EXPRESSION OF PATHOGENESIS RELATED PROTEINS BY PLANT GROWTH PROMOTING RHIZOBACTERIA IN CONTROLLING TARO LEAF BLIGHT

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KERALA, INDIA

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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LIST OF ABBREVATIONS AND SYMBOLS USED

%	Percentage
℃	Degree Celsius
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microlitre
µm	Micrometer
A	Absorbance
A260 nm	Absorbance at 260nm
A280nm	Absorbance at 280nm
AGE	Agarose Gel Electrophoresis
B	Bacteria
BLAST	Basic Local Alignment Search Tool for nucleotides
Bp	Base pair
Ctrl	Control
C. esculenta	*Colocasiae esculenta*
CA	Carrot Agar
cDNA	Complementary DNA
cm	Centimeter
Conc.	Concentration
CTCRI	Central Tuber Crops Research Institute
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide triphosphates
EDTA	Ethylene Diamine Tetra Acetic acid
et al.	And others
EtBr	Ethidium Bromide

FAO	Food and Agriculture Organization
Fig.	Figure
g	Gram
H	Hour
H_2O_2	Hydrogen peroxide
IAA	Indole Acetic Acid test
KB	King's B Medium
L	Liter
LA	Luria Agar
LAF	Laminar Air Flow chamber
LB	Luria Broth
M	Molar
m	Meter
mg	Milligram
min	Minute
ml	Milliliter
mM	Milli molar
mm	Millimeter
mRNA	Messenger RNA
NA	Nutrient Agar
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
nm	Nanometer
OD	Optical Density
P. *colocasiae*	*Phytophthora colocasiae*
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PGPR	Plant Growth Promoting Rhizobacteria
pH	Hydrogen ion concentration
POD	Peroxidase
PR	Pathogenesis Related
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room Temperature
RT-PCR	Real Time Polymerase Chain Reaction
s	Second
t	Tonne
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris EDTA
TLB	Taro Leaf Blight
U	Enzyme unit
UV	Ultra Violet
v/v	volume by volume
w	Weight
w/v	weight by volume

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INTRODUCTION

1. INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott) is an important staple or subsistence crop appreciated by millions of people from developing countries (Misra *et al*., 2008). It is a member of the Araceae family and is one among the oldest crops cultivated alongside most humid tropics and subtropics. It is a perennial tropical plant produced for its edible corms, leaves and petioles. It is consumed as a staple crop in West Africa, particularly in Ghana (Asraku, 2010), Nigeria (Bandyopadhyay *et al*., 2011) and Cameroon (Fontem and Mbong, 2011). All parts of the plant including corm, cormels, rhizome, stalk, leaves and flowers are edible and contain abundant starch (Bose *et al*., 2003). It is the fourteenth most consumed vegetable worldwide (Rao *et al*., 2010). In India, two types *viz*., *C. esculenta* var. *esculenta* (Dasheen type) and *C. esculenta* var. *antiqourum* (Eddoe type) are cultivated throughout the country. The crop is known by many common names *viz*., cocoyam, dasheen, colocasia, elephant's ear (plant and leaves), kalo etc.

Taro being the staple crop of many African countries, it is important to conserve it from extensive genetic erosion and more over the crop is under strain as it is infested by many fungal diseases (Caillon *et al*., 2006). Taro leaf blight caused by *Phytophthora colocasiae* Raciborski is the most destructive disease of taro (Raciborski, 1900). The disease begins as purple-brown water-soaked lesions on the leaf. Yellow liquid oozes from the lesions. These lesions then enlarge, join together and eventually destroy the entire lamina in 10-20 days. Free water collecting on older leaves, as well as high temperature and high humidity are conducive for the onset and spread of the disease and germination of the spores. The disease can be spread from plant to plant by wind and splashing rain. Spores survive in planting material for three or more weeks. It makes infected planting material as one common means of spreading the disease over long distances and from season to season.

Various approaches have been used to control taro leaf blight. Agronomic methods that have given partial success include careful choice of planting material,

planting density, planting resistant taro cultivars, intercropping taro with other crops rather than growing it as a sole crop (Nelson *et al*., 2011). Field removal of infected leaves has also been useful, but it is extremely laborious. In India, copper and metalaxyl fungicide showed a successful method in controlling *Phytophthora* (Misra *et al*., 2008). In Samoa, control has been achieved by an intensive spraying programme with Ridomil or Manzate, and more recently with phosphorous acid (Foschek). Chemical control is extremely tedious, expensive, and not totally effective and more over causes environmental pollution. An integrated control approach combining cultural and chemical methods seems to be the best at present to tackle the problem. The ultimate solution must lie in the breeding and release of resistant cultivars. The taro breeding programme in Bubia, Papua New Guinea, has already identified several promising lines in this regard. ICAR-CTCRI, regional centre at Bhubaneswar has developed a resistant variety, Muktakeshi.

Current trend is to explore beneficial microbes and organic amendments as alternative methods for disease control. In integrated disease management technologies, application of bio-agents is a strong and viable option for managing plant diseases. In recent years considerable attention has been paid to PGPR (Plant Growth Promoting Rhizobacteria) to replace agrochemicals (fertilizers and pesticides) for the plant growth promotion by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, crucial for soil fertility, bio-control of soil and seed borne plant pathogens and in promoting changes in vegetation.

PGPR are the major root colonizers, belonging to different genera and most reported strains are from species of *Pseudomonas, Bacillus* and *Serratia* etc. The strains of PGPR are known to survive both in the rhizosphere and phyllosphere. In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Bharathi *et al*., 2004). It is considered natural, eco-friendly and safe, and provides resistance against a

broad spectrum of pathogens. The use of PGPR has been reported for the control of various pathogens (Gutterson, 1990; Wei *et al*., 1991).

A proper understanding regarding the mechanism of action of various bioagents contributes to conquer the high inconsistency in pathogen suppression. Induction of defense inducing enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, chitinase, glucanase and phenols was reported in many crops. The PGPR application induces plant defense genes and priming the plant against pathogen attack. Even though, application of *Trichoderma* spp. is being recommended for TLB management, no serious attempt has been made with bacterial isolates. Identification of potent PGPRs with pathogen suppression and understanding inducible plant protection machinery of *P. colocasiae* after the application of PGPR involving differential expression of PR proteins may help in forming more precised utilization of bio-agents. This can end up as an efficient tool for the management of taro leaf blight and aid in the production of organic taro.

The plant system has revealed that defense responses are activated via signaling pathways mediated by endogenous signaling molecule such as salicylic acid, jasmonic acid, and ethylene. Activation of plant defenses is associated with changes in the expression of large number of genes. β-1,3-glucanases are able to catalyze the cleavage of the β-1,3-glucosidic bonds in β-1,3-glucan (Simmons, 1994). β-1,3-glucan is a major structural component of the cell walls of many pathogenic fungi (Wessels and Sietsma, 1981; Adams, 2004). Co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, tomato, tobacco, maize, soybean, wheat (Anguelova *et al*., 2001; De Loose *et al*., 1988; Cordero *et al*., 1994; Lawrence *et al*., 2000; Mauch *et al*., 1988; Takeuchi *et al*., 1990), rubber tree (Chye and Cheung, 1995), banana (Peumans *et al*., 2000) and rice (Yamaguchi *et al*., 2002).

The inoculation of PGPR increase various PR protein production *viz*., IAA, siderophore, cytokines, gibberlin production, activation of induced systemic resistance, activation of various lytic acids synthetic pathways like peroxidase, jasmonic acid, phenyl alanine lyase, salysilic acid, glucanase, chitinase etc, polysaccharide and

biofilm formation thus envisaging a highly mechanized protective agents against plant stresses (Gupta *et al*., 2015).

Trichoderma spp had been found to reduce TLB incidence. PGPR's have been recommended for the management of many *Phytophthora* diseases. However, no serious attempts had been made to exploit the potential of rhizobacteria to tackle the pathogen.

Hence the present study, "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight" was carried out with the following objectives.

1. To select efficient PGPR from the bacterial cultures maintained at microbial repository at ICAR- CTCRI for TLB management.
2. To characterize the isolates using biotechnological tools.
3. To study the differential expression of PR proteins in susceptible and tolerant varieties of taro (consequent to application of PGPR).

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 ROOT AND TUBER CROPS

Tropical root and tuber crops are known as the energy banks of nature serving as primary or secondary staple to meet the calorie needs of about one fifth of world's population in tropics and subtropics. Tropical root and tuber crops are third most important food crops after cereals and pulses (George *et al*., 2011). They serve as substitute for cereals due to higher carbohydrate and caloric content. Root and tuber crops are the main food of about 400 million people living in the tropics. Tropical tuber crops have the capacity to withstand adverse biotic and abiotic stresses and have potential to grow and yield even under low fertility conditions. The major tropical tuber crops consumed as food are cassava (*Manihot esculenta*), sweet potato (*Ipomoea batatas*), edible aroids (*Colocasia esculenta*), elephant foot yam (*Amorphophallus paeonifolius*) and yam (*Dioscorea* spp.) Tropical root and tuber crops including cassava, sweet potato, yams and aroids are relished as vegetables, used as raw materials for small scale industries, and consumed as staple food especially in the under developed countries (Ravi *et al*., 1996).

Tropical tuber and root crops are attacked by several diseases caused by fungi, bacteria, virus and phytoplasma causing yield reduction in the crops. In India, more than 80 diseases have been reported on these crops while the global figures are more than 200 (Anonymous, 1978). Among many diseases reported in India, only few diseases like Cassava mosaic diseases, tuber rot of cassava, leaf blight of colocasia and collar rot of elephant foot yam are of major importance and they cause considerably reduction in the yield. Yield reduction of cassava mosaic disease (25-80%), leaf blight of colocasia (25-50%) and collar rot of elephant foot yam (20- 100%) varies depending upon the nature of cultivars and influence of various weather factors (Palaniswami and Peter, 2008)**.**

2.2 TARO (*Colocasia esculenta* (L.) Schott)

Kingdom- Plantae

Phylum- Tracheophyta

Class- Liliopsida

Order- Arales

Family- Araceae

Tribe- Colocasieae

Genus- Colocasia

Specific epithet- esculenta

Botanical name- *Colocasia esculenta* (Linnaeus) Schott

      Taro (*Colocasia esculenta* (L.) Schott] is an significant tropical tuber crop, and member of the monocotyledonous family Araceae which has 1000 cultivars, grown for its edible corms, nutritious leaves and other traditional uses, serves as a staple food or subsistence food by millions of people in the developing countries in Asia, Africa, Caribbean Pacific Islands and Central America (Sharma *et al*., 2008). Taro ranks fourteenth among staple vegetable crops and in world, taro is grown in an area of 1,72,4182 ha producing 10,22,1960 tonnes during 2016-2017 (Rao *et al.*, 2010; Singh *et al*., 2012 and FAO STAT 2017). Taro is the second most important root staple crop after sweet potato in terms of consumption (Singh *et al*., 2006) and is ranked the fourth root crop after sweet potato, yam and cassava in terms of its production by weight (Bourke and Vlassak, 2004).

It is the fifth most consumed root vegetable around the globe (Mishra, 2010). It is a very robust vegetable that can grow taller than 1.18 m, greatly sought after as a food vegetable because of its big tuberous rhizomes also known as tubercles (Madeira *et al*., 2008). It is mainly grown for its cormels and leaves (Lokesh *et al*., 2014).Taro is cultivated as an important staple up to an altitude of 2,200 m (Bourke *et al*., 1998).

In India, taro is cultivated in the states like Assam, Manipur, Himachal Pradesh, Gujarat, Tamilnadu, Maharashtra, Kerala, Andrapradesh, Telanagana, Uttarakhand, Orissa, Bihar, West Bengal (Choudhary *et al*., 2011).Taro is an important tuber crop of Uttara Kannada District of Karnataka, India (Lokesh *et al*., 2014). In Kerala, it is mainly cultivated in Pathanamthitta, Kollam and Alapuzha districts. In Kerala, tuber crops representing 1.92% area of food crops during the year 2017-18 and Colocasia leads 1st position in area under the cultivation of tubers and the percentage of colocasia to the total area of tubers is 40.20 %. The area of taro during 2017-18 is 7,418 ha, in which Kollam district stands in 1st position with an area of 1,302 hectares followed by Pathanamthitta with an area of 1182 ha (Department of Economics and Statistics, 2017-2018).

The common names of taro cultivars in major languages in India are Arvi, (Hindi), Chempu (Malayalam), Seppankizghangu (Tamil), Kachchi (Kannada), Chamadumpa and Chemagadda (Telugu), Alu (Marati) and Kachu in Sanskrit and Bengali (Edison *et al.*, 2003). The taro cultivars are often named after the shape and nature of corms such as 'Panch mukhi' (five faced) in parts of Assam, White Gauria and Thamarakannan in Kerala, 'Sahasra mukhi' in Maharashtra, (Nusaifa Beevi, 2009).

There are several kinds of related species *viz*., swamp taro (*Cyrtosperma chamissonis*), tannia (*Xanthosoma sagittifolium*), giant taro (*Alocasia macrorrhiza*) which are confused with the ordinary or true taro (*Colocasia esculenta*) prevalent in India. *Colocasia* is usually cultivated by family agriculturists in traditional populations for their own consumption and for exchange for other products (Yalu *et al*., 2009; Singh *et al*., 2008).

There are two botanical varieties characterized by their corm shape and described as var. *esculenta* (dasheen type) which possesses a large cylindrical central corm and only few cormels; and var. *antiquorum* (eddoe type) which has a small globular central corm with several relatively large cormels arising from the corm. Of this, eddoe is more common in India. It has been suggested that of the two varieties, *C. esculenta* var. *esculenta* is diploid and var. *antiquorum* is triploid (Kuruvilla and Singh, 1981; Irwin *et al*., 1998, Asha Devi, 2012). It is generally accepted that the majority of triploids are of Asian origin (Matthews, 1990). But controversies are reported pointing out that all varieties included in var. *esculenta* and var. *antiquorum* are not diploid and triploid respectively (Sreekumari, 1992; Nusaifa Beevi, 2009).

Cytology has been used to define genetic diversity for many years (Kuruvilla and Singh, 1981; Coates *et al*., 1988; Gunman and Dongxiao, 1990). Taro could be separated into either diploid or triploid types with x = 14 as the basic number of chromosomes ie., 2n = 28 and triploids with 3n = 42 (Caillon *et al*., 2006; Asha Devi, 2012). Chromosome numbers reported for taro from various regions include 2n = 22, 26, 28, 38 and 42 (Onwueme, 1978). Plants with 3n = 42 were referred as *alowane* which is male, large plant and those of 2n = 28 were referred to as *alokine* that is female, short plant which is mainly cultivated by Solomon Island farmers (Jackson *et al*., 1977; Wang, 1983). Research works done in ICAR- CTCRI also showed that taro consisted of both diploids and triploids (Sreekumari, 1992; Nusaifa Beevi, 1992).

2.2.1. History

Linnaeus was the first to describe taro in 1752 as belonging to 2 species *Arum colocasia* and *Arum esculanta* (Hill, 1939). Later, Schott, 2000 established the species *Colocasia* which is supposed to have derived from colas an Egyptian word indicating taro it has higher mineral content and medicinal value compared to other tuber crops. It was mentioned in Chinese books that taro and was being grown in Egypt in the beginning of the Christian era as early as 100 B.C and arrived to east coast of Africa 2000 years ago (Whitney *et al*., 1939; Plucknett *et al*., 1970). Taro is thought to have

originated in South Eastern Asia and is later thought to spread eastward to South East Asia, China, Japan and Pacific Islands (Purseglove, 1972; Lebot, 1999). In, India, taro is thought to have originated in North Eastern parts (Kuruvilla and Singh, 1981). Spencer (1966) stated that taro and other edible arioids were distributed from east India to Formosa and the Solomon Islands. It grows optimally in humid areas (2,000 mm rainfalls) and is cultivated for its starchy corm (Sardos, 2012). It has been pointed out that taro was the principal crop in the highlands before to the introduction of sweet potato (*Ipomoea batatas*) 300-500 years ago (Clarke, 1977; Bayliss-Smith, 1982, 1985).

2.2.2. Importance

Taro is recommended for gastric patients and taro flour is a good baby food (Parthasarathy, 1986). Taro corms are rich in calcium, phosphate and vitamins A, B and C (Warid, 1973). The protein content in taro leaves is much higher than that in corms (Bradbury and Holloway, 1988). The corms and cormels are rich in starch, but low in fat and protein (Gopalan *et al*., 1977). Taro corms and leaves are also has medicinal values which includes in many ayurvedic preparations to reduce tuberculosis, ulcers, pulmonary congestion and fungal infection. Taro is also used as a traditional medicine with root extract used to treat rheumatism, while leaf extract is used for blood clotting at wound sites, neutralizing snake poison and as a purgative medicine (Thinh, 1997). The nutritional value of taro is much more superior to other tuber crops and potato in many constituents such as protein, minerals, fiber, phosphorus, iron, vitamin etc. (Misra and Chowdhury, 1997). In addition to its contribution to sustained food security in the domestic market, it also brings in export earnings (Revill *et al.*, 2005; Jianchu *et al.*, 2001).

Almost all parts of the taro plant are utilized; corms are baked, roasted, or boiled as a source of carbohydrates, leaves are consumed as a vegetable representing an important source of vitamins. Taro corms are rich in starch, which can be utilized in various industries for preparation of high fructose syrups and alcohol. The small

particle size of taro starch and the efficiency with which colour can be added to particles make the taro starch suitable for cosmetic dusting preparations such as face powders and other cosmetic powders. Griffin (1979) has documented the possibility of using taro starch in the production of biodegradable plastics. The taro mucilage which swells in water and becomes highly hydrated may be used as emulsifying, thickening and smoothing agent or cream suspensions and other colloidal food preparation. The tiny particle size starch of taro and the proficiency with colour can be added to particles, renders the taro starch a good candidate in cosmetic industry for preparations such as facial powders and other cosmetic powders (Nath, 2015). According to USDA National nutrient data base (USDA SR-21), nutrition value per 100 g taro consists of carbohydrate, protein, energy, total fat and dietary fibre in the amount of 26.46 g, 1.50 g, 112 Kcal, 4.1 g respectively and the vitamins such as folates, niacin, pantothenic acid, pyridoxine, riboflavin, thiamine, Vitamin A, Vitamin C, Vitamin E, Vitamin K in the amounts 22 µg, 0.600 mg, 0.303 mg, 0.283 mg, 0.025 mg, 0.095 mg, 76 IU, 4.5 mg, 2.38 mg and 1 µg respectively.

2.2.3. Varieties of taro

Taro varieties such as Sree Kiran (susceptible), Muktakeshi (tolerant), Sree Rashmi, Sree Pallavi, Satamukhi, Katyan, Pani Saru-1, Pani Saru -2, Topi. Telia, Thamarakannan, Jhankri, Sonajuli, Gyano, Mantri, Panchmukhi, Arvi. Kumar and Dubey (1996) reported screening of *Colocasia* genotypes for the disease. Several varieties of taro such as 'Ahina', 'Poonam Pat', 'Salem V', 'Bhadia Kachu', 'Naga Kachu', 'Pusa Sakin II' and 'Simla' are stated to be resistant to blight.

2.2.4. Morphology

Plants of the genus Colocasia are herbaceous, often with large leaves and bearing one or more underground stems or corms. Taro, the principal edible species, is a succulent, glabrous, perennial herb. The above ground portion of a taro plant is composed of large leaf lamina on long erect petioles. Taro possesses enlarged, starchy,

underground stems which are properly designated corms. These have been found to be highly variable with respect to hydration, size, color, and chemistry. The corm is composed, outwardly, of concentric rings of leaf scars and scales. It bears one or more smaller secondary cormels which arise from lateral buds present under each scale or leaf base (Miller, 1971).

