited by placental RNse inhibiter (Blackburn *et al*., 1977) or by vanadyl ribonucleoside complex (Puskar *et al*., 1982). Samples were incubated at 42ºC for 1h followed by 95ºC for 2 min to denature the RNA-DNA hybrids. The resulting cDNA was stored at -20ºC and was used further for RAPD assay.

5. 10.5 RAPD assay with cDNA

The synthesized cDNA was subjected to RAPD assay with the selected random decamer primers. However, no bands / amplified products were visualized after the reaction mix was subjected to agarose gel electrophoresis. This may be due to the annealing of the primers to the intron region of DNA which is not present in cDNA or may be due to certain inhibitory factors associated along with the RNA isolation especially the phenols present in the Trizole reagent. It may also be

concluded that a different set of primers should be used for future line of work so as to know whether any induction has occurred in the plant on addition of rhizobacteria at the molecular level.

5.11 SENSITIVITY OF PROMISING RHIZOBACTERIAL ISOLATES TO PLANT PROTECTION CHEMICALS AND FERTILIZERS

As ginger is a remunerative crop, farmers are adopting all available methods to increase the productivity of the crop where plant protection measures play an important role. Even after adapting to various cultural and chemical means, bacterial wilt of ginger continues to be a complex disease to manage. However, every effort has to be made to reduce the losses due to the disease by adopting suitable management strategy. Hence, while adopting integrated disease management practice using antagonists, it is imperative that pesticides including fungicides, insecticides, antibiotics and even fertilizers commonly used in ginger fields must be compatible with the biocontrol agents and further care must be taken to select a suitable combination. In this context, attempts were made to elucidate the compatibility of eight promising rhizobacterial isolates with fungicides, insecticides, antibiotics and fertilizers commonly used in ginger crop culture.

5.11.1 Antibiotics

The antibiotic sensitivity of promising six rhizobacterial isolates along with the reference cultures was tested. In general, the isolate showed varying levels of sensitivity towards the antibiotics. Oxytetracycline, Cephalxin and Nalidixic acid were compatible with all the isolates. Pencillin G and Ampicillin was compatible with all the isolate except one (RB-77). Tetracycline (0.05 ppm) was also compatible with the isolate RB-144 and RB-77. All other antibiotics tested showed inhibition indicating their incompatibility to various extents with Gentamicin showing maximum incompatibility. Such variation in the sensitivity of antibiotic to

rhizobacterial isolates belonging to fluorescent pseudomonads were reported by Samanta and Dutta (2004).

Since the proprietary antibiotic, Streptocycline, is widely recommended for the management of bacterial wilt of ginger, the compatibility of this antibiotic with the eight promising rhizobacterial isolates were also tested so as to know whether this chemical can be combined with the rhizobacterial isolates in the integrated management of the disease. Though, the isolates showed inhibition of growth, the extent of inhibition was comparatively less and also varied with the isolates indicating their partial compatibility. Among the isolates P.f2, RB-22, RB-144 and RB-77 in that order showed the least sensitivity to antibiotic. Akbar (2002) observed that the higher concentration of streptocyline inhibited *P. aeruginosa* to a slight extent.

5.11.2 Fungicides

A total of eight fungicides were tested to study the compatibility of these chemicals to the promising PGPR isolates along with the reference strains. It was observed that Master, Akomin-40, Bavistin, Captaf and Indole M-45 at different concentrations tested were compatible with the rhizobacterial isolates. Among the copper fungicides *viz*., Shield, Fytolan and Kocide, Fytolan followed by Kocide showed incompatibity towards majority of the isolates. While, the copper sulphate preparation, Shield, can be considered as compatible with the isolates. Further, it is to be noted that the isolate RB-144 and RB-11 were less sensitive to the copper fungicides. Hence, the compatible and partially compatible fungicides can be successfully integrated with the promising rhizobacterial isolates in the management of various disease of ginger. Further, as copper based fungicides reacted differently with the isolates, care should be taken while selecting the right isolate. A perusal of the literature revealed scanty information on the compatibility of rhizobacterial isolates of ginger to fungicides. Elkins and Lindow (1999) did not observe any detrimental effect of mancozeb on *P. fluorescens*. Similar results of compatibility of Mancozeb in addition to carbendazim were noticed by Mathew (2003). However,

according to Bhavani (2004), the antagonistic *Pseudomonas* spp. were compatible with Akomin-40, Indofil M-45 and Bavistin were compatible with the *Pseudomonas* spp. He also noticed higher inhibitory effect of Bordeaux mixture followed by Kocide and Fytolan to the bacteria. The compatibility of metalaxyl with *Pseudomonas* was noticed by Paul (2004).

5.11.3 Insecticides

In this study, none of the nine insecticides *viz*., Sevin, Target, Duralax, Rogor, Ekalux, Confidor, Phorate, Furadan and Marshall tested inhibited growth of the rhizobacterial isolates revealing their compatibility. Similar results were obtained by Bhavani (2004). He reported the compatibility of Phorate and lower dosages of Sevin, Ekalux, Nuvacron and Endosulfan with epiphytic *P. fluorescens* from cocoa pods. According to Thankamani *et al.* (2003) application of *P. fluorescens* along with Phorate and CoC spray resulted in significantly higher number of leaves, maximum length of roots, leaf area and total biomass of black pepper which indicated a synergistic effect of the insecticide and fungicide with the isolate of *P. fluorescens*. Mathew (2003) also noticed the compatibility of Imidachlorprid, Etofenprox, Chlorpyriphos and Triazophos at recommended doses with *P. fluorescens* isolated from cardamom. According to Paul (2004), rhizobacterial *Pseudomonas* strains from black pepper were compatible with Chlorpyriphos, Quinalphos, Dimethoate and Phorate. Hence, these insecticides can be safely applied to the crop for pest management without any deleterious effect to the rhizobacterial isolates.