Anatomically, the corm is composed of a thick, brown outer covering and starchy ground parenchyma. The root system of taro is adventitious and fibrous. All parts of the taro plant contain acrid principles, which are irritating to the mouth and esophagus which is destroyed by fermentation. The acridity is due to the presence of the calcium oxalate crystals in the taro plant. The density of crystals in corms may be as high as 120,000/cm (Wang, 1983).

2.2.5. Diseases of taro

Taro is susceptible to attack by over thirty pathogens. Phytophthora blight (*Phytophthora colocasiae*) and Pythium root and corm rot (*Pythium spp.*) are the most serious fungal diseases of taro.

The major fungal diseases infecting taro are *Phytophthora* Leaf Blight caused by *Phytophthora colocasiae*, Pythium Rot by *Pythium aphanidermatum*, Phyllosticta Leaf Spot by *Phylosticta colocasiophila*, Cladosporium Leaf Spot caused by *Cladosporium colocasiae*, Spongy Black Rot by *Botryodiplodia theobromae*, Fusarium Dry Rot caused by *Fusarium solani*, Corm rot by *Athelia rolfsii*, Brown leaf spot spot (or ghost spot) by *Cladosporium colocasiae*, Spongy black rot by *Lasiodiplodia theobromae*, White spot of taro by *Leptosphaerulina trifolii*, Corm and leaf spot by *Marasmiellus stenophyllus*, Orange leaf spot by *Neojohnstonia colocasiae*, Shot hole by *Phoma* spp, Leaf blotch by *Pseudocercospora colocasiae*, Corm soft rot by *Pythium* spp. etc (Carmichael *et al*., 2008).

Viruses are one of the most important pathogens along with TLB with some infections resulting in severe yield reductions and plant death. There are currently five viruses reported to infect taro majorly with varying distribution in taro growing region

and throughout the Pacific Islands i.e. *Dasheen mosaic virus* (DsMV), *Taro bacilliform virus* (TaBV), *Colocasia bobone disease virus* (CBDV), *Taro vein chlorosis virus* (TaVCV) and *Taro reovirus* (TaRV) (Revill *et al*., 2005).

2.2.6. Taro Leaf Blight (TLB)

According to Fisher *et al.* (2012) more than 600M people could be fed each year by halting the spread of fungal diseases in the world's five most important crops alone. Taro leaf blight (TLB) caused by *Phytophthora colocasiae,* an oomycete water mould, is one of the most devastating diseases of taro resulting in severe yield reductions and plant death. The pathogen causes circular, water soaked, necrotic spots on the leaves, followed by the collapse of the plant. During favorable conditions (intermittent rainy weather), the entire field is devastated within few weeks of the onset of infection. The disease is prevalent in all taro growing regions of the globe including India causing yield reductions of the magnitude of 30-50% (Jackson, 1999; Lebot *et al*., 2003; Misra *et al*., 2008; Singh *et al*., 2012).

Taro leaf blight infected by *Phytophthora colocasiae* Raciborski, contributes to the most critical and destructive fungal disease accountable for high loss in yield and eventually plant death (25 to 50%) of taro in India (Misra, 1997; Gadre and Joshi, 2003). To raise the complications, this pathogen also causes a severe post- harvest decay of taro corms (Jackson and Gollifer, 1975; Misra, 1997).

Disease starts with initial symptoms of small brown water soaked flecks on the leaf lamina that magnifies to appear as dark brown lesions, with a yellow margin. At night, the lesions expand by developing a 3-5 mm wide water-soaked margin (Misra, 2008). This margin dries out during the day and a newer water-soaked zone forms the following night (Fullerton and Tyson, 2001; Singh *et al*., 2012).The second state of infection is rapid destruction of the leaf, a petiole rot within a span of 10-20 days, or even less in many susceptible varieties. An obvious and characteristic feature of TLB is the formation lesions with colour varying with amber, bright-orange, or reddish-brown exudate, exuding from the upper and lower surface of the water-soaked margins.

The disease radically reduces the number of functional leaves and can result in yield reductions up to the intensity of 50% (Trujillo and Aragaki, 1964; Trujillo, 1967; Jackson, 1999). Masses of sporangia produced on the spreading margin of the lesion at night, imparts a white powdery appearance to the lesions. Spores are spread by wind driven rain and dew to the nearby plants and plantations and new planting materials thereby spreading diseases to other countries if strict quarantine measures are not undertaken (Gregory, 1983).

Onset of disease starts with monsoon, rainfall, dew, or guttation droplets and continues till the end of monsoon. Initial symptoms appears as small, water soaked lesions that increase in area and eventually spread to healthy plants and seen as large dark brown spots (Singh *et al*., 2012; Nelson *et al*., 2011). During night, the lesions expand by developing a 3-5 mm wide water-soaked margin. This margin dries out during the day and a newer water-soaked zone forms the following night (Fullerton and Tyson, 2001). Eventually leaf area is destroyed within few days. Under cloudy weather conditions with intermittent rains and temperature around 28^0C, the disease spreads at tremendous speed and the entire field gives a blighted appearance (Misra *et al*., 2007).

Causal organism

Phytophthora colocasiae
Kingdom- Chromista
Phylum- Heterokontophyta
Class- Oomycota
Order-Peronosporales
Family- Peronosporaceae
Genus- *Phytophthora*
Species- *Phytophthora colocasiae*

Phytophthora derived from Greek phyton, 'plant' and phthora, 'destruction'; 'the plant-destroyer' is a genus of plant-damaging oomycetes (water moulds), whose member species are capable of causing enormous economic

losses on crops worldwide, as well as environmental damage in natural ecosystems. The genus was first described by Heinrich Anton de Bary in 1875 (Brasier, 2009).

P. colocasiae is generally a diploid fungus and needs the existence of A1 and A2 mating types for production of sexual oospores. The oospore serves as the overwintering propagules and as a source of initial inoculum for spreading of the disease and renders genetic variability through possible new gene combination (Mc Donald, 2002; Nath, 2016). If the sexual spores are absent, the pathogen sustains as asexual clones in the infected plant or tubers. Mycelia of such infections produce many sporangia that are propagated by wind or rain-splashes.

2.2.7. History

Marian Raciborski was the first scientist to report *Phytophthora colocasiae*. The disease was first reported in India (Butler and Kulkarni, 1913) and reported from Java in 1900 and this disease alone brought a decline in taro cultivation and production (30-50%) in the Solomon Islands, Papua New Guinea, Philippines, Indonesia, China, Malaysia, Japan, India and countries of Africa and Caribbean. In India, leaf blight is reported to be a serious disease in many areas such as Kangra valley of Punjab which is now in Himachal Pradesh (Luthra, 1938), Assam (Chowdhury, 1944), Bihar (Anonymous, 1950), Himachal Pradesh (Paharia and Mathur, 1961) and other states (Prasad, 1982; Thankappan, 1985; Misra, 1999). In India, it is causing up to 50% yield loss (Misra, 1999; Misra *et al*., 2008) and TLB has been confirmed in many of the countries in the Pacific region most lately in Nigeria and West Africa (Bandyopadhyay *et al*., 2011). The geographic distribution of this disease is probably restricted to South-East Asia and the Pacific Areas (Holliday, 1980; Misra, 2008). Since taro is relatively used by local communities than international market, its disease management is neglected (Gregory, 1983).

The disease considerably diminishes the number of functional leaves leading to yield loss up to the magnitude of 50% (Trujillo and Aragaki, 1964; Trujillo, 1967; Jackson, 1977). The disease also spreads on to taro planting material and this fungus

has been identified on being alive for about 3 weeks post harvest on planting crops (Jackson, 1977). The incidence of taro leaf blight has been reported from different countries (Table 2.1).

Table 2.1 Occurrence of leaf blight of taro reported from different countries (cited

from Misra *et al.,* 2008)

Country	Reported by	Year
Indonesia	Raciborski	1900
India, Formosa	Butler and Kulkarni	1913
Fujian, China	Lin	1937
Sri Lanka	Park	1939
Taiwan	Sawada	1911
Marinas, Carolines and Burma	Anon	1943
Philippines	Gomez	1925
Malaysia	Thompson	1939
Hawaii	Parris	1941
Papua New Guinea	Shaw	1963
	Hicks	1967
	Putter	1976
Solomon Islands	Johnston	1960
Trust Territories of Pacific Islands	Trujillo	1971
Indonesia, Malaysia, Sarawak, Africa and Caribbeans	Anon	1978
Japan	CABI	1997
Equatorial Guinea, Ethiopia, Seychelles	Fernando Po, CABI	1997
Argentina	CABI	1997
Nigeria	Bandyopadhyay *et al*	2011
Ghana	Omane *et al*	2011

2.2.8. Management of the disease caused by *Phytophthora colocasiae*

Every year more than ten percent of the total crop yield is lost due to disease (Strange and Scott, 2005). There are several methods for managing leaf blight of *P. colocasiae* such as development of new agricultural practices, strict quarantine measures, breeding of resistant cultivars, cultural methods, use of pesticides and fungicides, screening of planting materials, genetic engineering but the use of tolerant cultivars seems to be most ideal and economical method (Misra, 2008). Resistant cultivars offer the best sustainable management strategy against taro leaf blight. There were relative positives with this technique as molecular studies clearly reported the existence of two distinct gene pools in Asia and the Pacific, with the diversity in Southeast Asia being far greater than that found in the Pacific (Lebot and Aradhya, 1991; Mace *et al.*, 2006). Leaf blight is a fast infecting and vastly destructive infection. Combining the managing patterns together with scrutiny can reduce and manage the disease incidence to a considerable level. Major management aspects to control taro leaf blight are discussed below.

1. Cultural control
2. Biological control
3. Fungicidal control
4. Use of resistant cultivars
5. Integrated disease management

2.2.8. 1. Cultural control

Disease management becomes effective if there is control in the inoculum level and humidity of field. Roughing or removal of infected leaves and the use of healthy corm as planting material along with crop rotation was found to be effective (Mundkur, 1949). Jackson *et al.* (1980) on the other hand found that removal of infected leaves did not help in reducing the disease incidence; wider than traditional spacing also did not reduce the blight incidence. Some provisional taro growers hamper the severe taro leaf blight infection by planting during dry season (Singh *et al.*, 2012).

Planting time can be shifted in the crucial stage of plant growth and optimum climatic conditions for disease development do not coincide with each other. In a field trial at the Regional Centre of CTCRI, Bhubaneshwar (India) studied the effect of planting time on the incidence of leaf blight and tuber yield, by Misra (1988, 1989, 1990) found that May planting gave highest tuber yield and escaped much of the damage caused by blight (Misra and Chowdhury, 1995).

2.2.8.2. Biological control

Several fungi and bacteria completely inhibited the growth of *P. colocasiae* (Misra *et al*., 2001). Some *Rhizobacteria* formed inhibition zones with *P. colocasiae* in dual plate culture (Anonymous, 2000). *Myrothecium roridum*, phylloplane microflora, *Streptomyces* were found as antagonistic to *P. colocasiae* studied by Narula and Mehrotra (1981, 1987). *In vivo*, the bacteria reduced the disease incidence up to 43%; *Streptomyces albidoflavus* reduced infection by 90-93% and *S. diastaticus* by 76%. *Botrytis cinerea* a fungus gave the best control of 33% reduction of plant infection. *Trichoderma viride*, *Trichoderma harzianum*, *Gliocladium virens* have potential antagonism against *P. colocasiae* (Sawant, 1995; Pan and Ghosh, 1997).

Mycoparasitic or hyperparasitic activities of *Trichoderma* species on *P. colocasiae* were brought by inducing morphological changes like coiling of hyphae, formation of haustoria-like structures, disorganization of host cell contents and penetration into host hyphae. *Rhizobacteria* completely inhibit the growth of *P. colocasiae in vitro. Trichoderma viride* effectively inhibited the population of *P. colocasiae* up to 88.88%, whereas *T. harzianum* and *T. pseudockei* reduced the population of *P. colocasiae* up to 77.77 and 88.88%, respectively (Mishra *et al*., 2008).

Veena *et al*. (2013) studied about the effect of vermicompost and vermiwash in controlling the taro leaf blight and collar rot of elephant foot yam disease. The vermicompost and vermiwash treated plants showed less than 10% TLB incidence and 0-50% collar rot incidence. The yield was increased in both the crops with respect to the application of vermicompost.

Nath *et al*. (2014) studied antagonistic potential of three *Trichoderma viz*., *Trichoderma viride*, *Trichoderma asperellum* and *Trichoderma harzianum* and found that *Trichoderma harzianum* was able to control *Phytophthora colocasiae in vivo*. Similarly, Ambuse and Bhale (2015) studied the efficacy of *Trichoderma* spp against sensitive and resistant isolates of *P. colocasiae* by dual culture method. *Trichoderma viride, T. harzianum, T. virens, T. koningii* and *T. pseudokoningii* species were used for antagonistic study. *Trichoderma viride* and *T. harzianum* showed 77.77% antagonism than others.

Nguemezi Tchameni *et al*. (2017) assessed the antagonistic effects of four different *T. asperellum* isolates against *Phytophthora megakarya* and their ability to enhance cacao growth and biochemical defence. All the isolates of *T. asperellum* were antagonistic to *P. megakarya*. In pot experiments, leaf number, plant height, shoots and root dry matter were significantly increased by *T. asperellum*. Amino acid and phenolic components content increased in either healthy or infected leaves from cacao plants inoculated with *T. asperellum*. The induction of specific amino acids such as alanine, glutamic acid and methionine play an important role in the adaptation of cacao plant to *P. megakarya* infection. These findings shows that *Trichoderma asperellum* could be used to improve the development of cacao plants and protect the plant against *Phytophthora megakarya*.

Shobha *et al*. (2019) used two strains of rhizobacteria, *Bacillus subtilis* and *Pseudomonas fluorescens*; two strains of endophytic fungi *Trichoderma viride* and *T. asperellum* for assessing systemic resistance against *Phytophthora capsici* for biological control of foot rot disease of pepper. Black pepper vines grown under greenhouse were challenge inoculated with *P. capsici* for analyzing the disease index, chitinase, peroxidase, phenylalanine ammonia lyase activity, polyphenol oxidase, β-1,3-glucanase assay and estimation of phenol content. *B. subtilis* and *T. viride* isolates reduced per cent disease incidence (20 and 18% as compared to untreated control) and increased the enzyme level also. Thus, *B. subtilis* and *T. viride* can be used as biocontrol agent for prevention of *P. capsici* infections in black pepper.

2.2.8.3. Fungicidal control

Copper fungicides showed to be very effective in successful controlling of taro leaf blight in many places like Fiji (Parham, 1949); India (Mundkur, 1949); Hawaii (Parris, 1941; Trujillo and Aragaki, 1964; Bergquist, 1972, 1974) and Solomon islands (Jackson *et al.*, 1980). Application of copper oxychloride at a time interval of 14-day significantly control the disease (Jackson and Gollifer, 1975). Dithane M-45, Polyram, Benlate, Perinox and Dyrene were also seen to be very effective in controlling taro leaf blight (Maheswari *et al.*, 1999). Metalaxyl was found to be one of the best candidates when used under different combinations by many scientists (Nelson *et al.*, 2011). Metalaxyl successfully inhibit the cellulolytic and pectinolytic enzymes formed by *P. colocasiae.* Metalaxyl, captafol and chloroneb were found to be effective in controlling *P. colocasia*e under *in vitro* and *in vivo* (Aggarwal and Mehrotra, 1987). The effect of fungicides in controlling leaf blight caused by *P. colocasiae* in *C. esculenta* revealed that 0.2% metalaxyl and mancozeb was the most effective treatment, followed by 0.2% captafol, bordeaux mixture (1% copper sulfate and lime) and 0.25% mancozeb (Bhattacharyya and Saikia, 1996).

Metalaxyl with copper gives excellent control of the disease when applied at 2-week interval (Cox and Kasimani, 1988). Das (1997) reported the efficacy of copper oxychloride, mancozeb, metalaxyl, captafol, ziram and Bordeaux mixture against leaf blight disease of taro var. *antiqourum.* Taro leaf blight controls are reported by using a fungicide spray (Forschek, a phosphorous acid-based product) to control the disease (Adams, 1999). Four sprays of zineb at 15-day intervals starting from the end of July to early August reduced the incidence of *P. colocasiae* in *C. esculenta* reported by *(*Sahu *et al.*, 1989). Spraying with metalaxyl at 3 kg/ha at 15-day intervals was highly effective in controlling the disease (Ghosh and Pan, 1991). Cox and Kasimani (1990) found that 5 applications of metalaxyl at 3- week intervals resulted in an increase of almost 50% in tuber yield. Leaf blight of taro has also been reported to be controlled by spraying 500 ppm of borax. Spraying of 500 ppm borax showed a significant

reduction in the leaf area damaged per plant by leaf blight and increased the corm/ cormel yield (Misra *et al*., 2007).

Nath *et al*. (2013) evaluated various fungicides *viz*., Metalaxyl, Samarth, Biofight and Akoton against Indian isolates of *Phytophthora colocasiae* causing leaf blight of taro. All the isolates were sensitive to metalaxyl. The most useful fungicide determined by *in vitro* method was evaluated *in vivo* for studying the pattern of inhibition before and after the disease development in detached taro leaf. Fungicide Samarth can be use as an substitute to metalaxyl for management of taro leaf blight.

Moise *et al*. (2018) evaluated the effect of *Trichoderma harzianum* (Edtm) and *Trichoderma aureoviride* (T4) as bio-control agents against *P. colocasiae*. The efficiency of metabolites in *P. colocasiae* was evaluated by poisoning method. The ability of the two antagonists to induce defense-related metabolites was done in pot experiments. In dual culture, the inhibition of the mycelia growth of the *P. colocasiae* was 34.77 and 41.77% for *T. harzianum* (Edtm) and *T. aureoviride* (T4), respectively. In pot cultures, *T. harzianum* (Edtm) and *T. aureoviride* (T4) significantly reduced (49.4 and 46.4%, respectively) the necrosis of taro leaf blight. Significant increase in the activities of PAL, polyphenol oxidase and polyphenol content was noted in healthy and infected taro plants.

2.2.8.4. Use of tolerant cultivars

Deshmukh and Chibber (1960) identified var. 'Ahina' as resistant to blight as it produced less no of sporangia compared to susceptible variety. Paharia and Mathur (1964) found var. 'Poonam Pat' as immune, 'Sakin V' as resistant. Misra (1988, 1989, 1990) screened 43 cultivars of *Colocasia* and cvs. 'Jankhri' and 'Muktakeshi' as highly tolerant to blight. The *P. colocasiae* glucan elicitor (Sriram *et al*., 2001) induce hypersensitive reaction in the tolerant cultivars like Muktakeshi and Jankhri this induced hypersensitive reaction was not elicited in the susceptible variety like Telia. Trujillo (1967) advocated the development of resistant varieties through breeding and selection, as resistance is already present in the Pacific area within the genus *Colocasia*.

Ho and Ramsden (1998) found that proteinase inhibitors were important factors in disease resistance in taro. The variety such as 'c-320', 'c-12', 'c-78' and 'Nadia local' obtained through breeding in ICAR-CTCRI, Trivandrum showed resistance of 66%, 33.33%, 30% and 26.31% respectively (Pillai *et al.*, 1993). The appearance of resistance genotypes in the population resulting from crosses between two partially susceptible genotypes was observed (Ivanicic *et al.*, 1995).