5.11.4 Fertilizers

As chemical fertilizer application is an integral part for achieving the maximum production of ginger, it is always desirable to use biocontrol agents for disease management compatible with the recommended fertilizers. Hence, the compatibility of Urea, Rajphos, Muriate of Potash (MoP), Ammonium sulphate and Factomphos with the eight rhizobacterial isolates were ascertained and it was found that all of them were compatible with the isolates indicating their tolerance to the

same. The compatibility of Rajphos, MoP and urea towards *P. fluorescens* was reported by Bhavani (2004).

5.12 *In vitro* SENSITVITY OF PLANT PROTECTION CHEMICALS AND FERTILIZERS TO *R. solanacearum*

Another study was conducted to find out the *in vitro* inhibitory effect of antibiotics, fungicides, insecticides and fertilizers on the growth of *R. solanacearum*. The same chemicals, which were used for testing the compatibility with antagonists, were used in this study also.

5.12.1 Antibiotics

Observations on the *in vitro* sensitivity of *R. solanacearum* to 11 different antibiotics showed that except for Cephalaxin, all the antibiotics at different concentrations inhibited the pathogen. It was noticed that the higher concentration of the antibiotic exerted maximum inhibition. Highest concentration of Streptocycline, Gentamicin, Kanamycin and Tetracycline exhibited maximum inhibition. The inhibitory effect of various antibiotics was studied by various workers. Rani (1994) observed the inhibitory effect of Ambistryn-S and Chloromycetin against *R.solanacearum* of ginger under *in vitro* conditions (Rani, 1994). She also observed that plants treated with Ambistryn-S, Terramycin and Chloromycetin showed minimum wilt incidence. Similarly, pre-planting and pre-storage treatment in Streptocycline (200 ppm) along with Dithane M-45 (0.25%) and Bavistin (0.1%) delayed the disease development. The effectiveness of Streptomycin and Streptopenicillin over other antibiotics against wilt pathogen of ginger both under *in vitro* and *in vivo* conditions has been well elucidated by Singh *et al*. (2000) whereas Penicillin G, tetracycline and plantomycin did not inhibit the pathogen at any of the concentrations tested. According to Sambasivam (2003), various isolates of *R.solanacearum* infecting ginger were resistant to Ampicillin and Rifampicin but sensitive to Chloramphenicol and Kanamycin. However, Carbenicillin, Nalidixic acid, streptomycin sulphate and Tetracycline showed a varied response with the

isolates. Similarly, several antibiotics like Oxytertacycline, Tetracycline, Penicillin G and Streptomycin inhibited *R.solanacearum* (Goorani *et al*., 1978). He *et al.* (1983) reported that all the strains of *P. solanacearum* were resistant to Penicillin, Viomycin and Chloramphenicol. Gunawan (1989) observed the suppression of *R. solanacearum* with Streptomycin sulphate. Likewise, Akbar (2002) reported that Ampicillin, Streptomycin sulphate and Kanamycin inhibited the pathogen whereas Rifampicin, Chloramphenicol and Oxytertacycline failed to restrict the growth of the pathogen. Kumar and Sarma (2004) noticed that all the ginger isolates tested were resistant to Tetracycline, Polymixin B sulphate and Chloramphenicol.

The inhibitory effects of Streptomycin and streptocycline on *Pseudomonas solanacearum* has been observed by many workers (Rangarajan and Chakravarti, 1969; Shivappashetty and Rangaswami, 1971). Paul (1998) observed that 250 and 500ppm concentration of Ambistryn, Oxytetracycline and Streptocycline inhibited *R.solanacearum* under *in vitro* conditions. The crop sprayed with Streptocycline solution (100 ppm) at regular intervals of 15 days also reduced the incidence of bacterial wilt in ginger (Dohroo, 2001). Likewise, seed rhizomes treated with Streptocycline 200 ppm for 30 min and shade dried before planting was found effective in reducing the wilt incidence (Anandaraj *et al*., 2005).

5.12.2 Fungicides

Amongst the eight fungicides tested, only the copper fungicides *viz.,* Fytolan, Kocide and Shield in that order showed inhibitory property towards *R. solanacearum*. Higher two concentrations of Fytolan and 0.2 per cent concentration of Kocide exhibited more inhibition of the pathogen. The lowest concentration of Shield did not show any inhibitory effect. The inhibitory effect of Bordeaux mixture towards the pathogen has already been reported (Rani, 1994). Similarly, the effectiveness of Bordeaux mixture, copper oxychloride, Thiride, Blue copper, Kocide, Nabem, Maneb, Dithane M-45, Captan, and Thiram against *R. solanacearum* has also been noticed by various workers (Severin and Kupferberg,

1977; Goorani *et al.,* 1978; Leandro and Zak, 1983; Jyothi, 1992; Akbar, 2002; Anandaraj *et al*., 2005)

5.12.3 Insecticides

A total of eight insecticides were evaluated against the pathogen as well and it was observed that all the insecticides tested were not inhibitory to the growth of *Ralstonia solanacearum.* Not much literature is available in this line.

5.12.4 Fertilizers

Contradictory to the result of effect of insecticides towards the pathogen, it was noticed that in general, all the fertilizers tested inhibited the pathogen. The highest concentration of Factomphos exhibited maximum inhibition followed by that of Rajphos, Urea and MoP. Thus results of the study clearly indicated that chemical fertilizer application has an indirect effect in checking the growth and multiplication of the pathogen. The usefulness of application of chemical fertilizers and soil ameliorants in checking the activity of the pathogen in ginger and other crops has also been documented. Vudhivanich (2002) noticed a decrease in the population of bacterial wilt pathogen of ginger in the soil amended with urea and calcium oxide and the decrease was attributed to toxicity of ammonium, ammonia and nitrate degraded from urea. Calcium oxide is added to convert the ammoniacal form into nitrite (NO_2) which is highly toxic to the growth of the pathogen. According to Hepperly *et al.,* (2004) addition of gypsum along with triple super phosphate at the time of planting ginger rhizomes helped in production of bacterial wilt free ginger rhizomes. Devi (1978) observed that sawdust along with urea combined with Agrimycin (200 ppm) showed reduction in wilt incidence of tomato. Moreover, application of urea and lime minimized the wilt incidence in solanaceous and cucurbitaceous vegetables. Combination of Actigard (acibenzola-S-methyl) with the S-H mixture (bagasse, rice husk, oyster shell powder, urea, KNO_3, calcium super phosphate and mineral ash) significantly reduced bacterial wilt of tomato caused by *R. solanacearum* (Anith *et al*., 2004).

5.13 MUTUAL COMPATIBILITY OF PROMISING RHIZOBACTERIA AND WITH REFERENCE CULTURES OF *Trichoderma* spp.

Even though, considerable progress in the development of effective biocontrol agent/s have been made over a period of time, application of these organism/s at times may not give the desired effect owing to failure in its adaptability to various ecological conditions. Hence, nowadays, focus is being made to develop consortia of useful bioagents selected from different areas with more adaptability to the different ecological niche to obtain maximum effect. In this context, the compatibility of the promising rhizobacterial isolates which promote growth and impart resistance to bacterial wilt was investigated. It was observed that all the eight isolates were compatible to each other in the *in vitro* study indicating the potential of these isolates as consortia for the management of the disease. As rightly pointed out, one approach to improve the efficacy and consistency of biological control against soil borne pathogens is to apply combinations of antagonistic micro organisms with different mechanisms of action (Bakker *et al*., 2003). According to Schippers (1992), inoculation of single strain of biocontrol agent only lead to a level of suppression and positive effects with single inoculants are often inconsistent and hence, it is postulated that in suppressive soils, a concerted action of several microbes and mechanism is responsible for the high consistent disease suppressiveness. There are several reports of biocontrol agents for plant disease having mixtures of fungi (DeBoar *et al.,* 1997 and Paulitz *et al.,* 1998), mixtures of bacteria (Mazzola *et al.,* 1995; DeBoar *et al*., 1997; Wei *et al.,* 1996) and most of these reports showed that combining antagonists resulted in improved biocontrol.

However, the result of the compatibility study of the rhizobacterial isolates with the reference culture of *Trichoderma viride* and *Trichoderma harzianum* revealed that all isolates except there *viz*., RB-22, RB-66 and P.f2 were compatible. The incompatible ones showed inhibition of the growth of the *Trichoderma* spp. Accordingly, the compatible five isolates could be used along with *T. viride* and *T. harzianum*, being recommended for the management of soft rot of ginger can be used together against the two soil borne disease *viz*., bacterial wilt and

soft rot of ginger. Similarly, Ochoa and Cotes (1998) and Larkin and Fravel (1998) reported synergistic effect of combined application of *P.fluorescens* and *Trichoderma* spp. in controlling soil pathogen. Results indicated that *T. viride* had compatibility with all the *P. fluorescens* isolates (Gholve and Kurundkar, 2004). Application of *T. harzianum* and *P. fluorescens* were recommended for promoting growth, suppressing the disease caused by pathogens in black pepper, ginger and cardamom (Jisha *et al.,* 2002). Increased vigour and yield in tomato due to application of PGPR, VAM and *T. harzianum* were reported by Varshney *et al.* (2002). Nevertheless, there are also reports of combinations of biocontrol agents that do not result in suppression of disease compared to that of inoculation of single antagonist (Sneh *et al.,* 1984; Miller and May., 1991; Dandurand and Knudsen, 1993).

5.14 CHARACTERIZATION OF PROMISING ANTAGONISTS

Attempts to identify the six promising rhizobacterial isolates *viz.,* RB-144 (Silent valley-1), RB-22 (Silent valley-4), RB-82 (Kalpetta-2), RB-66 (Kalpetta-3), RB-11 (Kalpetta-4) and RB-77 (Mananthavady-1) were carried out by studying the cultural, morphological and biochemical characters. It was observed that all of them were Gram –ve short rods and were positive to catalase, oxidase and arginine dihydrolase activity which clearly depicts that the isolates belong to the genus *Pseudomonas* as suggested in the Bergy's Manual of Systematic Bacteriology, Vol I (Staley *et al.,* 1989). Further, out of the six isolates, four isolates showed fluorescence under UV light and hence the isolates were grouped into fluorescent and non-fluorescent pseudomonads. The fluorescent and non-fluorescent pseudomonads were further subjected to various biochemical tests in order to identify them at the species level. It was noticed that the isolates did not produce levan from sucrose clearly indicating that they did not belong to the fluorescent species group '*chlororaphis*' and '*aureofaciens*'. Apart from RB-144, others were able to liquefy gelatin which differentiated the isolates from *Pseudomonas putida* as *P. putida* is characterized by their inability to liquefy gelatin and as a result, none of the fluorescent Pseudomonads belong to the species '*putida'*.