2.2.8.5. Integrated disease management

A farmer-friendly IDM package which includes for the management of the taro blight was developed (Misra *et al.*, 2001). In this package the short-duration crop with early planting i.e., in March, one protective spray with mancozeb (0.2%) at 45 days after planting followed by one spray with metalaxyl (0.05%) at 60 days after planting, intercropping with non-host crops like okra, use of disease free seed tubers and seed tuber treatment with *Trichoderma viride*. Use of tolerant cv. 'Muktakeshi' is also effective with mancozeb (0.25%) initially if the disease incidence occur and Ridomil MZ (0.2%) spraying after 15 days of mancozeb spray is found very successful (Mishra *et al.*, 2007). Benzyl amino purine mediated inhibitory effect of *P. colocasiae* was found *in vitro* by retardation of *P. colocasiae* to combat taro leaf blight was studied (Mishra *et al.*, 2008).

2.3. PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

The term 'rhizobacteria' implies a group of rhizosphere bacteria competent in colonizing the root environment (Kloepper *et al.*, 1991). These soil bacteria are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production (Chandler *et al.*, 2008; Ahemad *et al.*, 2009) by stimulating plant growth through mobilizing nutrients in soils, producing numerous plant growth regulators, protecting plants from phytopathogens by controlling or inhibiting them, improving soil structure and bioremediating the polluted soils by sequestering toxic heavy metal species and degrading xenobiotic

compounds (Braud *et al*., 2009; Hayat *et al*., 2010; Rajkumar *et al*., 2010; Ahemad and Malik, 2011; Ahemad, 2012).

The compounds secreted by plant roots ie., plant exudates *viz*., amino acid, organic acids, sugars, vitamins, enzymes and other gaseous molecules act as chemical attractants for a vast number of heterogeneous, diverse and actively metabolizing soil microbial communities. These exudates modify the chemical and physical properties of the soil and thus, regulates the structure of soil microbial community in the immediate vicinity of root surface (Dakora and Phillips, 2002; Ahmed and Kibret, 2014).

Plant growth promoting rhizobacteria (PGPR), are proficient to colonize the root surface, survive, multiply and compete with other microbiota, at least for the time needed to express their plant growth promotion/protection activities, promote plant growth (Kloepper, 1994). About 2-5% of rhizobacteria, when reintroduced by plant inoculation in a soil containing competitive microflora, exert a beneficial effect on plant growth and are termed as plant growth promoting rhizobacteria (Kloepper and Schroth, 1978).

Somers *et al*. (2004) classified PGPR based on their functional activities as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites) (Antoun and Prevost, 2005).

PGPR mediated plant growth promotion occurs by the alteration of the whole microbial community in rhizosphere niche through the production of various substances (Kloepper and Schroth, 1981) by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Glick, 2012).

Lee *et al*. (2015) screened out 78 *Bacilli* for the stimulation of ISR and found that *Bacillus amyloliquefaciens* strain HK34 showed disease reduction up to 99.1%

against *Phytophthora cactorum* when applied to root. An enhancement in expression of genes *PgPR10*, *PgPR5*, and *PgCAT* in plant leaves was also observed after treatment with HK34. These outcomes showed the ISR-eliciting potential of strain HK34.

Biocontrol capacity of two plant growth promoting rhizobacteria (PGPR) strains *viz*., *Pseudomonas fluorescens* and *Chryseobacterium balustinum* against blast diseases in rice paddy fields were studied by (Lucas *et al*., 2009). They both showed comparatively low disease incidence. They found that the most effective method was the combination of both strains inoculated to leaves suggested a biocontrol mediated protection, when applied to seeds, the disease incidence decreased up to 50%, suggesting systemic resistance which indirectly increased the rice productivity and quality.

The systemic resistance and growth promotion in pepper by an antibiotic producing *Bacillus vallismortis* strain BS07 was studied by (Jin Woe Park *et al*., 2013). Soil drench suppressed the soft root incidence significantly by reducing the percent disease lesion and there was a significant reduction in anthracnose infection caused by *Colleotrichum acutatum*.

Maleki *et al*. (2010) isolated 144 bacteria from cucumber rhizosphere and screened against *Phytophthora drechsleri,* causal agent of cucumber root rot, *in vitro* and greenhouse condition. On the basis of dual culture assays, eight isolates were selected for root colonization, PGPR and greenhouse studies. Among these isolates, isolate CV6 exhibited the highest colonization on the roots and promote plant growth under *in vitro* condition.

Rohini *et al*. (2016) studied about the phylloplane colonizing bacteria (PCB) and rhizosphere colonizing bacteria (RCB) individually and in combinations for plant growth promotion and control of Phomopsis leaf blight of brinjal (*Solanum melongena* L.). All RCB strains were characterized for their beneficial traits and their leaf and root colonizing ability were confirmed through SEM. Under greenhouse conditions, individual applications such as seed treatment with *Pseudomonas putida* Has-1/c (RCB) significantly increased the plant growth and leaf surface area, respectively.

Syed-Ab-Rahman *et al*. (2018) studied the bacterial isolates obtained from the rhizosphere of *Arabidopsis* and a plant less compost potting mix was screened for anti-oomycete activity against *Phytophthora capsici*, *P. citricola*, *P. palmivora* and *P. cinnamomi*. Three out of 48 isolates exhibited more than 65% inhibition. These strains, named UQ154, UQ156, and UQ202, are closely related to *Bacillus amyloliquefaciens*, *B. velezensis* and *Acinetobacter* sp., respectively, based on 16S rDNA sequence analysis.

The isolates were evaluated for their capability to fix nitrogen, solubilize phosphate, siderophore, indole acetic acid, cell wall degrading enzymes and biofilm production. The plant growth promoting activities were measured based on the germination percentage, root and shoot length, and seedling vigor of lettuce plants. Bacteria-inoculated *P. capsici* infected chili plants exhibited improved productivity based on CO_2 assimilation rates. The potential of bacterial isolates to control *Phytophthora* infection and promote plant growth.

Guo *et al*. (2019) studied bio-control of PGPR strain *Bacillus amyloliquefaciens* Ba168 against tobacco black shank (TBS) caused by *Phytophthora nicotianae* on tobacco. The study revealed the suppression potential of Ba168 strain.

Among plant growth regulators, indole acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth (Miransari and Smith, 2014). Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit (Shih-Yung, 2010). The microorganisms isolated from rhizosphere region of various crop have an ability to produce Indole acetic acid as secondary metabolites due to rich supply of substrates (Mohite, 2013). Up to 80% of rhizobacteria can synthesize indole acetic acid (IAA) colonized the seed or root surfaces is proposed to act in conjunction with endogenous IAA in plant to stimulate cell proliferation and enhance the host's uptake of minerals and nutrients from the soil (Vessey, 2003).

Tryptophan is an amino acid commonly found in root exudates, has been identified as main precursor molecule for biosynthesis of IAA in bacteria (Etesami *et al*., 2009).

Bacillus pumilus isolate yielded 5.6 mg/biomass IAA co-production along with a potent BLIS (bacteriocin like inhibitory substance) production (Zaghiyan *et al*., 2012). *Bacillus subtilis, B. megaterium*, *B. subtilis* subsp. *Subtilis* and *Pseudomon as* sp where inoculated to peanut root, produced indole-3-acetic acid, hormone and biofilms that clearly promote peanut root growth (Yuttavanichakkul *et al*., 2012).

Das *et al*. (2016) done an attempt to screen and identify PGPR traits in bacteria which were isolated and characterized from rhizospheric soils. Fourteen pure cultures were selected for *in vitro* screening of isolates for their plant growth promotion activities. The 14 isolates were able to produce IAA as well as positive for Phosphate solubilization and Ammonia Production whereas negative for Cyanide production. With the addition of tryptophan from 50 to 500 μg/ml the production of IAA was increased.

2.4. PATHOGENESIS RELATED (PR) PROTEINS

A group of plant coded proteins induced by different stress stimuli, named "pathogenesis-related proteins". PR protein in plants was first discovered and reported in tobacco plants infected by *tobacco mosaic virus* (Van Loon and Van Kammen, 1970). The term PR was coined Antoniw in 1980. Several monocot and dicot plants have been found to produce PRs through a ubiquitous reaction during pathogen attack (Lee, 2011; Upadhyay *et al*., 2014). These pathogenesis related responses and inhibition of fungal growth because of these proteins proved their defensive functions in the plant (El-kereamy *et al.*, 2011).

Classification of PR proteins (PR-1 to PR-17) based on the antifungal effect, Chitinase and β-1,3-Glucanase activity. Plant reactions to various threats including physical, chemical and biological stresses, such as wounding, exposures to salinity, drought, cold, heavy metals and pathogen attacks, like fungi, bacteria and viruses which involve the activation of set of genes, encoding different proteins such as physical strengthening of the cell wall through lignification, suberization and callose

deposition inducing production of phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins (Agrios, 1997).

Induction of systemic resistance in Panax ginseng against *Phytophthora cactorum* by native *Bacillus amyloliquefaciens* HK34 was studied by (Lee *et al*., 2009). Leaves from the plant treated with HK34 induced systemic resistance against *P. cactorum.* The percentage of disease control was 85.6% by inducing the production of small acidic intracellular proteins (PR10 gene expression), thaumatin and osmotin like proteins (PgPR 5 gene expression) and reactive oxygen species (ROS) mediated out brust to increase cellular redox reactions to maintain homeostasis (PgCAT gene expression).

El-Kereamy *et al*. (2009) studied the expression analysis of a plum pathogenesis related 10 (PR10) protein during brown rot infection also increased the production of small acidic intracellular protein by the expression of PgPR 10 genes which combated the brown root infection. Similar studies done by Kim *et al*. (2008) to combat rice pathogen, and Liu and Ekramoddoullah (2006), in pepper for antiviral pathway Park *et al*. (2004) and in *Zea mays* (Xie *et al*., 2010). Defense genes induced by pathogens and abiotic stress in Panax ginseng was studied by Lee *et al*. (2011). He analyzed the production of thaumatin and osmotin like proteins for combating the pathogens and protecting the plant. Similar studies were done in Panax ginseng (Kim *et al*., 2009; Vigers *et al*., 1991).

Purev *et al*. (2010) studied about the isolation of a novel catalase gene from Panax ginseng and analysed the response of this gene to various stresses and identified the production of ROS to wake up the antioxidative machineries like catalase by upregulating PgCAT expression. Miller *et al*. (2010) performed similar studies for plants under drought and salinity stresses to increase the PgCAT signaling. Apel and Hirt (2004); Sathiyaraj *et al*. (2011) performed studies in the same aspect by increasing the antioxidative machineries to reduce biotic and abiotic stresses.

2.5. ENZYMES

The use of antagonistically important *Bacillus* species is increasing very rapidly. *Bacillus* species have a inimitable ability to replicate quickly, resistant to unfavorable environmental conditions and is used as a biocontrol agent for a vast spectrum of host. Volatile compounds produced by *B. subtilis* also play an important part in plant growth promotion and eliciting plant defence mechanism by triggering the induced systemic resistance (ISR) in plants (Compant *et al*., 2005). The US Food and Drug Administration (US FDA) declared *B. subtilis* as GRAS organisms for its use in food processing industries (Denner and Gillanders, 1996). Endosporic and enzymatic products of *B. subtilis* were found highly active against many fungal pathogens (Shafi *et al*., 2017).

Durairaj *et al*. (2018) studied seven fungal genera isolated from infected ginseng root rot samples and reported that *Pseudomonas aeruginosa* and *Bacillus stratosphericus* strains inhibited the mycelial growth of fungal phytopathogen.

2.5.1. Chitinases (E.C. 3.2.1.14)

Chitin, a linear polymer of β-1,4-N-acetylglucosamine (GlcNAC), is the second most abundant biopolymer on the planet. Chitinases (E.C. 3.2.1.14) are enzyme that mediates the breakage of a bond between C1 and C4 of two consecutive N acetyl-D-glucosamine monomers of chitin. They are widely distributed in nature, occurring in bacteria, fungi, animals and plants. Chitinases have the ability to degrade chitin directly to low molecular weight chitooligomers, which serve a broad range of industrial, agricultural, and medical functions such as elicitor action and anti-tumor activity (Yuli, 2004). Chitinases have also attained a lot of attention as they are thought to play a key role in mosquito control and plant defense systems against chitin-containing pathogens (Hamid *et al*., 2013).

A number of bacteria have the ability to produce chitinases, including *Streptomyces*, *Alteromonas*, *Escherchia*, *Aeromonas*. *Bacillus* and *Serratia* which produces four different types of chitinases. Most of the bacterial chitinases, which have

been isolated and sequenced so far, are included in family 18 of the glycosyl hydrolases; with the exception of a chitinase (C-1) isolated from *S. griseus* that belongs to the family 19 of the glycosyl hydrolases (Hamid *et al*., 2013). Bacterial chitinases are active over a wide range of pH and temperatures, depending on the source of the bacteria from which they have been isolated. The production of chitinases in bacteria is mainly for the degradation of chitin and its utilization as an energy source. Endochitinase from *Streptomyces violaceusniger* (Shekhar *et al*., 2006). Bacterial chitinases show a broad range of isoelectric points ranging from 4.5 to 8.5 (Hamid *et al*., 2013).

Chitin is a major cell wall component of fungi. Fungal chitinases show a high amino acid homology with class III plant chitinases (Hayes *et al*., 1994). Serine/threonine rich-region, chitin-binding domain, and C-terminal extension region is absent in most of the fungal chitinases, and these seem to be unnecessary for chitinase activity because naturally-occurring chitinases that lack these regions are still enzymatically active (Hamid *et al*., 2013). Fungal chitinases have been divided into fungal/plant chitinases, which correspond to class III chitinases and show similarity to class V chitinases from plants, fungi, and bacteria (Takkaya *et al*., 1998).

Plant chitinases are usually endo-chitinases capable of degrading chitin, a major constituent of certain fungal cell walls as well as inhibit fungal growth (Broekaert *et al*., 1988; Schlumbaum *et al*., 1986) which are localized in vacuole and other chitinases such as class 3 are localized outside the cell. Extra cellular chitinases block the growth of hyphae and release fungal elicitors which then induce additional chitinase biosynthesis and further defense reaction (Barber *et al*., 1989; Mauch and Staehelin, 1989). Chitinase, together with β-1,3-glucanase participate in the plant defense system against fungal pathogens. Chitin and β-1,3-glucanase are major components in the cell wall of many fungi and there is possibility of plant chitinase and β-1,3- glucanase target fungi cell wall components as substrate and has antifungal function (Abeles *et al*., 1970; Pegg, 1988).

Chitinase are classified into two categories, endochitinases and exochitinases. Endochitinases produce chitotrose, chitotetraose and upon cleavage and exochitinase produces di-acetylchitobios and monomer N-acetylglucosamine upon catalysis (Cohen-Kupiec and Chet, 1998).

Plant chitinases are produced as pathogenesis related proteins in plant self defense in response to the attack of phytopathogens, or by contact with elicitors such as chitooligosaccharides or growth regulators such as ethylene (Hamid *et al*., 2013). The chitinases of plants are generally endochitinases of smaller molecular weight as compared to the chitinases of insects (Hamid *et al*., 2013).

Purified chitinase inhibited growth of only one fungal species whereas a combination of chitinase and another PR protein, β-1,3-glucanase, inhibited the growth of all fungi tested showing a synergism in activities (Mauch *et al*., 1988). Various studies have shown that chitinase expression against phyto-pathogen systems is higher and induction is stronger in the resistant varieties in comparison to susceptible varieties in the sugar beet (Nielsen *et al*., 1993), wheat (Anguelova *et al*., 2001) and tomato varieties (Lawrence *et al*., 2000) but no difference in the induction timing or amounts of PR-protein in resistant and susceptible cultivars of cotton (McFadden *et al*., 2001). However, quick response in the resistant cultivars might affect the cell wall of germinating fungal spores, releasing elicitors leading to the expression of PR-genes and disease resistance.

Wang *et al*. (2004) studied the application of electrospray ionization mass spectrometry in rapid typing of fengycin homologues produced by *Bacillus subtilis*, *B. amyloliquefaciens* V656 synthesized two types of chitinases enzymes and both significantly inhibited *Fusarium oxysporum* growth.

2.5.2. Glucanase (E.C.3.2.1.6)

Plant β-1,3-glucanases are pathogenesis-related (PR) proteins, which belong to the PR-2 family of pathogenesis-related proteins and are believed to play an important role in plant defense responses to pathogen infection. β-1,3-glucanases are also found

in yeasts, actinomycetes, bacteria, fungi, insects and fish (Boller, 1985; Pan *et al.*, 1989). β-1,3-glucanases are able to catalyze the cleavage of the β-1,3-glucosidic bonds in β-1,3-glucan (Simmons, 1994). β-1,3-glucan is a another major structural component of the cell walls of many pathogenic fungi (Wessels *et al.*, 1981; Adams, 2004). *Phytophthora infestans* is an oomycete pathogen that causes late blight of potato and tomato. Oomycetes have a cell wall that is comprised of 80-90% β-1,3-glucan. Syntheses of these enzymes can be induced by pathogens or other stimuli. β-1,3-glucan is unlike chitinases, the substrate for β-1,3-glucanases is widespread in plants and therefore these enzymes may have other physiological functions as well as in plant defense.

β-1,3-glucanases have direct effect in defending against fungi by hydrolyzing fungal cell walls, which consequently causes the lysis of fungal cells. β-1,3-glucanases was showed to have an indirect effect on plant defense by causing the formation of oligosaccharide elicitors, which elicit the production of other PR proteins or low molecular weight antifungal compounds, such as phytoalexins (Keen and Yoshikawa, 1983; Ham *et al.*, 1991; Klarzynski *et al.*, 2000). β-1,3-glucanase genes have been reported in a number of plants, including tobacco (De Loose *et al.*, 1988), soybean (Takeuchi *et al.*, 1990) , rubber tree (Chye and Cheung, 1995), banana (Peumans *et al.*, 2000) and rice (Yamaguchi *et al.*, 2002). There are different β-1,3-glucanase genes in different plant species. More than 14 β-1,3-glucanase genes have been reported in tobacco plants, (Leubner-Metzger and Meins, 1999). Plant β-1,3-glucanases having size from 30-40 kDa, with both acidic and basic isoforms.

β-1,3-glucanases can degrade the fungal cell wall by disrupting hyphal tips, especially in combination with a chitinase (Mauch *et al.*, 1988). β-1,3-glucanases, digest fungal cell walls, leading to the release of oligosaccharide elicitors which stimulate the production of PR proteins and other defense-related molecules (Ryan and Farmer, 1991). β-1,3-glucanases usually expressed at low concentration in plants, but when plants are infected by fungal, bacterial or viral pathogens, β-1,3-glucanases enzyme concentration increases dramatically. Plant β-1,3-glucanases are induced not

only by pathogen infection, but also by other factors such as salicylic acid abscisic acid, methyl jasmonate, ethylene and gibberellins. Stress factors like wounding, drought, exposure to heavy metals, air pollutant ozone and ultraviolet radiation can stimulate synthesis of β-1,3-glucanases in some plants (Akiyama *et al*., 2004; Zemanek *et al*., 2002; Fecht-Christoffers *et al*., 2003; Sandermann *et al*., 1998; Thalmair *et al*.,1996). These various factors often appear to interact, resulting in a dynamic response to biotic, as well as abiotic stimuli.

Nazeem *et al*. (2008) studied the expression of pathogenesis related proteins in black pepper (*Piper nigrum L.*) in relation to Phytophthora foot rot disease. They reported that the β -1,3 glucanase was increased a high amount in the tolerant variety 'Panniyur-1'. An increase in the level of PAL and peroxidase was also reported which may have role in over expression of glucanase. The chitinase enzyme was not produced during the infection. The cell wall of the *Phytophthora* does not contain chitin and may explain why the plant did not express chitinase enzyme activity upon infection by *P. capsici.*

Potato leaves infected with *P. infestans* have shown high glucanase activity. This enzyme solubilizes elicitor active glucan molecule from the fungal cell wall and also induces other defense enzymes (McDowell and Dangal, 2000). Infection of *Capsicum annuum* with *P. capsici* led to the accumulation of β -1,3-glucanases and it was much more pronounced in the resistant species (Egea *et al*., 1999).

B. subtilis strains MDSR7, MDSR11 and MDSR14 were evaluated. MDSR7 and MDSR14 cause a significant reduction in soil pH and enhanced microbial respiration as well as b-glycosidase, dehydrogenase, auxin production and microbial biomass-C in the rhizosphere of wheat and soybean. All the strains significantly enhanced the availability and assimilation of zinc which enhances the plant growth (Ramesh *et al*., 2014).

Chandrasekaran *et al*. (2017) studied Expression of β-1,3-glucanase (GLU) and phenylalanine ammonia-lyase (PAL) genes and their enzymes in tomato plants induced after treatment with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv.

vesicatoria. Before sowing, the seeds of tomato was treated with antagonist *Bacillus subtilis* CBR05, and the fourth week seedling was challenged with *Xanthomonas cam pestris* pv. *vesicatoria* (XCV), activities of plant-defense enzymes β-1,3 glucanase (GLU, 42.5%) and phenylalanine ammonia-lyase (PAL; 93.9%) were significantly higher than in control plants at 72 h after inoculation with XCV.

2.5.3. Peroxidase (E.C. 1.11.1.7)

Peroxidase (POD) is an oxidoreductase that catalyzes a reaction in which hydrogen peroxide acts as the acceptor and another compound acts as the donor of hydrogen atoms (Adams *et al*., 1978). Higher plants contain ferriprotoporphyrin peroxidases, which are one of the three major classes of peroxidases. The enzyme is reported to exist in both soluble and membrane-bound forms (Robinson *et al*., 1999). The enzyme can be found in vacuoles, tonoplast, plasmalemma, and inside and outside the cell wall and has a variety of functions.

POD is involved in plant hormone regulation, defense mechanisms, indole acetic acid degradation during maturation and senescence of fruits and vegetables, decolourisation of waste (Jadhev *et al*., 2009), treatment of waste water containing phenolic compounds (Dalal and Gupta, 2007) and lignin biosynthesis (Onsa *et al*., 2004). Because of its multiple functions, the enzyme is commonly found as several isoenzymes in plants.

PODs comprise one important class of PR proteins (PR-9) implicated in these "defense responses," in which an important role is to catalyze the formation of phenolic radicals at the expense of H_2O_2 (Gaspar *et al*., 1986). PODs may also oxidize phenolic monomers to form lignin (Siegel, 1954; Mader *et al*., 1980; Grisebach, 1981), function in H_2O_2 production (Elstner and Heupel, 1976; Mader *et al*., 1980), and metabolize indole acetic acid (Endo, 1968; Mato *et al*., 1988).

In plants, reactive oxygen species (ROS) are continuously produced predominantly in chloroplasts, mitochondria and peroxisomes. Development of an antioxidant defense system in plants protects them against oxidative stress damage

either by partial suppression of ROS production or by the scavenging of ROS already produced in plants (Murgia *et al.,* 2004). Antioxidants protect cells from the oxidative damage and thereby reduce the risk of cell damage (Smitha and Sudha, 2011). Major ROS-scavenging enzymes of plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR). Together with the antioxidants ascorbic acid and glutathione (Noctor and Foyer, 1998), these enzymes provide cells with highly efficient machinery for detoxifying O_2^- and H_2O_2. Takeda *et al.* (1998, 2000), reported that ascorbate peroxides belongs to the class I heme-peroxidases that is found in higher plants, chlorophytes red algae (Sano *et al.*, 2001), and members of the protest kingdom (Shigeoka *et al.*, 1980b; Wilkinson *et al.*, 2002).

Higher level of plant growth-promoting hormones (GA3 and IAA) and defence-related enzymes (peroxidase (PO), polyphenol oxidase (PPO) and superoxide dismutase) were detected in plants treated with PGPR plants (Chowdappa *et al*., 2013).

Ability of antinemic/antifungal *Bacillus* spp. for the production of plant growth hormones and thereby triggering defense enzymes was studied by Sarangi *et al*., 2017. The triggering of defence enzymatic activity of PPO is confirmed in tomato challenged with *M. incognita* and coinoculated with *B. weihenstephanensis* (TSB4) as a measure to contain root knot nematode disease. The study inferred that the *B. weihenstephanensis* (TSB4) enhance the defence enzymatic activity of PO and increase its activity by 50%. There was significant enhancement in PAL activity of tomato followed by the soil application of *B. weihenstephanensis* (TSB4) evaluated for the management of *M. incognita*.

Biocontrol agent *Bacillus amyloliquefaciens* LJ02 induces systemic resistance against cucurbits powdery mildew was investigated by Li *et al*., 2015. LJ02FB can effectively diminish the occurrence of cucurbits powdery mildew. When treated with LJ02FB, cucumber seedlings produced significantly elevated amount of superoxide dismutase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, free salicylic acid (SA) and expression of one pathogenesis-related (PR) gene *PR-1,* secrete

resistance-related substances into rhizosphere that inhibit the germination of fungi spores and the growth of pathogens as compared to that of the control.

Peroxidase enzyme activity of rhizobacteria in shallots bulbs to induce resistance of shallot towards bacterial leaf blight was studied by Yanti *et al*., 2015. The introduction of shallot bulbs with rhizobacteria *Bacillus*, *Stenotrophomonas* and *Serratia marcescens* could increase plant resistance to disease of bacterial leaf blight. Peroxidase activity in roots and leaves of shallots was analyzed after being introduced by the rhizobacteria and inoculated with pathogenic bacteria (Xaa). Peroxidase activity was increased tenfold. The activity of peroxidase in the roots is higher than in the leaves. Isolates PK2RP3 is rhizobacteria isolate with the highest peroxidase activity.

Serratia marcescens known as rhizobacteria isolated from onion and can induce resistance in *Arabidopsis* plants against *Cucumber Mosaic Virus* (Ryu *et al*., 2003). Bacterization of betelvine cut with *Serratia marcescens* NBRI1213 induces phenylalanine ammonia lyase, peroxidase, and polyphenoloxidase activities in leaf and root (Lavania *et al*., 2006). Peroxidase activity could inhibit pathogenic infection due to lignification that inhibits the pathogen entry (Silva *et al*., 2004).

Plant growth-promoting rhizobacteria mediate induced systemic resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae was studied by Chithrashree et al., 2011*. Among the seven strains tested as fresh suspensions, talc and sodium alginate formulations under laboratory and green house conditions, maximum germination of 86% was recorded after seed treatments with fresh suspension of *Bacillus subtilis* GBO3. Seed treatment with fresh suspensions of *Bacillus pumilus* SE34 and GBO3 followed by challenge inoculations with *Xoo* increased accumulation of phenylalanine ammonia lyase, peroxidase and polyphenol oxidase compared to untreated control seedlings increasing the systemic resistance in plants.

Induction of plant antioxidant system by interaction with beneficial and pathogenic microorganisms was studied by Helepciuc *et al*., 2014. Cucumber plantlets treated with bacterial isolates having antifungal activity *viz*; *Bacillus licheniformis* B40,

Bacillus amyloliquefaciens Bw, *Bacillus* sp. Bw1, *Pseudomonas aeruginosa* P14 and fungus *Pythium debaryanum* Hesse suspensions were examined for antioxidant system activation. Potential alteration in superoxide dismutases, catalases, guaiacol peroxidases and lignin production was seen and increased the number of superoxide dismutase and catalase isoforms, and also the enzymatic activity of these antioxidants.

Wang *et al*. (2014) investigated *Bacillus cereus* AR156 induced resistance to *Colletotrichum acutatum* associated with priming of defense responses in loquat fruit and inferred that treatment with *B. cereus* AR156 enhances the defence related activities such as PAL, chitinase, β-1,3 glucanase, PO and PPO and stimulated amassing of hydrogen peroxide.

Akram and Anjum (2011) investigated the resistance elicitation ability of *Bacillus fortis* 162 and *B. subtilus* 174 against *Fusarium* wilt of tomato and showed that both strains induced systemic resistance in tomato plants. They also observed the increased level of phenylalanine ammonia lyase (PAL), PPO and PO in bacterial-treated tomato plants and both strains significantly reduced tomato *Fusarium* wilt.

Characterization of systemic resistance in sugar beet elicited by a nonpathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent was investigated by Bargabus *et al*., 2002. The experiment showed an increased activity of chitinase, β-1,3-glucanase and peroxidase, all pathogenesis-related proteins and accepted indicators of systemic resistance whose combined effect reduced *Cercospora beticola* Sacc.

Association of some plant defense enzyme activities with systemic resistance to early leaf blight and leaf spot induced in tomato plants by azoxystrobin and *Pseudomonas fluorescens* was investigated by Anand *et al*., 2007. *Pseudomonas fluorescens* (10 kg ha^{-1}) was evaluated for their efficacy in inducing defense enzymes in tomato against *Alternaria solani* and *Septoria lycopersici*. The activity of defense enzymes peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), β-1,3 glucanase, chitinase, catalase total phenols was found to be increased in azoxystrobin and *P. fluorescens*-treated tomato plants.

Chen *et al*. (2000) studied defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum* and inferred that when cucumber roots were treated with *Pseudomonas corrugata* 13 or *Pseudomonas aureofaciens*, PO increased as the disease developed on the roots and these accumulations peaked 4±6 days after pathogen inoculation. They also indicated that the peroxidase isomer forms in cucumber roots induced by rhizobacteria were different from that in roots infected with *P. aphanidermatum* suggesting that the mechanisms of PO activation by the rhizobacteria may be different from those of pathogen infection.

Jayaraj *et al*. (2004) studied *B. subtilis* strain AUBS1 for systemic resistance induction against sheath blight of rice by its foliar application under greenhouse conditions and found a significantly increased level of PO and phenylalanine ammonia lyase (PAL). *B. subtilis*-treated leaves were found to have accumulation of PR proteins, increased levels of thaumatin and β-1,3-glucanases which together play an important role for induction of resistance in rice plant.

The work done by Konappa *et al*. (2016) was focused on the role of defense related enzymes in imparting resistance to tomato plants against *Ralstonia solanacearum*. The biocontrol agent Lactic acid bacterium (LAB) was used against the bacterial wilt caused by *R. solanacearum*. Tomato seedlings were raised from LAB pretreated seeds, were challenge inoculated with *R. solanacearum*, harvested at different time intervals (0-72 h) and assayed for defense enzyme activity. Treatment of tomato seedlings with LAB induced a significant amount of Peroxidase (POX), Polyphenol oxidase (PPO), Phenylalanine ammonia lyase (PAL), total phenolics and β-1,3-glucanase activities. The activities of PAL, POX, PPO and β-1,3-glucanase showed maximum at 24 h, 24 h, 32 h and 24 h respectively after challenge inoculation. The LAB treated seeds showed increase in germination percentage (6%) and seedling vigour index (259) compared with control. LAB exhibited 61.1% of disease reduction of bacterial wilt in tomato.

2.5.4. Phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5)

Phenylalanine ammonia-lyase (PAL) catalyses the non-oxidative deamination of phenylalanine to *trans*-cinnamate (Kim and Hwang, 2014). The conversion of the amino acid phenylalanine to *trans*-cinnamic acid is the entry step for the channeling of carbon from primary metabolism into phenylpropanoid secondary metabolism in plants (Hyun *et al.*, 2011). The levels of secondary metabolites such as phenylpropanoids are controlled in response to environmental cues (Dixon and Paiva, 1995; Payyavula *et al.*, 2012). PAL is one of the few amino acid-transforming enzymes which has a prosthetic group dehydroalanine which is post-translationally modified amino acid which plays a key role in catalysis of the activation of the amino group of phenylalanine to form a better leaving group than NH_3. PAL amino acid sequences contain a serine residue that is completely conserved among different species, and is presumed to be associated with the active site of the enzyme (Hyun *et al.*, 2011).

Phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine (Vogt, 2010). The evolutionary emergence of the phenylpropanoid pathway in plants is an important adaptation that enables plant defence against abiotic and biotic stresses (Ferrer *et al.*, 2008).

Fungi and plants are able to synthesize phenylalanine via the shikimic acid pathway. L-Phenylalanine, derived from the shikimic acid pathway, is used directly for protein synthesis in plants or metabolized through the phenylpropanoid pathway (Hyun *et al.*, 2011). Phenylpropanoid compounds are precursors to a wide range of phenolic compounds, such as flavonoids, isoflavonoids, anthocyanins, plant hormones, phytoalexins, and lignins (Dixon and Paiva, 1995; La Camera, *et al.*, 2004, Karthikeyan *et al.*, 2005). PAL is an inducible enzyme that responds to biotic and abiotic stresses such as pathogens, UV irradiation, and low temperature (Dixon and Paiva, 1995; MacDonald and D'Cunha, 2007). The interaction between the pathogen and the host plant induces some changes in cell metabolism, primarily activity of enzymes, particularly phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol

oxidase (PPO), lipoxygenase (LOX), superoxide dismutase (SOD), and β-1,3 glucanase (Ngadze *et al*., 2011).

PAL plays an important role in plant defence; it is involved in the biosynthesis of salicylic acid (SA), an essential signal involved in plant systemic resistance (Mauch-Mani and Slusarenko, 1996; Nugroho *et al.*, 2002; Chaman *et al.*, 2003). *PAL* gene expression responds to a variety of environmental stresses, including pathogen infection, wounding, nutrient depletion, UV irradiation and extreme temperatures (Edwards *et al.*, 1985; Liang *et al*., 1989a, b; Huang *et al.*, 2010; Payyavula *et al.*, 2012; Jin *et al.*, 2013). In *Arabidopsis thaliana*, PAL is encoded by a small gene family with four members, denoted *PAL1−PAL4* (Raes *et al.*, 2003; Huang *et al.*, 2010). PAL enzymes from many sources, especially from monocots and certain fungi, have activity towards L-tyrosine and can produce *trans-p*-coumaric acid (Hyun *et al*., 2011).

Phenylalanine ammonia-lyase (PAL) has a crucial role in secondary phenylpropanoid metabolism and is one of the most extensively studied enzymes with respect to plant responses to biotic and abiotic stress (Kim and Hwang, 2014). The presence of PAL has been reported in diverse plants including certain algae, including *Dunaliella marina*, fungi, and a few prokaryotic organisms, including *Streptomyces* (Xiang and Moore, 2002). In plants, PAL activity has been detected in many species, representing monocots, dicots, gymnosperms, ferns, and lycopods. In fungi, PAL activity has been detected only in a few basidiomycetes and deuteromycetes, and in one ascomycete, *Nectria cinnabarina*. There have been no reports of PAL in animals (Hyun *et al*., 2011). The presence of introns has been reported in both plant and fungal PAL genes. Plant PAL genes generally contain only one intron, while yeast PAL genes have five or six introns (Hyun *et al*., 2011).

Characterization and evaluation of *Bacillus amyloliquefaciens* strain WF02 regarding its biocontrol activities and genetic responses against bacterial wilt in two different resistant tomato cultivars was studied by Huang *et al*., 2016. They found that the mortality rate of the plants reduced from 16% to 3% after inoculation with bacteria and inferred that PGPR-associated plant defenses, and the expression of PAL, PR1a,

LOX and ACO were the reason for this decreased mortality. A three fold and seven fold increase in PAL enzyme was seen in Micro-Tom and L390 respectively.

Bacillus thuringiensis strain 199 can induce systemic resistance in tomato against *Fusarium* wilt. Akram *et al*. (2013) investigated the potential of *Bacillus thuri ngiensis*strain 199 to induce systemic resistance in tomato against *Fusarium* wilt. They inoculated *Bacillus* strain to some two week old tomato seedling, and they were eventually challenged with *Fusarium* strain. The plants inoculated with bacillus showed less symptoms than the uninoculated ones. A significant increase of 1.3, 1.8 and 1.4 fold in polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and peroxidase (PO) was observed in comparison with untreated control.

Solanki *et al*. (2012) studied the characterization of mycolytic enzymes of *Bacillus* strains and their bio-protection role against *Rhizoctonia solani* in tomato and reported that root treatment of *Bacillus* strains showed superior defense during pathogen suppression in terms of chitinase, glucanase, peroxidase, poly phenol oxidase, phenylalanine ammonia-lyase activity and total phenolic content in leaves of tomato. All these enzymes linked enzymes accumulation which was studied with plant protection and greenhouse results indicated that *B. amyloliquefaciens* MB101 and *B. subtilis* MB14-treated plants offered 69.76 and 61.51% disease reductions, respectively, over the infected control which gives the importance of these organisms to be a potential biocontrol agent.

Effects of *Pseudomonas aureofaciens* on defense responses in soybean plants infected by *Rhizoctonia solani* was studied by Jin *et al*., 2011, whose main objective was to investigate the ability of the plant growth-promoting rhizobacterium, *Pseudomonas aureofaciens* to induce plant defense systems. They identified ascorbate peroxidase (APX) and phenylalanine ammonia lyase (PAL) activities of *R. solani* treated roots were increased by 75.1% and 23.6%, respectively.

Liang *et al*. (2011) studied the induction of resistance in cucumber against seedling damping-off by plant growth-promoting rhizobacteria (PGPR) *Bacillus megaterium* strain L8. They reported that the levels of PAL activity in the roots

displayed a wave like induction pattern after treatments with *B. megaterium* and showed two peaks at 5 and 11 days respectively.

Endophytic *Bacillus* species confer increased resistance in cotton against damping off disease caused by *Rhizoctonia solani* (Rajendran and Samiyappan, 2008). The treatment with endophytic bacterial bioformulation increased the level of β-1,3 glucanase, peroxidase, poly phenol oxidase, phenylalanine ammonia lyase and phenol.

Chandhra *et al*. (2007) studied the change in phenylalanine ammonia lyase activity and isozyme patterns of polyphenol oxidase and peroxidase by salicylic acid leading to enhance resistance in cowpea against *Rhizoctonia solani.* Increase in PAL activities was observed specifically in UPC-4200 when plants were exposed with *Rhizoctonia solani* spores.

Podile and Laxmi (1998) studied seed bacterization with *Bacillus subtilis* AF1 increases phenylalanine ammonia-lyase and reduces the incidence of *Fusarium* wilt in pigeon pea and that reported that *Bacillus subtilis* AF1 induced an increase in PAL and POD activities in pigeon pea.

Chen *et al*. (2000) defense enzymes induced in cucumber roots by treatment with plant growth-promoting rhizobacteria (PGPR) and *Pythium aphanidermatum* and inferred that when cucumber roots were treated with *Pseudomonas corrugata* 13 or *Pseudomonas aureofaciens*, phenylalanine ammonia-lyase (PAL) activity was stimulated in root tissues in 2 days and this activated accumulation lasted for 16 days after bacterization and PAL activity increased as the disease developed on the roots.

Bacillus subtilis strain BSCBE4 and *Pseudomonas chlororaphis* strain PA23 (*P. aureofaciens*) were found out to be important and effective biocontrol agents against *Pythium aphanidermatum*, the causal agent of damping off of hot pepper (Nakkeeran *et al*., 2006). The two bacterial strains induced development of plant defence related enzymes including phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, phenol content, suppressed incidence of damping off and increased growth of hot pepper seedlings.