The isolate RB-22 alone produced a blue-black pigmentation in the Hi Assorted media for pyocyanine production, thus revealing its affinity to '*aeruginosa'* group. Except RB-144, all isolates reduced nitrate. Further, all the fluorescent and non-fluorescent isolates were subjected to sugar utilization tests and it was observed that the fluorescent Pseudomonads RB-22, RB-82 and RB-66 utilized glucose, dextrose, mannose, mannitol and fructose, clearly pointing out that these isolates belong to fluorescent group 'biovar III' as these are even unable to produce levan from sucrose which is a unique character differentiating from other biovars of fluorescent group. These characteristic features are also in line with the reference cultures of *Pseudomonas* (P.f1 and P.f2). It was noticed that among the non-fluorescent pseudomonads *viz.,* RB-144 and RB-77, RB-144 utilized dextrose and fructose while RB-77 utilized only maltose which is an important criterion of *Pseudomonas pseudoalcaligenes*. However, both the isolates could be merely designated as only non fluorescent pseudomonads, as they require further investigation to confirm the species level.

Hence, based on the above characters and also in comparison with the characters as described in Bergys' Manual, from among the six isolates, RB-22 was tentatively identified as ***Pseudomonas aeruginosa***; RB-692, RB-1516 and RB-11 as ***Pseudomonas fluorescens*** biovar III and RB-77 and RB-144 could be barely identified by their genus and hence they are designated as a **'non-fluorescent pseudomonads'**.

In conclusion, it is noticed that the eight rhizobacterial isolates have proved to play a major role in plant growth promotion in ginger as well as in inducing systemic resistance against the bacterial wilt pathogen. Among them, isolate RB-11 identified as *Pseudomonas fluorescens* biovar III performed well both under field and *in vitro* conditions. The enhanced plant growth and yield of ginger treated with this isolate could be due to the higher production of growth promoting hormones and making available insoluble form of 'P' from soil as well as by suppression of other deleterious pathogens by production of inhibitory substances like ammonia, antibiotics and siderophores. The significant reduction of bacterial

wilt incidence by rhizobacterial isolates especially RB-11 could be attributed to the enhanced synthesis of phenols, amino acids, proteins and defense related enzymes such as peroxidase, polyphenol oxidase, catalase and phenylalanine ammonia lyase which might have played a role in inducing systemic resistance in ginger against the pathogen. However, investigation on the potential of the isolate in different agro climatic situation needs to be ascertained so as to confirm its efficacy in inducing resistance against bacterial wilt disease. Moreover, induced systemic resistance provides ecofriendly and economically viable disease management strategy which is currently needed for many of our spice crops to meet the global standard and to compete in the global market in the coming years.

Summary

6. SUMMARY

Bacterial wilt is considered as one of the major constraints in the cultivation of ginger in Kerala. The seed and soil borne nature of the pathogen and its wide host range makes the management of the disease less effective. Considering the serious nature of the disease, the present investigation was undertaken to harness the potential of plant growth promoting rhizobacteria (PGPR) in promoting growth of ginger as well as in inducing systemic resistance against the bacterial wilt disease. The salient findings of the present study are summarized below:

1. The bacterial wilt pathogen was isolated and its pathogenicity established. The cultural, morphological and biochemical characters confirms that the pathogen is *Ralstonia solanacearum* belonging to biovar III.
2. Enumeration of rhizosphere microflora of ginger from different locations of various districts revealed that the total microbial population was high in Silent valley tracts as well as in Waynad areas.
3. Out of the 163 rhizobacterial isolates, 45 were found antagonistic to the pathogen. Among them, 20 isolates showed prominent zone of inhibition of above 15mm against the pathogen.
4. Out of the 20 rhizobacterial isolates put forth in the pot culture experiment, only nine showed pronounced effect in increasing all the biometric characters in addition to the yield of ginger apart from the two reference cultures of *Pseudomonas fluorescens.*
5. The 11 rhizobacterial isolates including the reference cultures of *P. fluorescens* were tested for growth promotion and disease suppression under *in vitro* based on various parameters *viz*., antagonistic index, vigour index, hydrogen cyanide, ammonia and IAA production, ‘P’ solubilization and each were scored as per a modified standard score chart. It was observed that from among the 11 isolates including the reference cultures, six of them showed an inhibition zone of above 20mm and were therefore score as 3. The isolates were bioassayed for the ability to promote seedling growth and it was

observed that all the isolates except for the reference culture of P.f2 were given a score of 2. The isolates were tested for production of volatiles like ammonia and HCN and it was found that none of them were cyanogenic in nature, therefore scored as 1. Except for reference culture of P.f2 and RB-82, all isolates showed a high production of ammonia. The highest 'P' solubilization was noticed with RB-144 and least in RB-151, RB-71 and RB-77. With respect to IAA production, RB-22 produced the maximum quantity of IAA and the least by RB-69.