Chakraborty *et al*. (2016) used *Pseudomonas aeruginosa* KUCd1 strain to improve plant growth and reduce *Phytophthora* infection in brinjal under *in vivo* conditions. The induced systemic resistance against *Phytophthora nicotianae* and elicitated rapid defence response (several fold increase in the activity of various defense-related enzymes; PAL, POD, PPO and CAT) protecting the plant from pathogen and also protection from disease. The study shows the potential use of biocontrol agent against *P. nicotianae*.

2.5.5. Total Phenols

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins (Blainski *et al*., 2013). Phenols, sometimes called phenolics, are one of the main secondary metabolites present in the plant kingdom (Khathiwora *et al*., 2010). Phenolics are synthesized when plant pattern recognition receptors recognize potential pathogens (Newman *et al*., 2007; Ongena *et al*., 2007; Schuhegger *et al*., 2006; Tran *et al*., 2007) by conserved pathogen-associated molecular patterns (PAMPs), leading to PAMP-triggered immunity (Zipfel, 2008). As a result, the progress of the infection is restricted long before the pathogen gains complete hold of the plant (Bittel and Robatzek, 2007; Nicaise *et al*., 2009).

Plants respond to pathogen attack by accumulating phytoalexins, such as hydroxycoumarins and hydroxycinnamate conjugates (Karou *et al*., 2005; Mert-Turk, 2002). The synthesis, release and accumulation of phenolic in particular, salicylic acid (Boller and He, 2009; Koornneef and Pieterse, 2008; Lu, 2009; Tsuda *et al*., 2008) are central to many defence strategies employed by plants against microbial invaders.

Akram *et al*. (2016) screened bacillus strains for their ability to induce systemic resistance against fusarium wilt of tomato under both split root system and field conditions. Fourteen bacillus strains were used for initial screening of resistance induction under green house conditions. There was an increase in quantities of defense related biochemicals as total phenolics, PO, PPO and PAL enzymes were examined to

document induced systemic resistance (ISR) phenomenon in tomato plants under influence of these bacterial inducers. Two *Bacillus* strains *viz*., *B. fortis* IAGS162 and *B. subtilis* IAGS174 provided maximum control over fusarium wilt under split root system.

Calorimetric assays were also done and prove highly significant for defense related biochemicals in tomato plants under the influence of these two bacterial strains. Talc based formulations of these two strains were prepared to check their efficacy under field conditions. These not only provided protection against fusarium wilt, but also markedly enhanced growth and fruit yield of plants under field conditions. The study revealed the importance of these microbial organisms for suppression of Fusarium wilt and growth promotion in our agriculture system.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The study entitled "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight" was carried out at the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2014 - 2016. Details regarding to the experimental materials and protocols used in the study are elaborated in this chapter.

3.2 CULTURE COLLECTION AND MAINTANENANCE

Eighty four bacterial isolates and virulent isolates of taro leaf blight pathogen, *Phytophthora colocasiae* were collected from the microbial repository, Division of Crop Protection, ICAR-CTCRI. All these isolates were purified and maintained on Nutrient Agar (Appendix I) and Carrot agar (Appendix I) respectively. All the bacterial isolates were obtained from rhizosphere of tuber crops from different parts of the country. The bacterial isolates used for the study are listed below (Table 1).

Media used

Bacteria: Nutrient Agar, Luria Bertani Agar, King's B Base Agar

Fungi: Potato Dextrose Agar, Carrot Agar

Glass wares

Conical flask, screw capped bottles, pipettes, petri dishes, test tubes.

Equipments

Vortex mixer, Laminar Air Flow chamber, BOD incubator.

Table 1. Details of isolates used for the study

S. No.	Isolate	Place of collection	Crop
1	13-7	Chullimannor	Cassava
2	13-8	Anchal	Elephant foot yam
3	13-9	Anchal	Elephant foot yam
4	13-10	Anchal	Elephant foot yam
5	13-11	Anchal	Elephant foot yam
6	13-12	Anchal	Elephant foot yam
7	13-14	Mohanpur	Elephant foot yam
8	13-15	Kalyani	Taro
9	13-16	Rajendranagar	Taro
10	13-18	Mohanpur	Elephant foot yam
11	13-19	Sreekariyam	Cassava
12	13-20	Sreekariyam	Cassava
13	13-22	Orissa	Sweet Potato
14	13-24	Orissa	Sweet Potato
15	13-26	Sreekariyam	Cassava
16	13-27	Sreekariyam	Cassava
17	13-28	Arunachal Pradesh	Taro
18	13-29	Orissa	Sweet Potato
19	13-30	Orissa	Sweet Potato
20	13-31	Sreekariyam	Cassava
21	14-7	Sreekariyam	Taro
22	14-28	Sreekariyam	Taro
23	14-32	Malappuram	Cassava
24	14-33	Mankada	Elephant foot yam
25	14-34	Sreekariyam	Cassava

S. No.	Isolate	Place of collection	Crop
26	14-35	Malappuram	Elephant foot yam
27	14-36	Mankada	Cassava
28	14-37	Malappuram	Elephant foot yam
29	14-38	Malappuram	Elephant foot yam
30	14-39	Malappuram	Elephant foot yam
31	14-40	Kurathoor, Malappuram	Elephant foot yam
32	14-41	Attapadi	Cassava
33	14-42	Attapadi	Cassava
34	14-43	Attapadi	Cassava
35	14-44	Malappuram	Elephant foot yam
36	14-45	Attapadi	Cassava
37	14-46	Sreekariyam	Elephant foot yam
38	14-47	Sreekariyam	Elephant foot yam
39	14-49	Sreekariyam	White yam
40	14-51	Sreekariyam	White yam
41	14-52	Sreekariyam	White yam
42	14-53	Sreekariyam	White yam
43	14-54	Sreekariyam	White yam
44	14-55	Sreekariyam	White yam
45	14-56	Sreekariyam	Cassava
46	14-57	Sreekariyam	Cassava
47	14-58	Sreekariyam	Taro
48	14-59	Sreekariyam	Taro
49	14-60	Kovvur	Elephant foot yam
50	14-61	Sreekariyam	Taro
51	14-62	Kovvur	Elephant foot yam

S. No.	Isolate	Place of collection	Crop
52	14-63	Kovvur	Elephant foot yam
53	14-64	Anchal	Elephant foot yam
54	14-65	Sreekariyam	Taro
55	14-66	Sreekariyam	Taro
56	14-67	Sreekariyam	Taro
57	14-68	Sreekariyam	Cassava
58	14-69	Sreekariyam	Cassava
59	14-70	Sreekariyam	Cassava
60	14-71	Sreekariyam	Cassava
61	14-72	Sreekariyam	Cassava
62	14-73	Ranchi	Taro
63	14-75	Ranchi	Elephant foot yam
64	14-76	Sreekariyam	Cassava
65	14-77	Sreekariyam	Taro
66	14-80	Sreekariyam	Taro
67	MPNA1	Sreekariyam	Taro
68	RRNA2	Sreekariyam	Taro
69	RSNA7	Sreekariyam	Taro
70	mRNA3	Sreekariyam	Taro
71	MSNA8	Sreekariyam	Taro
72	MSNA9	Sreekariyam	Taro
73	RB9	Kollam	Taro
74	RB26	Kollam	Cassava
75	EN16	Pathanamthitta	Cassava
76	EN22	Pathanamthitta	Elephant foot yam
77	rSKB1	Sreekariyam	Taro

S. No.	Isolate	Place of collection	Crop
78	rSKB5	Sreekariyam	Taro
79	mSKB1	Sreekariyam	Taro
80	mRKB1	Sreekariyam	Taro
81	mPKB2	Sreekariyam	Taro
82	RSLB5	Sreekariyam	Taro
83	mSLB1	Sreekariyam	Taro
84	mSLB7	Sreekariyam	Taro

Screening of bacterial isolates against *P. colocasiae*

The bacterial isolates were screened for their antagonistic potential against *P. colocasiae* (1) by adopting direct confrontation/dual culture method (2) antibiosis test for production of diffusible inhibitory metabolites by candidate microbes effective against pathogen and (3) antibiosis test for production of volatile compound by candidate microbes inhibiting the pathogen through production of inhibitory compound.

3.2.1 Direct confrontation/ Dual culture method

All the 84 bacterial isolates were evaluated for their antagonistic activity against the pathogen under *in vitro* conditions following the dual culture technique as described by Skidmore and Dickinson, 1976. Five mm discs of pathogen were taken from the edge of an actively growing fungal colony with a cork borer and placed in the centre of fungal agar plate and test microbe was streaked on either side of the disc. Plates incubated with the pathogen alone served as the control. Three replications were kept in each case and the culture plates were observed constantly, the radial growth of the pathogen was recorded at an interval of 24 h. The percentage inhibition was worked out as follows.

I = C-T*100/C

I = Percentage inhibition

C = Radial growth of pathogen in control plates

T = Radial growth of pathogen in dual culture plates

3.2.2 Antibiosis test for production of diffusible components

The test was carried out using cellophane paper method described by Dennis and Webster, 1971a. Cellophane paper was cut and sterilized in an autoclave at 121^0C and 15 lbs for 15 min. PDA (Appendix I) was poured into plates and were allowed to cool. Sterilized cellophane papers were carefully overlaid. Each candidate bacterial culture was streaked in the centre of the cellophane paper. Three replicates were kept for each isolate. The plates were incubated at room temperature for 48 h. Then, the cellophane paper along with bacterial growth was carefully removed under aseptic condition. Mycelial discs of 5 mm were cut from the actively growing cultures of virulent *P. colocasiae* isolate. The radial growth of pathogen was recorded after every 24 h and compared with the growth in control plate. Based on this, percentage inhibition of pathogen, if any was calculated.

3.2.3 Antibiosis test for production of volatile compounds

The test was carried out by slightly modifying the sealed petri dish technique described by Dennis and Webster, 1971b. For this test; the candidate bacteria and the target pathogen were inoculated on Nutrient Agar and Carrot Agar media respectively. The lids of the inoculated petri plates were removed; the open ends of the bottom portions of plates were joined and sealed with paper tape. All these steps were done in LAF chamber. Three plates were kept for each isolate and incubated at room temperature until the growth of target pathogen completed 90 mm. The radial growth of pathogen was recorded after every 24 h and compared with the growth in control plate. Based on this, percentage inhibition of pathogen, if any was calculated.

3.2.4 Production of IAA

Indole acetic acid (IAA) production by the bacteria was measured as described by Ahmad *et al*. (2005) with some modifications. Bacterial cultures were grown for 4

days in 30 ml autoclaved Luria broth (Appendix I) with L-tryptophan (1mgml^{-1}) at 28°C. Fully grown cultures were centrifuged at 4000 rpm for 15 min. The supernatant (2 ml) was mixed with two drops of ortho phosphoric acid and 4 ml of the Salkowski reagent (mixture of 50 ml, 35% of perchloric acid and 1 ml, 0.5 M $FeCl_3$ solution) and development of pink colour in the mixture indicated IAA production and its concentration was determined using a spectrophotometer at 540 nm wavelength against a standard curve.

3.2.4.1 Preparation of standard of IAA

The following steps were carried out to make the standard for IAA production.

1. One gram of IAA in 100 ml 1 N NaOH.
2. Taken 2 ml from step 1 added to 100 mL (broth) - working standard.
3. 200 µl W.S to 1800 µl broth = 0.04 mg of IAA.
4. 400 µl W.S to 1600 µl broth = 0.08 mg of IAA.
5. 600 µl W.S to 1400 µl broth = 0.12 mg of IAA.
6. 800 µl W.S to 1200 µl broth = 0.16 mg of IAA.
7. 1000 µl W.S to 1000 µl broth = 0.2 mg of IAA.
8. Blank 2 ml un-inoculated broth.
9. Taken the spectrophotometer reading at 540 nm.

The following isolates were selected for further study based on disease suppression as well as IAA production.

13-14, 14-33, 14-54, 14-68, 14-69, 14-70, 14-71, 14-72, RRNA2, RSLB5.

3.3 IDENTIFICATION OF POTENT ISOLATES

The isolates which showed consistent high inhibitory potential were selected for further study.

3.3.1 Molecular characterization of the potent isolates using 16S rRNA sequencing

Materials used

Bacteriological media

Luria Bertani (LB) broth

Equipments and glass ware

Centrifuge, water bath, incubator, UV transilluminator, Gel documentation system, PCR machine, Laminar air flow, micropipettes, micro tips, gel cutter, QIA Quick gel extraction kit, conical flasks, beakers, measuring cylinder, glass rod.

Chemicals and solvents

Agarose, Ethidium bromide, Isopropanol, Phenol, Chloroform, Ethanol, Sodium Dodecyl Sulfate (SDS), Sodium chloride, Deoxy nucleotide triphosphare (dNTP)

Enzymes and buffers

Taq buffer, TAE buffer, TE buffer, Proteinase K, Ribonuclease, Taq polymerase.
Methodology followed

3.3.1.2 Isolation and purification of bacterial DNA

The bacteria cultures were inoculated in LB broth (Appendix I) and incubated overnight for extracting genomic DNA. A portion of the culture, *viz*; 1.5ml was transferred to eppendorf tube and centrifuged at 12,000 rpm for 10 min and discarded the supernatant. The pellets were collected and re-suspended in 400 µl TE buffer (Appendix II) and vortexed. To this, 50 µl of 10% SDS (Appendix II) and 20 µl of Proteinase K (Appendix II) were added and incubated for 1 h at 37ºC. Pipetted in and out the contents many times using micropipettes. The upper aqueous layer was transferred to a new tube and reextracted twice with 500 µl phenol chloroform (1:1) and twice with 500 µl chloroform. Centrifuged the sample each time at 12,000 rpm for 10 min. Add 25 µl of NaCl and 1 ml of 95% ethanol and mixed gently to precipitate the DNA and again centrifuged at 12,000 rpm for 10 min. Discarded the supernatant and dried the pellet. Then re-suspended the DNA in 50 µl TE buffer and stored at -20^{0}C.

3.3.1.3 Quality Check of DNA

To check the quality of the DNA, Agarose Gel Electrophoresis (Bio Rad, CA, USA) was performed. For this, 1X TAE buffer (Appendix II) was prepared from 50X TAE (Appendix II) stock solution and 0.6 g of agarose (Himedia) dissolved in 60 ml of 1X TAE buffer was used to obtain 1% agarose and this solution was boiled at 60^0C in microwave oven till the agarose is completely dissolved. It was allowed to cool to 40^0C and 0.5 µg/ml ethidium bromide (EtBr) (Appendix II) was added to intercalate with the DNA sample and was mixed thoroughly with gentle swirling without the formation of bubbles. This was casted on a gel tray with comb bound on both sides with cellophane tape. After the agarose gel was solidified, the comb was removed and the gel was placed on the electrophoretic buffer tank containing 1X TAE buffer. The sample was prepared by mixing 5 µl of DNA and 1 µl of loading dye (bromophenol blue) on cellophane tape and loaded in the well. A single well was loaded with 3 µl of 1Kb DNA ladder (Genei, Bangalore). Then 1X TAE buffer was added as electrophoresis buffer to the buffer tank. The electrodes (anode and cathode) were connected to the respective slots in the power pack and electrophoresed (BIO RAD Power Pac HV, USA) at 60 V for 1 hr. The negatively charged DNA moved towards the anode through the gel. When the dye reached the end of the gel, the run was terminated and taken from the apparatus. The gel image was captured in a gel documentation system (Alpha Innotech, USA).

3.3.1.4 PCR Amplification of genomic DNA

The genomic DNA isolated from bacteria was used for PCR amplification. By using 16SrRNA gene primers, the 16SrRNA was amplified from the genomic DNA. The PCR was carried out using the following primer sequence and components of the reaction mix were as follows:

8F (forward primer) sequence : 5'AGAGTTTGATCCTGGCTCAG3'

1492R (reverse primer) sequence : 5'CGGCTACCTTGTTACGACTT3'

25 µl reaction mix (35 cycles)

Taq buffer   : 2.5 µl x 11 = 27.5 µl

dNTP mix   : 0.5 µl x 11 = 5.5 µl

Taq polymerase   : 0.5 µl x 11 = 5.5 µl

Forward primer   : 0.5 µl x 11 = 5.5 µl

Reverse primer   : 0.5 µl x 11 = 5.5

Nuclease free water : 19 µl x 11 = 209 µl

DNA   : 1.5 µl

Total volume   : 25 µl

The reaction mix was prepared and vortexed (Labnet vortex mixer, USA) and flashed down. Aliquot 23.5 µl of the reaction mix was taken into PCR tubes and added 1.5 µl of the DNA sample into each tubes and vortexed the content and flashed down the tubes also. The DNA template was amplified in Agilent Technologies sure Cycler 8800 (Agilent Technologies, USA). PCR was performed using the following cycle program. The initial denaturation was at 94^0C for 2 min, 10 sec denaturation step at 94^0C for 1 min, 30 s, annealing at 49^0C for 30 s and extension step at 72^0C for 2 min. There were 35 cycles and the final extension step was at 72^0C for 10 min. The PCR amplification of 16SrDNA was confirmed along with Gene Ruler 1kb plus DNA ladder (Thermo Scientific, USA) by running the amplification product in 1.5% agarose gel (Appendix II). The gel was visualized under UV light and the image was captured using Gel documentation system using Alpha Imager (Alpha Innotech, USA).

3.3.1.5 Elution of 16S rDNA

The amplified product was cut using a gel cutter and eluted using the QIA quick gel extraction kit (Quiagen). The major steps performed were as follows,

The DNA band amplification was excised from the gel cutter. An empty eppendrof tube was weighed using an electronic precision balance and the gel slice was put in to it and the weight was taken again. Then added thrice the volume of buffer QG to one volume of gel (i.e. added 300 µl of buffer to 100 mg of gel). Incubated at 50^0C

for 10 minutes or till the gel slice has completely dissolved. To help dissolving the gel it was mixed by vortexing the tube every 2-3 min during the incubation. When gel slice dissolved completely, checked the colour of the mix as yellow. Added 1 gel volume of isopropanol to the sample and mixed well by vortexing and then placed in a QIA quick spin column in a 2 ml collection tube which is provided along with the kit. To bind DNA, applied 750 µl of the sample to the QIA quick column and centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the QIA quick column was placed back in the same collection tube. Added 0.5 ml (500 µl) of buffer QG to QIA quick column and centrifuged for 1 minute again and discard the flow through. In washing step, added 750 µl of buffer PE to QIA quick column and kept for 2 min and centrifuged at 13,000 rpm for 1 min. discarded the supernatant and centrifuged the QIA quick column for an additional 1 min at 13,000 rpm for removing the excess residues. Air dried the samples by placing a new 1.5 ml eppendorf tube for removing excess of ethanol. After 30 minutes, taken back the tubes and eluted the samples using buffer EB (Elution buffer). The buffer was pre-warmed at 55^0C for 10 min. 30 µl of elution buffer was added to the center of QIA quick membrane and incubated at room temperature for 2 minute and centrifuged at 13,000 rpm for 1min. Finally, the eluted 16SrDNA was stored at -20ºC.

3.3.1.6 Checking of eluted DNA in agarose gel

The eluted DNA samples were checked in 1.2% agarose gel. Weighed 0.9 gm of agarose and dissolved in 80 ml of 1X TAE buffer and melted and added ethidium bromide (EtBr) and poured into the casting tray. After the gel was solidified, the comb was removed and the gel was placed on the electrophoretic buffer tank containing 1X TAE buffer. The samples (4 µl) were mixed with the gel loading dye (Appendix II) and loaded in the wells and 3 µl of 1 Kb DNA ladder also. The gel was run at 60 V for 1 hr and viewed the image using a gel documentation system (Alpha imager, Innotech, USA).