6. The plant growth promoting index (PGPI) of 11 rhizobacteria including the reference cultures of *P. fluorescens* were calculated based on the above six parameters and it was observed that four isolates *viz*., RB-22, RB-144, RB-11 and RB-82 showed a PGPI of above 70 and the lowest index in RB-151.
7. TLC analysis of growth regulators produced by the 11 isolates including the reference cultures revealed that though all the cultures produce auxin and their related compounds, only two isolates *viz*., RB-141 and RB-11 produced both gibberellic acid as well as auxins.
8. Secondary metabolites like salicylic acid, antibiotics and siderophores were estimated.
    a. It was observed that all isolates produced salicylic acid in varying amounts with the maximum by the two reference cultures followed by the isolates RB-11 and RB-22.
    b. The maximum number of antibiotics was produced by the isolate RB-22 comprising of pyoluteorin, pyrrolnitrin, pyocyanin and unidentified metabolite and this was closely followed by RB-144, RB-66, RB-11 and P.f2 which produced three antibiotics. The antibiotic 2, 4 DAPG was produced by RB-66, RB-11 and P.f2 apart from certain unidentified ones.
    c. With respect to siderophore production, four cultures *viz*., RB-22, RB-82, RB-66 and RB-11 apart from the reference cultures showed fluorescence under UV light, however, maximum zone in CAS plates was detected in the isolate RB-22 and RB-11.

9. All the rhizobacterial isolates were able to sustain their population even upto three months.
10. All the 11 rhizobacterial isolates were again assessed under *in planta* conditions and it was observed that among the various isolates, RB-11 favoured early sprouting of ginger.
11. Seed treatment followed by soil application and foliar spray of the rhizobacterial isolates were found to increase the growth and yield of ginger. The effect was comparatively more with the isolate RB-11.
12. The least natural incidence of shoot borer was observed in plants treated with RB-11 and RB-66, rhizome rot with plants in RB-11 and no incidence of bacterial wilt with RB-82 and RB-11.
13. The least incidence of bacterial wilt on challenge inoculation with the pathogen was noticed in plants bacterized with RB-11 at different intervals of observation.
14. In general, higher accumulation of phenolics occurred in plants treated with the rhizobacterial isolates. However, more increase in phenol content after challenge inoculation was observed in plants treated with RB-11 and RB-82.
15. The number and type of phenols varied from one to five from before inoculation of the pathogen to 5 DAI. Five phenols were detected in samples of RB-22, RB-11 and RB-71 at 5 DAI. Salicylic acid, catechol and hydroquinone were the common phenols identified apart from certain unidentified ones.
16. The total protein estimated by spectroscopy varied in all treatments before and after challenge inoculation. However, the maximum protein content was observed in plants in T_3 (RB-82), T_1 (RB-144) and T_5 (RB-11) before and after five days of challenge inoculation.
17. The total amino acid content though varied in all the treatments before and after challenge inoculation, the maximum amino acid at 5 DAI was observed in plants in treatment T_5 (RB-11).
18. The type and number of amino acids varied from before to five days after challenge inoculation. Before inoculation, rhizobacterial treated plants showed the presence of three amino acids while only two were noticed in

control. However, at 3 DAI, the number varied from 3 to 5. Proline was observed only in T_5 and the concentration of phenylalnine was also more in T_5 compared to other treatments. Though there was not much change in the number of amino acids at 5DAI, there was a change in the type of amino acids.

19. After the second pot culture experiment, eight isolates including the reference cultures which showed a promising effect under *in planta* studies as well as under *in vitro* conditions were further used for the third pot culture experiment which included RB-144, RB-22, RB-82, RB-66, RB-77 and RB-11 and these isolates were assayed for the defense related enzymes.
20. Appreciable increase in peroxidase activity over time was observed in all the rhizobacterial treated plants after challenge inoculation. However, Native PAGE analysis revealed six isoforms of peroxidase in plants treated with RB-144, RB-22, RB-82, RB-66, RB-11 and P.f1 whereas only three isoforms were noticed in control.
21. All the rhizobacterial isolates had a profound influence in increasing polyphenol oxidase activity. Native PAGE analysis revealed four isoforms of PPO in rhizobacterial treatments while only three were detected in Control and plants grown as per PoP.
22. The rhizobacterial treatments and challenge inoculation with the pathogen triggered more activity of phenylalanine ammonia lyase in ginger than those in control.
23. Protein profile of plants in various treatments inoculated with pathogen revealed a total of 10 bands in treatments RB-144, RB-22, RB-82, RB- and RB-11 while the control and PoP were short of a band.
24. Chlorophyll content varied among treatments where the highest total chlorophyll content were in plants treated with T_3 (RB-22) followed by T_5 (RB-11).
25. NPK analysis revealed that the highest nitrogen content was in T_7 (RB-77), phosphorous (P) content in plants treated with T_5 (RB-11) and the maximum potassium (K) content in T_5 and T_8.