3.3.1.7 Sequencing and identification of isolates

The gel elutes of PCR products (10 µl) each along with the primer (15µl), for ward 8F primer as 5'AGAGTTTGATCCTGGCTCAG3' and reverse 1492R primer as 5'CGGCTACCTTGTTACGACTT3' were sent to RGCB (Rajiv Gandhi Center for Biotechnology), Thiruvananthapuram for sequencing.

3.4 GROWTH PROMOTION STUDY

Growth promotion of the selected isolates was evaluated using cowpea seedlings.

Pot culture study on growth promotion (Cowpea)

A pot culture experiment was conducted in the net house to evaluate the growth promotion activity of bacterial isolates. The potting mixture containing sand: soil: cow dung in the ratio (1:1:1) was used for the study. The pots were filled with the mixture approximate weighing 2 kg and labeled separately with the isolate number. The cowpea variety (Kanakamani) released by KAU was collected from the Farming System Research Station, Kottarakkara, Kollam. The isolates were grown on KB (Appendix I) and NA broth (Appendix I). Three replicates were kept for each isolate. The bacterial cultures were centrifuged at 5000 rpm for 10 min. The pellets were resuspended in 20 ml sterile water and pooled all 3 samples into a measuring cylinder and made upto 100 ml and poured into a conical flask. OD value of the samples was measured at 630 nm using spectrophotometer. Seeds were soaked overnight in bacterial suspension and the seeds soaked in sterile distilled water served as control. Drained the excess suspension/water and planted 10 seeds/ pot. Ten pots were kept for each isolate and growth parameters of cowpea seedlings *viz.*, days taken for seed germination, germination (%), shoot length (cm), root length (cm), number of leaves, leaf area were taken at 15, 30, 45 days after sowing.

Selection of bacterial isolate:-Based on the disease suppression, IAA production and the growth promotion in cowpea seedlings, the isolate was selected and from molecular characterization, it was confirmed that the isolate is safe to use.

3.5 STUDY ON THE DIFFERENTIAL EXPRESSION OF ENZYMES

The study was conducted using Muktakeshi and Sree Kiran, tolerant and susceptible taro varieties respectively, released by ICAR- CTCRI.

The study was conducted under net house condition as per the treatment details given below. Plastic pots were used for growing the plants. Potting mixture was prepared as mentioned earlier and sterilized using autoclave. Approximately 20 kg of potting mixture was filled into plastic pots. The bacterial isolate was cultured on nutrient broth (50 ml) and kept for 48 hours in the shaker. Bacterial culture in broth was centrifuged at 5000 rpm for 10 minutes. The pellet was re-suspended in 25 ml sterile water. OD value of the sample was measured at 630 nm using spectrophotometer. Then medium size corms were washed under running tap water for 5 min and surface sterilized with 1% sodium hypochlorite solution by keeping the corms in solution for 5 minutes. Later the corms were rinsed three times with sterile distilled water. The excess water was allowed drain and the corms were dried under room condition. The corms were then soaked in bacterial suspension ($2x10^{-7}$) and the corms soaked in sterile water served as control. The corms were kept overnight in suspension. Drained the excess suspension/water and planted each tuber/pot. Ten pots were kept for each treatment inside the net house. The bacterial inoculum was incorporated to the plant as soil application using dolomite based bacterial inoculum. Ten gram of the mixture was applied to pots. The bacterial inoculum was also given as foliar spraying to the plants.

Treatment details

Sree Kiran (Susceptible)

T1- Plants with PGPR

T2- Plants with *Phytophthora colocasiae*

T3- Plants with PGPR + *Phytophthora colocasiae*

T4- Control

Muktakeshi (Tolerant)

T5- Plants with PGPR

T6- Plants with *Phytophthora colocasiae*

T7- Plants with PGPR + *Phytophthora colocasiae*

T8- Control

3.5.1 Preparation of *P. colocasiae* preparation

Floating disc method described by Nath *et al.* (2016) was adopted to prepare sufficient inoculum to conduct the experiment. Five leaf discs (5x5 cm) of taro were floated in sterile distilled water in 200 mm glass petri plates and inoculated with a mycelial disc excised from the margins of actively growing cultures of *P. colocasiae.* The leaf discs were incubated at 25ºC in the dark and examined daily for symptom development. The lesion diameter was recorded 4 days after inoculation. The infected region was excised and again submerged in sterile water. The whole setup was incubated at 4ºC for 30 minutes and was transferred to room temperature. A drop of the solution was observed under microscope for zoospore release. This zoospore suspension was used to artificially inoculate the leaves for further enzyme assay.

3.5.2 Artificial inoculation

The plants were placed inside a moist chamber and 50 µl zoospore suspension was placed on the third leaf and covered with plain agar plug. The inoculum along with mycelial plug was covered with wet cotton and cello tape was used to hold the inoculum in place. Humidity was maintained in the chamber throughout the period to facilitate the pathogen attack.

The leaf samples were collected at different intervals, 0h, 6h, 12h, 24h, 2 days, 4 days and 8 days and stored at -80ºC for doing enzyme assay and differential expression of proteins using standard protocols.

3.6 QUANTIFICATION OF DEFENSE ENZYMES

The enzymes, Peroxidase, Chitinase, Glucanase, Phenylalanine Ammonia Lyase (PAL) and total phenol were assayed as per the standard protocols.

3.6.1. Peroxidase

Enzyme Extraction

The activity of peroxidase was estimated using the protocol by Sadasivan and Manikam, 2008. One gram of leaf sample was homogenized in 0.1 M Potassium phosphate buffer, pH 6.0 (Appendix III) (1:10, w/v) in a chilled pestle and mortar and centrifuged the homogenate at 16,000 g for 20 min at 4°C. The supernatant was used as enzyme source.

Enzyme Assay (modified Summer and Gjessing, 1943)

Pipetted out 1 ml of o-dianisidine, 0.5 ml of H_2O_2, 1 ml of phosphate buffer and 2.4 ml of distilled water into a test tube. For the preparation of blank excluded H_2O_2 and added 0.5 ml water. Incubated at 30^{0}C and started the reaction by adding 0.2 ml of enzyme. After 5 min, the reaction was stopped by adding 1 ml of 2 N H_2SO_4. The absorbance was read at 430 nm.

Calculation

The specific activity was expressed as units/min/mg or per g weight of sample considering one unit of enzyme as an increase in OD by 1.0 under standard condition.

3.6.2. Phenylalanine ammonia lyase

Plant sample of 1 g was homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 (Appendix IV), containing 1.4 mM of 2- mercaptoethanol and 50 mg of insoluble polyvinylpyrrolidene (PVP). The resulting extract was filtered through cheese cloth and the filtrate was centrifuged at 20,000 rpm for 15 min at 4ºC and the supernatant was used as enzyme source. The activity of L-phenylalanine ammonia-lyase (PAL; E.C. 4.3.1.5) was determined by the method Dickerson *et al* (1984). The reaction mixture containing 0.5 ml of the enzyme extract was incubated with 0.5 ml boric acid-borax buffer (pH 8.8) and 0.5 ml of 12 Mm L-phenylalanine in the same

buffer for 30 min at 30ºC and spectrophotometric reading was recorded at 290 nm. The amount of trans-cinnamic acid synthesized was calculated using the extinction coefficient of 9630 M^{-1}. The enzyme activity was expressed as nmol trans-cinnamic acid per min per g of sample.

Calculation

9.63/ millimole = 9.63 x 10^{-3} x 10^{-3} per nanomole

Enzyme activity = nanomole/minute/gram tissue

9.63 x 10^{-6} nm is equivalent to 1unit

Total volume of the assay system = 2.8 ml

The enzyme activity per 30 minute = OD x 2.8 x 5/ (9.63 x 10^{-6} x 0.4 x 1g)

Enzyme activity per minute = OD x 2.8 x 5 x 10^6 x 10 / (9.63 x 4 x 30)

= 121149.2 x OD nM/Min/g tissue

3.6.3. Assay for β-1,3-glucanase (E.C. 3.2.1.6)

β-1,3-glucanase activity was estimated using the procedure of Koga *et al* (1988). Leaves (1 g) were homogenized in 5 ml of 0.05M sodium acetate buffer (pH 5.0) (Appendix V) at 4^0C. The extract was filtered through cheese cloth and the filtrate was centrifuged at 10,000 rpm for 15 min at 4^0C and the supernatant was used in the enzyme assay. The assay mixture contained 900 μl of suitably diluted enzyme and 100 μl of laminarin solution (100 mg/ml) in 1 ml sodium acetate buffer (50 mM, pH 5.2). The mixture was incubated at 40^0C for 30 min and the released reducing sugar was measured as glucose equivalents (Somyogyi, 1952). One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that produce one mg of NAG per hour under given assay conditions.

3.6.3.1 Estimation of reducing sugars (Nelson and Somogyi method, 1952)

0.2 ml of the aliquot was pipetted out from the homogenate, the volume was made up to 1ml in each tube using distilled water. To that, 1 ml of the copper reagent was added and then placed in a boiling water bath for 20 min. On cooling, 3 ml of the arsenomolybdate reagent was added to each tube. The volume was then made upto 20

ml with distilled water. The OD of the blue colour was measured at 520 nm. A standard graph was prepared using different concentration of D-glucose solution under identical condition.

The calculations are given below.

Test = 1 ml buffer + 900 μl enzyme extract + 100 μl laminarin solution (10 mg/ml)

Total = 2 ml

Incubation time = 30 minutes

Constant factor = 345 μg

Sample taken for Somogyi method = 0.2 ml

Amount of reducing sugar in 0.2 ml sample = OD of test x 345 μg

Amount released by 2 ml assay system = OD of test x 345 x 2/0.2

= OD of test x 345 x 10 μg

Amount of reducing sugar released by 900 μl enzyme extract = OD of test x 345 x 10 μg

Amount released by 5 ml i.e., 1 g tissue = OD of test x 345 x 10 μg x 5/0.9

Amount released by 1 g tissue in 1 h = OD of test x 345 x 100 x 5x 60/9 x 30 μg

= OD of test x 345/9 mg

= OD of test x 38.33 mg

3.6.4. Assay for Chitinase

3.6.4.1 Preparation of colloidal chitin

Colloidal chitin was prepared using the method by Berger and Reynold (1958). Weighed 5 g of chitin (Sigma Aldrich), stirred well with 30 ml of concentrated HCl and kept overnight at 4ºC. Prepared 250ml of 50% chilled ethanol, added the soup of the HCl mixture slowly while stirring the ethanol. Centrifuged the ethanol at 10,000 rpm for 10 minutes at room temperature and washed the pellet thoroughly using sterile distilled water three times and centrifuged. The resulting pellet was weighed and was dissolved in sterile distilled water to make a concentration of 7 mg/ml.

3.6.4.2 Assay for chitinase

The reaction mixture contained colloidal chitin solution (7mg/ml), 1.0 ml of sodium acetate buffer of pH 5.2 and 1 ml of suitably diluted enzyme. After incubation at 50°C for 1 hr, the released reducing sugar was measured as N-acetylglucosamine (NAG) equivalents by the Somogyi- Nelson method (1952). One unit of chitinase activity was defined as the amount of enzyme that produces 1 mg of NAG per hour under given assay conditions.

3.6.4.3 Estimation of reducing sugar (modified Nelson and Somyogyi method)

Solution A:- 25 g anhydrous sodium carbonate, 25 g sodium potassium tartarate, 200 g sodium sulphate in 800 ml of distilled water and make up to 1L.

Solution B:- Copper sulphate pentahydrate (30 g) was dissolved of in 200 ml of distilled water containing 4 drops of concentrated H_2SO_4.

Solution C:- 50 g of ammonium molybdate was dissolved in 90 ml of distilled water and added 42 ml of concentrated H_2SO_4. Dissolved 6 g of sodium arsenate heptahydrate in 50 ml of water and added this solution to ammonium molybdate. The volume was adjusted to 1L.

Solution D:- 1ml of reagent B to 25 ml of reagent A.

Solution E:- Diluted solution C, 5 fold (50 ml to 250 ml) with distilled water before use.

0.2 ml aqueous sample containing up to 50 mg of reducing sugar equivalents were taken in test tubes. 0.5 ml solution D was added to it and mixed well in a vortex mixer. Then the tubes were kept in boiling water for 20 minutes and were cooled to room temperature. The tubes were stirred for about 10 seconds until the CO_2 was completely released. 3 ml of solution E was added to each tube and vortexed for 10 seconds. The solution was allowed to stand for 10 minutes and was stirred again. The absorbance was measured at 520 nm. A blank was prepared by using water (0.5 ml) in place of the sample (0.5 ml). A standard curve was prepared using appropriate amounts

of reducing sugar (e.g. glucose 0-50 mg/test). The enzyme activity was defined as the mg sugar released/ g fresh tissue/ hr. The calculations are given below.

Test = 1 ml colloidal chitin (7 mg/ml) +1 ml buffer + 1 ml enzyme extract

Total = 3 ml

Incubation time = 1 hour

Constant factor = 345 µg

Sample taken for Somogyi method = 0.2 ml

Amount of reducing sugar in 0.2 ml sample = OD of test x 345 µg

Amount released by 3 ml assay system = OD of test x 345 x 3/0.2

= OD of test x 345 x 15 µg

Amount released by 1000 µl enzyme extract = OD of test x 345 x 15 µg

Amount released by 5 ml i.e., 1 g tissue = OD of test x 345 x 15 µg x 5/1

Sugars/ g tissue/h = OD of test x 345 x 45/1000 mg

= OD of test x 15.525 mg

3.6.5. Total phenol content

Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One g of sample was homogenized in 10 ml of 80% methanol using pestle and mortar. Centrifuged the homogenate at 10,000 rpm for 20 min and collected the supernatant. In a test tube, 1 ml of methanolic extract was added to 5 ml of distilled water and 250 µl of Folin- Ciocalteu reagent (1N) and the solution was kept at 25ºC. After 3 min, 1 ml of 20% sodium carbonate solution and 1 ml of distilled water was added and mixed properly. The tube was placed in boiling water for 1 min, cooled and measured the absorbance of the developed blue colour using UV-visible spectrophotometer at 650 nm against a reagent blank. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteu reagent with a phenol solution and expressed as catechol equivalents mg^{-1} tissue weight.

Evaluation of growth promotion and disease suppression potential of isolate 13-14 in taro

The effect of PGPR isolate 13-14 on plant growth and disease suppression was studied using Sree Kiran and Muktakeshi. Grow bags containing approximately 20 kg potting mixture was used for planting taro varieties. Treatment details are given below. Here also the cormels were treated with the bacterial inoculum and applied to the soil. Six plants were kept for each treatment.

Treatment details

Sree Kiran (Susceptible)

T1- Plants with bacterial isolate, 13-14

T2- Plants with *Phytophthora colocasiae*

T3- Plants with bacterial isolate, 13-14 + *P. colocasiae*

T4- Control

Muktakeshi (Tolerant)

T5- Plants with bacterial isolate, 13-14

T6- Plants with *P. colocasiae*

T7- Plants with bacterial isolate, 13-14 + *P. colocasiae*

T8- Control

Various growth parameters of taro plants were measured

      a. Number of days taken for sprouting

      b. Sprouting (%)

      c. Height of the plant (cm)

      d. Number of leaves

      e. Leaf length (cm)

      f. Leaf breadth (cm)

Parameters measured during harvest were as follows

      a. Weight of cormels (g)

      b. Weight of mother corm (g)

      c. Number of cormels

d. Number of mother corm

The disease intensity was scored using 0-5 scale at regular intervals and the PDI was calculated using the formula

PDI = Total score x100

No of leaves assessed x Max score

Scale used for recording PDI (Lakshmipriya *et al*., 2016)

0- No disease

1- <10% incidence

2- 11-25% incidence

3- 26-50% incidence

4- 51-75% incidence

5- 76-100% incidence

RESULTS

4. RESULTS

The present study entitled the "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight" was carried out during the period 2014-2016 in the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram.

4.1 CULTURES

Cultures of eighty four bacterial isolates collected from different parts of the country and *Phytophthora colocasiae* were obtained from microbial repository, Division of Crop Protection, ICAR-CTCRI. All the bacterial isolates were purified and maintained on nutrient agar (NA) or King's agar B (KB) slants and *P. colocasiae* was maintained on carrot agar (Plate 1) in refrigerator for the study.

4.2 SCREENING OF BACTERIAL ISOLATES AGAINST *P. COLOCASIAE*

A total number of 84 bacterial isolates were screened for identifying the most potent isolate (PGPR) against the taro leaf blight pathogen *P. colocasiae* by different methods *viz.*, dual culture/direct confrontation, diffusible and volatile metabolites production methods.

4.2.1 Direct confrontation/ Dual culture method

The maximum inhibition was shown by the isolate MRNA3 (68.36%) and the least inhibition was with the isolate, MPNA1 (2.07%) (Table 2.). Maximum number of isolates (42) showed 51-60% inhibition against the pathogen (Fig. 1 & Plate 2, 3, 4). Twenty six isolates showed >60% inhibition against the pathogen. Out of this, five isolates (14-60, 14-61, mRNA3, RSLB5, mSLB1) showed 65-70% of inhibition. Only 16 isolates showed <50% inhibition and out of this, three organisms *viz.*, MPNA1, rSKB5 and mSLB7 showed <10% inhibition.

4.2.2 Diffusible metabolite production method

In general, the isolates showed less percentage of inhibition in this method. The maximum inhibition was shown by the isolate RRNA2 (65.24%) and the least

inhibition was with the isolate, 14-34 (15.33%). Twelve isolates showed more than 50% mycelial growth inhibition. Out of this 12, six isolates *viz.*, 13-7, 13-14, 13-30, 14-68, 14-69, RRNA2 showed 60-65% of inhibition; four isolates showed 55-60% of inhibition and two isolates showed 50-55% of inhibition against *P. colocasiae*. Maximum number of isolates (26 numbers) showed inhibition in the range of 20-30% (Fig. 2). Only three isolates *viz.*, 13-14, 14-98 and 14-69 could show >60% inhibition in dual culture and diffusible metabolite production methods adopted for screening the isolates. Similarly, seven isolates *viz.*, 13-7,13-15,14-33, 14-70, 14-71, 14-72 and RRNA2 showed >50% inhibition by both the methods.

4.2.3 Volatile compounds production method

The maximum inhibition was shown by the isolate 14-33 (58.71%) and the least was with the isolate, rSKB5 (21.73%). Compared to other two methods, more number of isolates showed higher percentage of inhibition by this method. Thirty isolates showed >50% inhibition and none of the isolates showed <20% inhibition (Fig. 3 & Plate 5). Out of 30 isolates which showed >50% inhibition, twenty one bacterial isolates showed 55-60% inhibition and nine isolates showed 50-55% of inhibition. Eight isolates *viz.*, 13-14, 14-33, 14-68, 14-69, 14-70, 14-71, 14-72 and RRNA2 showed >50% inhibition in all three tested methods.