26. Treatment with rhizobacteria had no significant effect on the oil content of ginger rhizome, but there was a significant effect in the oleoresin content with the maximum in RB-144 which was on par with all treatments except control and PoP.
27. An attempt was made to elucidate the molecular mechanism of induction of systemic resistance in ginger by synthesizing cDNA and was subjected to RAPD assay. However no amplification was observed with cDNA with the selected 12 primers of OPE and OPF series which had shown amplification with DNA.
28. Sensitivity of eight promising rhizobacterial isolates with fungicides, antibiotics, insecticides and fertilizers was assessed.
    a. Among antibiotics, oxytetracycline, cephalaxin, nalidixic acid were compatible with all the isolates. Penicillin and Ampicillin inhibited only RB-77. Rifampicin and Gentamicin showed maximum incompatibility with all isolates. Tetracycline was compatible with RB-144 and RB-77. The higher dose of Streptocycline were incompatible with all rhizobacterial isolates, however its recommended and lower doses were compatible with the isolate to a certain extent.
    b. All the eight isolates were compatible with fungicides *viz*., Master, Akomin-40, Bavistin, Captaf and Indole M-45 at different concentration tested. However, Fytolan and Kocide showed incompatibility towards majority of isolate. The copper sulfate fungicide, Shield, was found compatible with all the isolates.
    c. All the insecticides tested *viz*., Sevin, Target, Duralax, Rogor, Ekalux, Confidor, Phorate, Furadan and Marshall were compatible with all the rhizobacterial isolates.
    d. All the fertilizer *viz*., urea, MoP, Rajphos, ammonium sulphate and factomphos were compatible with all the rhizobacterial isolates.
29. *In vitro* inhibitory effect of antibiotics, fungicides, insecticides and fertilizers on growth of *R. solanacearum* was tested.
    a. All the antibiotics except Cephalaxin were inhibitory to the pathogen.

b. Copper fungicides tested inhibited the pathogen. The higher two concentrations of Fytolan and 0.2 per cent of Kocide showed more inhibition of the pathogen.
c. All the insecticides tested were not inhibitory to the pathogen.
d. Among fertilizers, Factomphos exhibited maximum inhibition of the pathogen followed by Rajphos, urea and MoP.

30. The mutual compatibility among the rhizobacterial isolates was tested. All of them were compatible to each other.
31. The compatibility study of rhizobacterial isolates along with the reference cultures of *Trichoderma viride* and *Trichoderma harzianum* revealed that except for *P. aeruginosa* (RB-22), *P. fluorescens* (RB-66) and the reference culture of *P.fluorescens* (P.f2), the rest of the isolates were compatible with both *Trichoderma* spp.
32. Based on the cultural, morphological and biochemical characters coupled with characters of reference cultures of *P.fluorescens*, RB-22 was tentatively identified as *Pseudomonas aeruginosa*, RB-82, RB-66 and RB-11 as *Pseudomonas fluorescens*. However, RB-77 and RB-144 was identified upto their genus level and therefore categorized as non fluorescent pseudomonads.

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Appendix

APPENDIX–I

1. Triphenyl tetrazolium chloride media (TTC) (pH 6.8)

Peptone	:	10 g
Casein hydrolysate	:	1.0 g
Glucose	:	5.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

Autoclave 5 ml of 1% Triphenyl Tetrazolium chloride.

2. Nutrient agar media (NA) (pH 7.2)

Peptone	:	20 g
Beef extract	:	1.0 g
NaCl	:	5.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

3. Martin's rose bengal streptomycin agar medium (MRBA)

Dextrose	:	10.0 g
Peptone	:	5.0 g
KH_2PO_4	:	1.0 g
$MgSO_4$	:	0.5 g
Agar	:	20.0 g
Rose Bengal	:	0.03 g
Streptomycin	:	30.0 mg (added aseptically)
Distilled water	:	1000 ml

4. Thornton's agar media (TT) (pH 7.4)

Mannitol	:	1.0 g

Asparagine	:	0.5 g
K_2HPO_4	:	1.0 g
KNO_3	:	0.5 g
$MgSO_4$	:	0.2 g
$CaCl_2$	:	0.1 g
NaCl	:	0.1g
$FeCl_3$	:	0.002
Agar	:	20.0 g
Distilled water	:	1000 ml

5. Kenknights agar medium (KK) (pH 7.0)

Dextrose	:	1.0 g
KH_2PO_4	:	0.1 g
$NaNO_3$	:	0.1 g
KCl	:	0.1g
$MgSO_4$	:	0.1g
Agar	:	20.0 g
Distilled water	:	1000 ml

6. Kings'B media (KB) (pH7.2)

Peptone	:	20 g
Glycerol	:	10 ml
K_2HPO_4	:	1.5 g
$MgSO_4$ $7H_2O$	:	1.5 g
Agar	:	20.0 g
Distilled water	:	1000 ml

7. Soil extract agar media (SEA)

Glucose	:	1.0 g
KH_2PO_4	:	0.5 g
Soil extract	:	100 ml

Agar	:	20.0 g
Distilled water to	:	1000 ml

Autoclave 1000g of garden soil with 1000ml of tap water for 30 min. A small amount of $CaCO_3$ is added and the soil suspension is filtered through filter paper.

8. Methyl red agar media (MRA) (pH 7.0)

Beef extract	:	3.0 g
Peptone	:	5.0 g
Methyl red	:	150 mg
Distilled water	:	1000 ml

Dissolve 150mg methyl red in 10ml water and sterilize separately and add after autoclaving the medium.

9. Crystal violet agar media (CVA) (pH 7.0)

Beef extract	:	3.0 g
Peptone	:	5.0 g
Crystal violet	:	4.0 ml
Distilled water	:	1000 ml

Prepare stock solution of crystal violet (0.05%) (w/v) and filter sterilize and add after sterilization of medium.

10. Peptone water (pH 7.0)

Peptone	:	10.0 g
NaCl	:	15.0 g
Distilled water	:	1000 ml

11. Pikovaskya's medium (pH 7.0)

Glucose	:	10.0 g
$Ca(PO_4)_3$	:	5.0 g
NH_4SO_4	:	0.5g
KCl	:	0.23g
$MgSO_4$	:	0.1g
$MnSO_4$	:	trace

$FeSO_4$	:	trace
Yeast extract	:	0.5g
Agar	:	20.0 g
Distilled water	:	1000ml

12. Luria Bertani broth (LB) (pH 7.2)

Tryptone	:	10.0 g
Yeast extract	:	5.0 g
Glucose	:	1.0 g
NaCl	:	10.0 g
Distilled water	:	1000ml

13. Glucose mineral medium (pH 7.2)

Glucose	:	30.0 g
NH_4SO_4	:	2.0 g
KH_2PO_4	:	3.0 g
Mg SO_4	:	0.5 g
Distilled water	:	1000ml

14. Casaminoacid broth (pH 6.8)

Peptone	:	10.0 g
Casein hydrolysate	:	1.0 g
Glucose	:	5.0 g
Distilled water	:	1000ml

15. Nutrient broth glucose media (NBG) (pH 7.2)

Peptone	:	5.0 g
Beef extract	:	1.0 g
Yeasty extract	:	2.0 g
NaCl	:	5.0 g
Glucose	:	20 .0 g
Distilled water	:	1000ml

APPENDIX–II

Composition of modified Fiss Minimal Medium for assay of siderophores

The following stock solutions were prepared, autoclaved ($FeSO_4$ was steam sterilized) and stored at 4°C.

a. **Potassium phosphate (KH_2PO_4) and Asparagine solution:** 0.524 per cent solution of KH_2PO_4 and L-Asparagine were prepared by dissolving 5 g of KH_2PO_4 and 5 g of L-Asparagine in Millipore water to make a final volume of 954 ml. The pH was adjusted to 6.8 with a solution of 6.0 M NaOH.

b. **Glucose solution:** 50 per cent solution of glucose was prepared by dissolving 50 g of glucose in Millipore water to make a final volume of 100 ml.

c. **Manganese sulphate ($MnSO_4$) solution:** 0.001 per cent solution of $MnSO_4$ was prepared by dissolving 0.001 g $MnSO_4$ in Millipore water to make a final volume of 100 ml.

d. **Magnesium sulphate ($MgSO_4$) solution:** 0.4 per cent solution of $MgSO_4$ was prepared by dissolving 0.4 g $MgSO_4$ in Millipore water to make a final volume of 100 ml.

e. **Zinc chloride ($ZnCl_2$) solution:** 0.005 per cent solution of $ZnCl_2$ was prepared by dissolving 0.005 g $ZnCl_2$ in Millipore water to make a final volume of 100 ml.

f. **Ferrous sulphate ($FeSO_47H_2O$) solution:** 1mM solution of $FeSO_4$ was prepared by dissolving 0.0278 g $FeSO_4.7H_2O$ in Milli pore water to make a final volume of 100 ml. The solution was subjected to tyndallisation.

Modified Fiss minimal medium contained: 5.03 gl^{-1} KH_2PO_4, 5.03 gl^{-1} Asparagine, 5.0 gl^{-1} glucose, 40 mgl^{-1} $MgSO_4$, 100 μgl^{-1} $MnSO_4$, and 500 μgl^{-1} $ZnCl_2$. Iron-restricted modified Fiss minimal medium was prepared by adding 139 μgl^{-1} $FeSO_4$ to the final medium (0.5 μM). High iron modified Fiss minimal medium was prepared by adding 5.56 mgl^{-1} $FeSO_4$ to the final medium (20 μM).

APPENDIX-III

I. Reagents for isozyme analysis

a. 30 % Monomer solution

30% Acrylamide	: 60.0g
60% Bisacrylamide	: 1.0g

b. 4X Resolving buffer

1.5M Tris base	: 36.3g
Distilled water	: 200ml
pH	: 8.8

c. 4X Stacking gel buffer

0.5M Tris base	: 3.0g
Distilled water	: 50ml
pH	: 6.8

d. Ammonium per sulphate : 10 %

A. Preparation of separating / resolving gel mix (8 per cent)

30% monomer solution	: 2.7 ml
4X resolving buffer	: 2.5 ml
Distilled water	: 4.69 ml
Ammonium persulphate	: 100 µl
TEMED	: 10 µl

B. Preparation of stacking gel mix (6 per cent)

30% monomer solution	: 0.67 ml
4X stacking gel buffer	: 1.25 ml
Distilled water	: 3 ml
Ammonium persulphate	: 25 µl
TEMED	: 10 µl

II. Buffers used in Native Polyacrylamide Gel Electrophoresis (Native PAGE)

A. Sample Buffer (5X)

Tris HCl (pH 6.8)	: 5.0ml
Sucrose	: 0.5g
Mercaptoethanol	: 0.25 ml
Bromophenol blue	: 1.0 ml
Distilled water to	: 10.0 ml

Dilute to 1X and use.

B. Electrode buffer

0.05M Tris	: 6.0g
0.192M Glycine	: 14.4g
Distilled water to	: 1000ml

APPENDIX-IV

I. Reagents for DNA isolation as per Doyle and Doyle (1987)

A. Extraction Buffer (4X)

Sorbitol : 25.6 gm

Tris : 48 gm

EDTA disodium salt : 7.4 gm

Distilled water : 1000ml

pH : 7.5

The chemicals were dissolved in 600 ml sterile distilled water. The pH was adjusted to 7.5 and final volume was made up to 100 ml with distilled water and then autoclaved.