Table 2. Mycelial growth inhibition shown by bacterial isolates against *P. colocasiae* in different methods of screening

S. No.	Isolate number	% of inhibition (dual culture method)	% of inhibition (diffusible metabolite production)	% of inhibition (volatile production)
1	13-7	51.06	60.81	42.56
2	13-8	54.16	41.22	53.26
3	13-9	52.79	45.00	56.28
4	13-10	58.65	44.37	47.13
5	13-11	46.67	42.49	53.61
6	13-12	60.85	26.67	43.61
7	13-14	64.43	60.60	53.39
8	13-15	62.50	59.20	42.95
9	13-16	57.82	45.43	46.28
10	13-18	56.67	30.00	43.61
11	13-19	63.20	22.51	48.75
12	13-20	44.17	23.00	46.13
13	13-22	52.08	49.33	43.14
14	13-24	53.47	39.33	42.28
15	13-26	55.00	43.94	44.47
16	13-27	56.95	45.20	46.94
17	13-28	53.35	38.43	56.94
18	13-29	61.81	44.23	49.90
19	13-30	47.50	60.33	43.61
20	13-31	60.41	41.25	29.06
21	14-7	52.08	35.24	26.66
22	14-28	61.12	34.28	57.23
23	14-32	49.36	33.33	26.00
24	14-33	58.65	50.57	58.71
25	14-34	19.84	15.33	45.14
26	14-35	52.08	30.25	24.40
27	14-36	56.00	46.25	43.62
28	14-37	56.96	43.75	31.33
29	14-38	62.00	50.53	40.13
30	14-39	52.75	41.33	29.00
31	14-40	56.95	38.75	34.47
32	14-41	47.91	36.50	57.04
33	14-42	53.47	31.36	27.40
34	14-43	56.95	41.52	47.12

S. No.	Isolate number	% of inhibition (dual culture method)	% of inhibition (diffusible metabolite production)	% of inhibition (volatile production)
35	14-44	59.35	36.33	53.90
36	14-45	59.04	18.21	28.15
37	14-46	63.53	42.36	43.23
38	14-47	56.25	29.51	47.01
39	14-49	53.12	27.33	34.85
40	14-51	51.39	40.39	53.91
41	14-52	63.53	25.33	40.13
42	14-53	61.12	32.44	57.24
43	14-54	61.73	36.61	54.98
44	14-55	61.14	44.44	43.62
45	14-56	57.29	29.35	47.14
46	14-57	62.50	42.95	31.23
47	14-58	60.68	33.33	57.22
48	14-59	58.15	42.63	40.12
49	14-60	63.35	42.50	57.42
50	14-61	62.50	30.55	56.57
51	14-62	62.63	29.37	43.23
52	14-63	57.34	38.33	43.33
53	14-64	60.53	35.69	57.33
54	14-65	54.00	41.13	57.27
55	14-66	56.00	43.00	43.62
56	14-67	52.68	38.25	38.18
57	14-68	65.32	60.00	56.51
58	14-69	64.73	61.13	57.12
59	14-70	65.91	57.33	56.33
60	14-71	62.94	59.62	57.00
61	14-72	64.12	59.31	56.93
62	14-73	56.00	20.23	43.66
63	14-75	59.00	37.50	52.23
64	14-76	56.68	24.33	55.90
65	14-77	52.00	37.99	49.14
66	14-80	57.34	25.33	57.04
67	MPNA1	2.07	20.18	34.83
68	RRNA2	58.65	65.24	56.20
69	RSNA7	42.63	22.22	47.23
70	mRNA3	68.36	25.70	43.90
71	MSNA8	59.04	30.28	57.13

S. No.	Isolate number	% of inhibition (dual culture method)	% of inhibition (diffusible metabolite production)	% of inhibition (volatile production)
72	MSNA9	63.09	27.84	47.92
73	RB9	55.56	25.10	37.90
74	RB26	45.00	28.15	48.37
75	EN16 KB	58.65	28.11	51.52
76	EN22 KB	47.91	23.55	45.42
77	rSKB1	42.75	27.50	43.70
78	rSKB5	4.33	21.33	21.73
79	mSKB1	34.46	26.66	40.26
80	mRKB1	37.49	28.07	43.99
81	mPKB2	35.01	22.20	54.85
82	RSLB5	67.61	42.53	56.81
83	mSLB1	66.1	29.33	45.73
84	mSLB7	8.85	27.33	40.13

Plate 1. *Phytophthora colocasiae* grown on Carrot agar medium
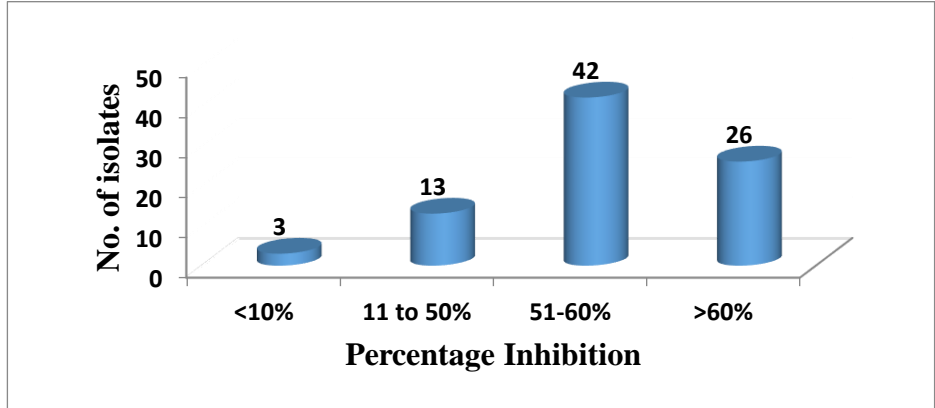
Figure. 1 Number of isolates under different groups based on inhibition percentage in dual culture method
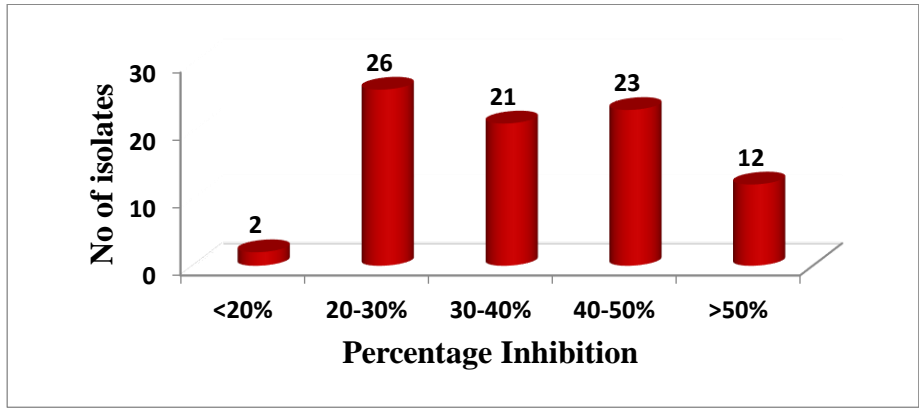
Figure. 2 Number of isolates under different groups based on inhibition percentage in diffusible metabolite production method
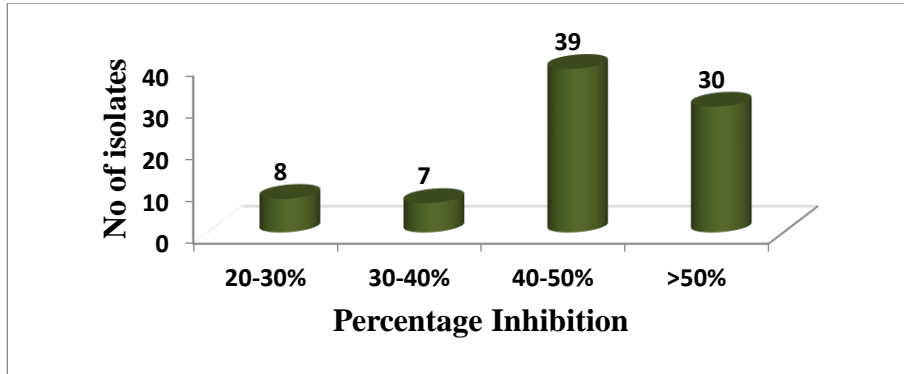
Figure. 3 Number of isolates under different groups based on inhibition percentage in volatile production method
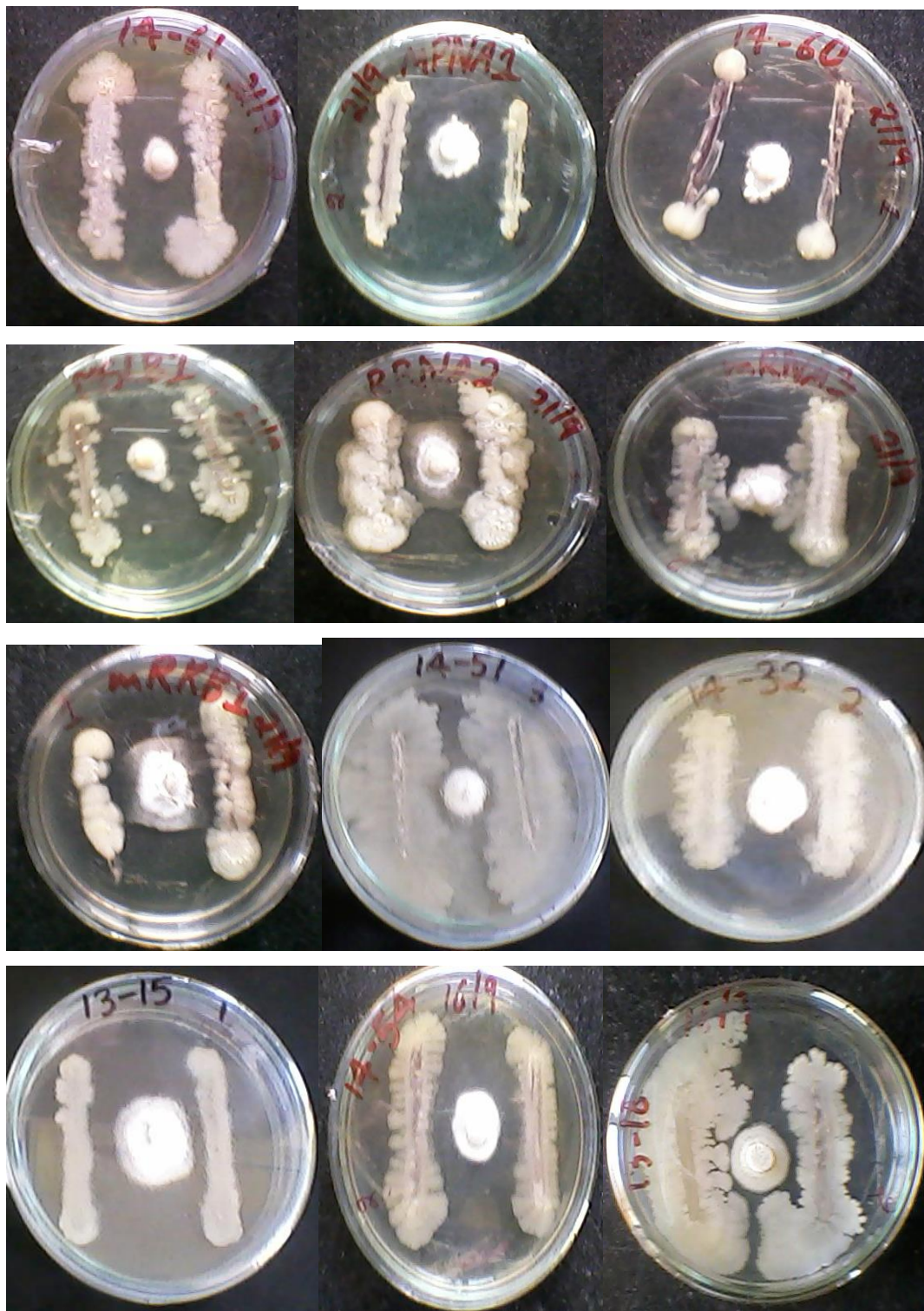
Plate 2. Screening of bacterial isolates against *P. colocasiae* by dual culture method
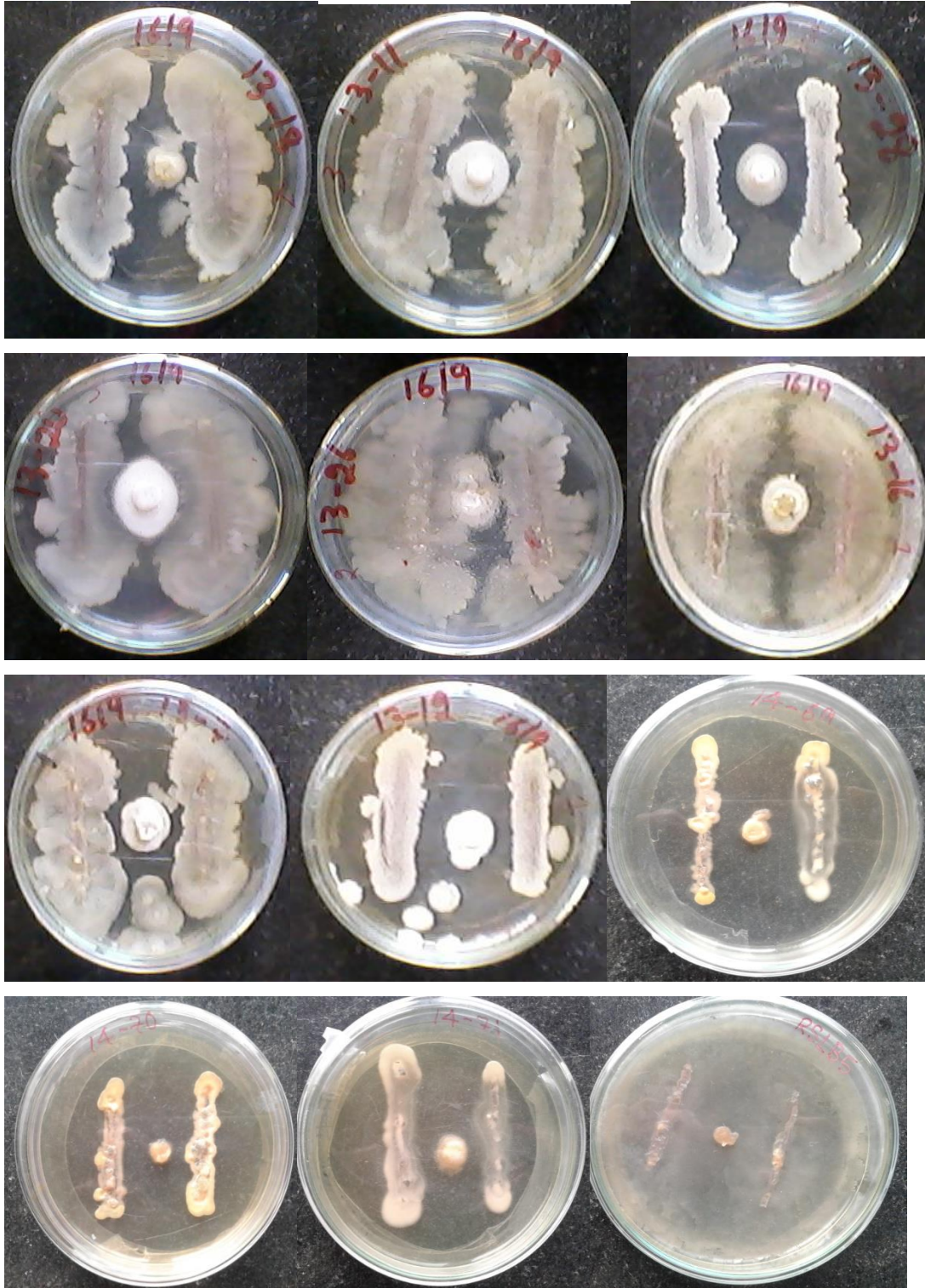
Plate 3. Screening of bacterial isolates against *P. colocasiae* by dual culture method
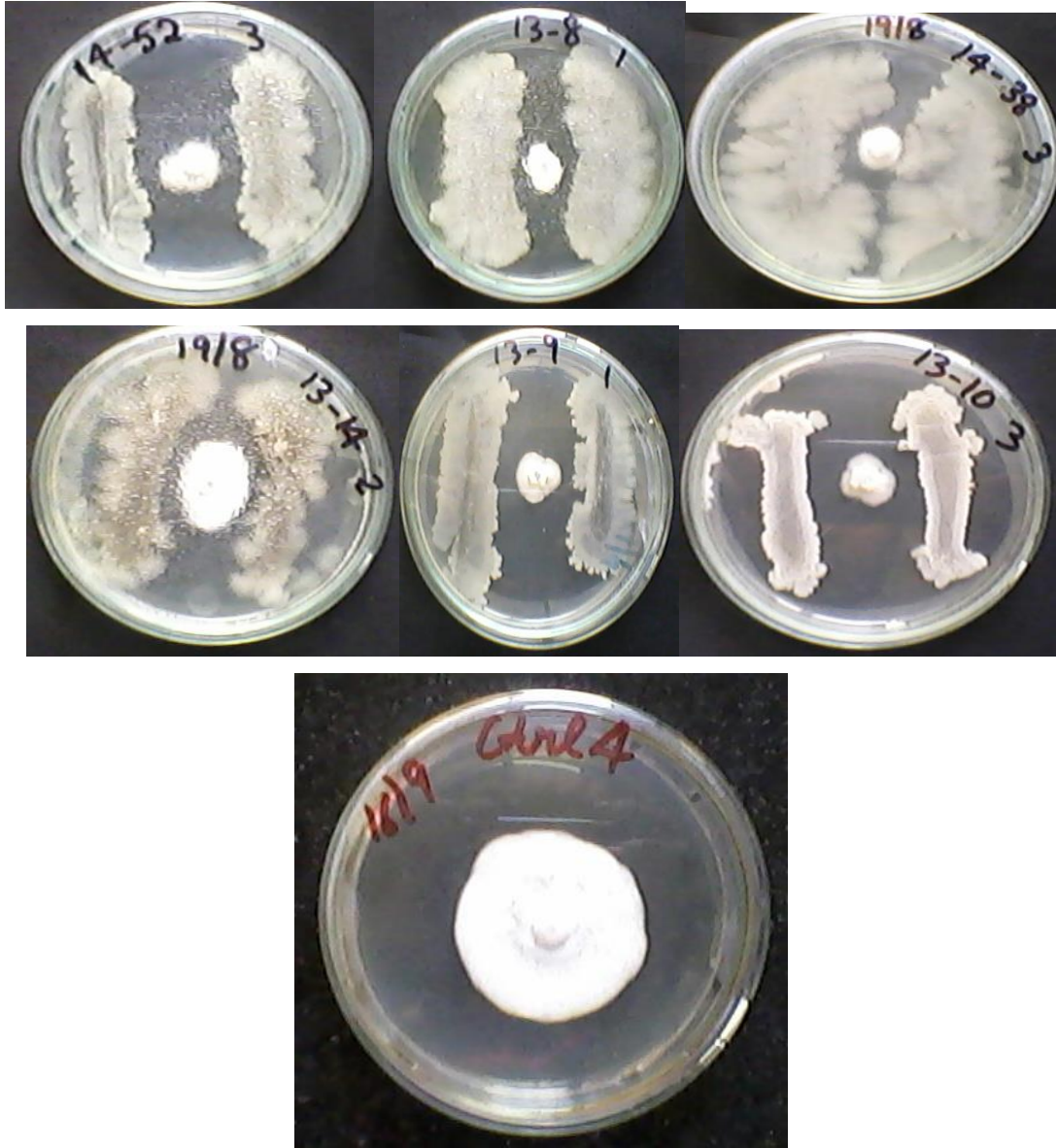
Plate 4. Screening of bacterial isolates against *P. colocasiae* by dual culture method
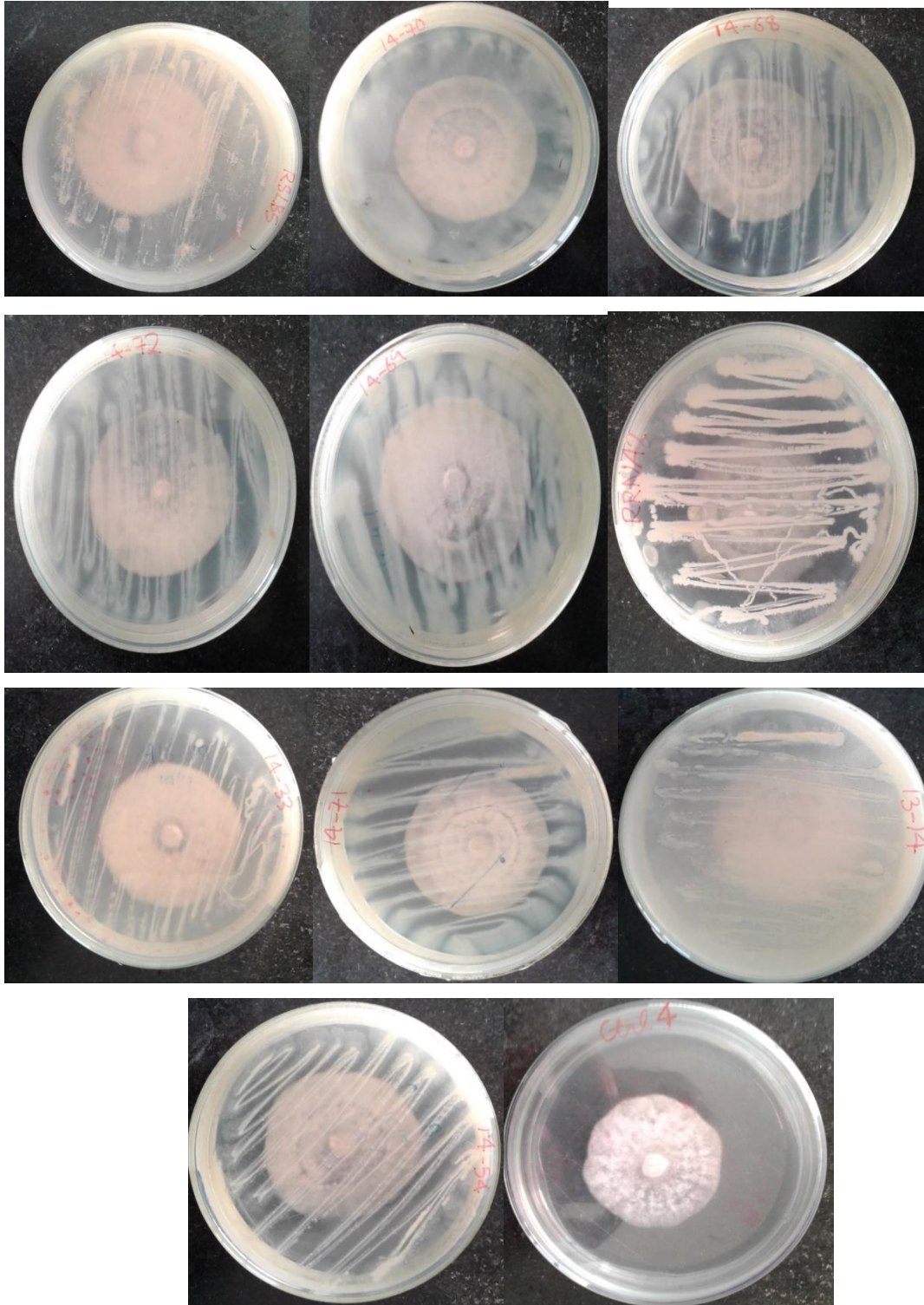
Plate 5. Volatile metabolite production by bacterial isolates against *P. colocasiae*