B. Lysis Buffer

1 M Tris (pH 8) : 200 ml

0.25 M EDTA : 200 ml

CTAB : 20.0 gm

5M NaCl : 400 ml

Distilled water to : 1000 ml

Cetyl Trimethyl Ammonium Bromide (CTAB) was dissolved in 20 ml sterile distilled water. To this the required volumes of stock solutions are added.

C. TE Buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Tris-HCl 1.0 M (pH 8.0) : 1.0 ml

EDTA 0.25 M (pH 8.0) : 0.4 ml

Distilled water : 98.6 ml

Autoclaved and stored at room temperature.

II. Buffer and dye used in Agarose Gel Electrophoresis

A. 6X Loading / Tracking dye

Bromophenol blue	: 0.25 %
Xylene cyanol	: 0.25 %
Glycerol	: 30 %

The dye was prepared and kept in refrigerator at $4^{o}C$

B. 50X TAE buffer (pH 8.0)

Tris base	: 242.0 g
Glacial acetic acid	: 57.1 ml
0.5 M EDTA (pH 8.0)	: 100 ml
Distilled water to	: 1000 ml

The solution was prepared and stored at room temperature

Abstract

PLANT GROWTH PROMOTING RHIZOBACTERIA MEDIATED INDUCED SYSTEMIC RESISTANCE AGAINST BACTERIAL WILT IN GINGER

By

RESHMY VIJAYARAGHAVAN

ABSTRACT OF THE THESIS

submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy in Agriculture

Faculty of Agriculture
Kerala Agricultural University

Department of Plant Pathology
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR-680 656
KERALA, INDIA

2007

ABSTRACT

The pathogen causing bacterial wilt of ginger was isolated and identified as *Ralstonia solanacearum* biovar III based on its cultural, morphological and biochemical characters coupled with pathogenicity. Rhizosphere microflora of ginger from different locations of Thrissur, Waynad and Palakkad districts were isolated. Altogether, 163 rhizobacterial isolates were selected from these areas and their antagonistic activity against the pathogen assessed. Out of 163 isolates, only 45 showed antagonistic reaction. Further, study of these antagonists based on zone of inhibition resulted in selection of 20 isolates. The effect of these 20 isolates in promoting the growth of ginger was studied in pot culture in comparison with three reference cultures of *P. fluorescens* and *B. subtilis.* Result of this experiment revealed that only 11 isolates including the two reference cultures of *P. fluorescens* had growth promoting effect as evidenced in terms of yield and yield attributing characters of ginger. Factors which impart growth promotion in ginger by these isolates were assessed by estimating the inhibition zone, vigour index, hydrogen cyanide, indole acetic acid, ammonia production and 'P' solubilzation and based on that, PGPR index of the isolates was worked out. In addition to that, production of salicylic acid, antibiotics and siderphore by the isolates, the secondary metabolites which are known to play a role in disease suppression were assessed. The isolates varied in their ability to produce salicylic acid. Isolates RB-22 followed by RB-11, RB-144 and RB-66 produced more number of antibiotics which include pyoluteorin, pyrrolnitrin, 2,4DAPG etc. Similarly, isolate RB-22 and RB-11 produced maximum siderophores.

The potential of these 11 rhizobacterial isolates in imparting resistance against the disease was assessed in another pot culture experiment by estimating phenol, proteins and amino acid content of ginger upon challenge inoculation. Here also, the isolates showed a profound effect on growth and yield of ginger especially by those plants bacterized with RB-11. There was no natural incidence of bacterial wilt in plants treated with RB-11 and RB-22. Upon challenge inoculation also, plants bacterized with RB-11 showed the least incidence. In general, rhizobacterial treated

plants contained more amount of phenol, protein and amino acids than untreated ones. Upon challenge inoculation with the pathogen, the rate of increase of these compounds in rhizobacteria treated plants was more than that of control during different intervals of observations.

A third pot culture experiment was conducted to assess the effect of rhizobacterial treatments on defense related enzymes of ginger upon challenge inoculation. Here, eight most promising ones including the reference cultures were used. In general, the study revealed more activity of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in rhizobacterial treated plants that too after challenge inoculation. Native PAGE analysis revealed six isoforms of PO and four isoforms of PPO in a majority of the rhizobacterial treated plants whereas only three were noticed in control. Similarly, difference in the protein profile of rhizobacterial treated plants and control was noticed. Chlorophyll, NPK and oil and oleoresin content varied among treatments where the highest was observed in rhizobacterial treated plants. An attempt was made to elucidate the molecular mechanism of induced systemic resistance (ISR) in ginger by synthesizing cDNA and was subjected to RAPD assay. However, no conclusive evidence on ISR was observed.

The compatibility of eight rhizobacterial isolates including the two reference cultures with antibiotics, fungicides, insecticides and fertilizers were assessed which revealed variation in their sensitivity. Moreover, mutual compatibility of the rhizobacterial isolates and compatibility with *Trichoderma* spp. were also studied and it was observed that all bacterial isolates were mutually compatible. However, *Pseudomonas aeruginosa*, *P. fluorescens* (RB-66) and the reference culture of *P .fluorescens* (P.f2) were found incompatible with the *Trichoderma* spp. The promising six rhizobacteria isolates were identified based on cultural, morphological and biochemical characters and also in comparison with that of reference culture of *P. fluorescens*. They were tentatively identified as *Pseudomonas aeruginosa* (RB-22), *Pseudomonas fluorescens* (RB-82, RB-66, RB-11) and the remaining two, RB-144 and RB-77, as non-fluorescent Pseudomonads.