4.3 PRODUCTION OF IAA

All 84 bacterial isolates grown in culture medium amended with tryptophan, developed pink colour in the mixture indicating IAA production. The IAA production was detected by using Salkowski reagent and the concentration was determined using the spectrophotometer at 540 nm wavelength (Table 3). The bacterial isolates tested had different abilities in secreting IAA levels. The IAA production ranged from 0.30 μgml^{-1} to 16.80 μgml^{-1}. The maximum IAA production was noted with the isolate 13-14 and the lowest concentration of IAA produced by the isolate MSLB1. Out of 84 isolates tested, 14 isolates produced >10 μgml^{-1} of IAA (Fig. 4). Among eight isolates which showed >50% mycelial growth suppression in all three methods, 4 isolates *viz.*, 13-14, 14-33, 14-71 and RRNA2 showed >10 μgml^{-1} IAA production also.

Table 3. IAA production shown by bacterial isolates under study

S. No.	Isolate	IAA production (µg/ml)	S. No.	Isolate	IAA production (µg/ml)
1	13-7	3.94	43	14-54	12.25
2	13-8	3.75	44	14-55	4.91
3	13-9	6.62	45	14-56	9.99
4	13-10	2.73	46	14-57	7.28
5	13-11	6.04	47	14-58	4.97
6	13-12	9.05	48	14-59	3.56
7	13-14	16.80	49	14-60	7.81
8	13-15	4.22	50	14-61	6.51
9	13-16	11.59	51	14-62	7.70
10	13-18	9.96	52	14-63	10.23
11	13-19	2.76	53	14-64	9.21
12	13-20	3.72	54	14-65	9.30
13	13-22	3.28	55	14-66	8.88
14	13-24	7.75	56	14-67	11.31
15	13-26	8.28	57	14-68	3.28
16	13-27	3.53	58	14-69	7.12
17	13-28	11.31	59	14-70	8.03
18	13-29	11.53	60	14-71	10.79
19	13-30	5.38	61	14-72	7.39
20	13-31	7.86	62	14-73	8.66

S. No.	Isolate	IAA production (µg/ml)	S. No.	Isolate	IAA production (µg/ml)
21	14-7	3.20	63	14-75	9.30
22	14-28	7.59	64	14-76	9.60
23	14-32	5.08	65	14-77	7.20
24	14-33	11.92	66	14-80	8.83
25	14-34	7.26	67	MPNA1	4.99
26	14-35	9.99	68	RRNA2	16.52
27	14-36	8.19	69	RSNA7	5.35
28	14-37	11.37	70	mRNA3	1.24
29	14-38	8.41	71	MSNA8	4.30
30	14-39	10.15	72	MSNA9	5.88
31	14-40	3.26	73	RB9	7.56
32	14-41	2.76	74	RB26	10.07
33	14-42	7.70	75	EN16 KB	4.17
34	14-43	3.09	76	EN22 KB	4.63
35	14-44	8.00	77	rSKB1	4.39
36	14-45	7.37	78	rSKB5	8.06
37	14-46	7.78	79	mSKB1	9.02
38	14-47	4.77	80	mRKB1	4.77
39	14-49	8.74	81	mPKB2	5.05
40	14-51	6.65	82	RSLB5	14.04
41	14-52	7.45	83	mSLB1	0.30
42	14-53	3.23	84	mSLB7	5.32
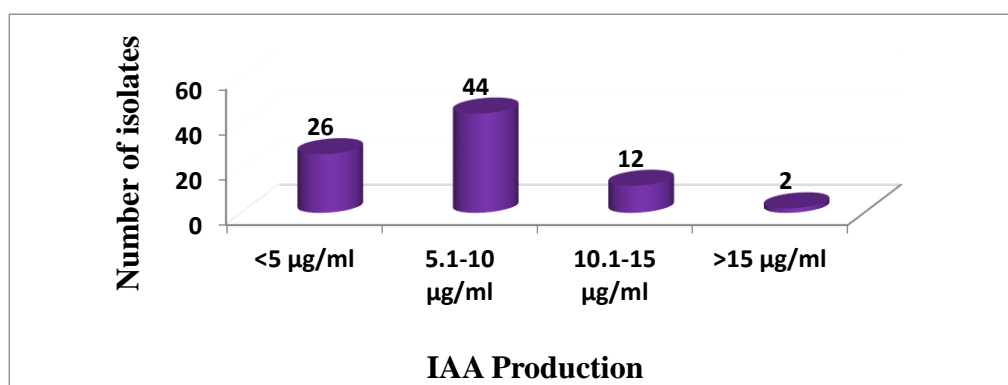
Figure. 4 Number of isolates under different groups based on IAA production

4.4 IDENTIFICATION OF POTENT ISOLATES

4.4.1 Molecular characterization of the potent isolates

The bacterial DNA was extracted from the selected potent isolates and the purity of DNA was checked using (1%) agarose gel electrophoresis. The gel image was captured in a gel documentation system. Clear bands were visualized under UV.

4.4.2 PCR Amplification of genomic DNA

The genomic DNA isolated from bacteria was used for PCR amplification. The PCR reaction was carried out by using 16SrRNA gene primers (8F forward primer 5'AGAGTTTGATCCTGGCTCAG3'and 1492R reverse primer 5'CGGCTACCTTG TTACGACTT3') for 35 cycles. The PCR amplification of 16SrDNA was confirmed along with Gene Ruler 1 kb plus DNA ladder by running the amplification product in 1.5% agarose gel. The size of the amplified product was approximately 1500 bp. The gel was visualized under UV light and the image was captured using Gel documentation system using Alpha Imager (Plate 6).
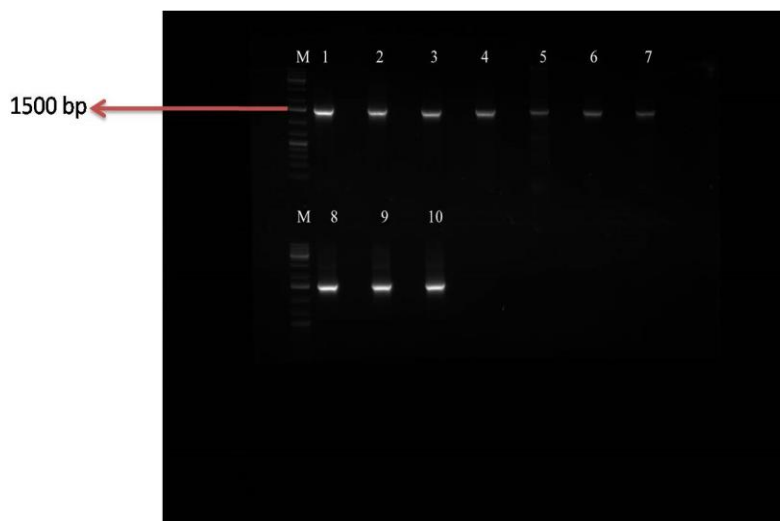
Plate 6. PCR amplification of 16S rDNA of the potent isolates

M-Ladder 1kb plus, 1-RRNA2, 2-14.54, 3-14.71, 4-14.72, 5-14.68, 6-14.69, 7-14.70, 8-14.33KB, 9-13.14KB, 10-RSLB5

4.2.3 Elution of 16SrDNA

Elution of genomic DNA was done using the QIA quick gel extraction kit method.

4.2.4 Eluted DNA check in agarose gel

The eluted DNA samples were checked in 1.2% agarose gel. The gel was visualized under UV light and the image was captured using Gel documentation system using Alpha Imager.

4.2.5 Sequencing and identification of isolates

The PCR amplified sample was effectively purified using QIA quick gel extraction kit and was sequenced at RGCB (Rajiv Gandhi Center for Biotechnology), DNA Finger printing Lab, Thiruvananthapuram. The bacterial isolates (Fig. 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14) were identified as *Bacillus amyloliquefaciens* (13-14, 14-33), *Pseudomonas aeruginosa* (14-68, 14-69, 14-70, 14-71, 14-72), *Bacillus cereus* (14-54, RRNA2) and *Bacillus licheniformis* (RSLB5).

4.2.6 Alignment and Scoring

The nucleotide sequence obtained from RGCB was analyzed using the Bio Edit v7.0.9 sequence was searched for sequence similarity with BLAST (NCBI) tool. Gene annotations were done using the available sequence in NCBI databases. All annotations were based on BLAST searches with a score threshold of ≥ 200 for BLASTN and e-values of 0.0 with a minimum of 95-100% identified. The BLAST hit matched the query cover up of 100% (Table 4). All the sequences were submitted at NCBI (APPENDIX VI).

Sequence of the isolate 13-14 (*Bacillus amyloliquefaciens*)

TAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTT
TGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCG
GCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGA
GAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTCGGATCGTAAGGCTCTGGTTGTTAANGGGAAGAAAGTGCCGTTCAAATAGGGCGG
CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTT
AAGTCTGATGTGAAAGNCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTT
GAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGT
GTTANGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGNGGCCCGCACAAGCGGTGGAGCA
TGTTGTTNAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAATC
CTAGAGATAGGACGTCCCCTTCGGGGGCAGGATGACAGGTGGTGCATGGTTGTTCTCAGC
TCGTGTCGTGGAGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATTTTAATTGCCA
GCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACA
AAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGC
AGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACC
CGAAGT

Sequence of the isolate 14-33 (*B. amyloliquefaciens*)

CCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGA
TAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAGACATA
AAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTAGCGGAGCGGCCCGCCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTG
TTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGCACNTTGACGGTACCTANCCAGA
AAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCG

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GAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGG
CTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAA
TTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGAC
TCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAG
TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGNGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTNCG
GGGGCAGAGTGACAGGGTGNGTATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGNGTTA
AGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAG
GTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTA
TGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGT
TAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGA
AGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGT

Sequence of the isolate 14-54 (*B. cereus*)

TGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAA
CATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGAC
CCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGCTTTCGGGTCGGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCCTAGTTGGANA
AGCTGGCACCTTGGACGGTACCTAACCAGAAAGCCNCGGCTAACCNTGTGCCAGGGGCC
GCGGTAATACGTAGGTGGCAAGCCTTATCCGGAATTATTGGGCGTAAAGNCCGCGCNGGT
GGTTCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGAGAGGGGTCATTGGAAACTGG
GAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAG
ATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGT
GCTAAGTGTTAGAGGGGTTTCCGCCCTTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGC
CTGGGGAGTACGGCCGCAAGGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCATGTGGTTTAATTSGAAGCAACGCGAAGAA

Sequence of the isolate 14-68 (*Pseudomonas aeruginosa*)

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG
ATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATC
TTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGC
CTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAAGG
CCTGATTCCAGCCATGCCGCGTGTGTGAAGAAACGGTCTTCGGGATTGTAAAGCAACTTT
ANAGTTGGGAGGNAAGGGCAGTNAAGTTAATACCTTGCTGTTTTGACGTTACCCAACAGA
ATAAGCACCGGNCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGT
GGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGG
CGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCT
TAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA
CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAAAGATGGATTGGTGCCT
TCGGGAACTCAGACACAGGTGCTGCATGGGCTGTCGTCAGCTCGTGTCGTGAGATGNTTG
GGNTTTAAGTNCCCGGTTAACGAGCGCNAACCCTTGTCCTTAGTTACCAGCAACCTCGGG
ATGGGCACCTCTAATGGGAGCNACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACG
TCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAG
GGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGT
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AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGA
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Sequence of the isolate 14-69 (*P. aeruginosa*)

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG
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CTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGCTACAATGGGCGGAAA
AGCCTTGATCCAAGGCCATGCCGCGTGTGTGAAGAAGGTCTTCCGGGATTGTAACAAGCA
CTTTAAGTTTGGGAGGAAAGGGCAGTAAAGTTAAATACCCTTGCTGGTTTTGACGGTTAC

CAAACAGAATAAGCACCGGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGGATG
TGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAG
AGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGT
GGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCT
TGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAA
GGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGNTTTAAT
TCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGA
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TGAGATGTTGGGTTAAGTCCCGGTAACGGAGCGGCAACCCTTGTCCTTAGTTACNCAGCC
ACCTCGGGATGGGCCACCACTCTTAAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTG
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GGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAA
TGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCTCCAGAAG

Sequence of the isolate 14-70 (*P. aeruginosa*)

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG
ATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATC
TTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGC
CTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAAGC
CTGATTCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCAACTTTAAGT
TGGGAGGGAAAGGGCAGTAAAGTTAAATACCTTGGCTGTTTTGACGTTACCAAACAGAAT
AAAGCACCGGGCTAACCTTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTT
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AACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGC
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GGGGTTTAAGTCCCGTAACGAGCGGCAACCCTTGTCCTTAGTTACTCAGCACCTTCGGGTT
GGGCACCTTCTTAAGGAGACTGCCTTGGTGACAAACCGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGG
TTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCT
GCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAA

Sequence of the isolate 14-71 (*P. aeruginosa*)

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG
ATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATC
TTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGC
CTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGGCGGAA
AAGCCTGATTCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTA
AGTTGGGAGGAAAGGGCAGTAAAGTTAATACCCTTGCTGTTTTGAACGTTACCCAACAGA
ATAAGCACCGGGCTAACCTTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTT
TAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGNGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTG
GTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAA
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TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGAT
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AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGC
CTTCGGGAAACTCAGACACAGGTGCTGCATGGCTGGTCGTCAGCTCGTGGTCGGTGGAGG
ATGTTTGGGGTTTAAAGTCCCGTAACGGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTC
GAGGTGGGGCCACCTTCCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACA
AAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGC
AGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCC
AGAAGTA

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Sequence of the isolate 14-72 (*P. aeruginosa*)

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGG
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CTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGA
CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGCGCGAAAAA
GGCNCTGATGCCAGCCATTGGCCCGGCGTGTGTGAAGAAGGTCTTCGGATTGTACAAGCA
CTTTAAGTTGGGAGGGAAGGGCAGTAAAGGTTAATACCTTGCTGTTTTGACGTTACCCAA
CAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCG
TTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTG
GTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAA
GGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGAT
CTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA
AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGGTG
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TGGTTTGGGGTTTAAGTCCCGTAAACGAGCGCAATCCCTTGTCCTTAGTTACCAAGCACCT
CGGGATGTGGGCAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGA
CGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAA
AGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCA
GAAGTA

Sequence of the isolate RRNA2 (*B. cereus*)

TGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAA
CATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGAC
CCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGGANAAG

CTGGCACCTTGACGGTACCTAACCAGAAAGCCNCGGCTAANTACGTGCCAGGCAGCCGCG
GTAATACGTAGGTGGCAAGCCTTATCCGGAATTATTGGGCGTAAAGNCCGCGCNGGTGGT
TCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGAGAGGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATA
TGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGC
TAAGTGTTAGAGGGGTTTCCGCCCTTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCT
GGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTSGAAGCAACGCGAAGAA

Sequence of the isolate RSLB5 (*B. licheniformis*)

TAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCT
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TCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAAC
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CCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATT
GGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGC
TGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAG
CACTCCCCCTGGGGAGTACGGTCGCAAGACTGGGGAACAATTG

Table 4. Identity of the isolates based on BLAST analysis

S. No	Isolate Name	Species identified	Query coverage	Percentage Identity	Accession number of the NCBI BLAST	Accession number of NCBI submitted
1	13-14	*Bacillus amyloliquefaciens*	100%	98.41%	KM036068.1	MN589742.1
2	14-33	*B. amyloliquefaciens*	100%	98.97%	MG892875.1	MN589743.1
3	14-54	*B. cereus*	100%	97.36%	CP016316.1	MN588090.1
4	14-68	*Pseudomonas aeruginosa*	100%	98.04%	CP033686.1	MN555434.1
5	14-69	*P. aeruginosa*	100%	97.40%	CP034368.1	MN555441.1
6	14-70	*P. aeruginosa*	100%	98.00%	HM439422.1	MN555445.1
7	14-71	*P. aeruginosa*	100%	98.00%	HM439393.1	MN555450.1
8	14-72	*P. aeruginosa*	100%	97.83%	KY549643.1	MN555461.1
9	RRNA2	*B. cereus*	100%	98.16%	HQ236057.1	MN588083.1
10	RSLB5	*B. licheniformis*	100%	99.88%	JN998718.1	MN598652.1
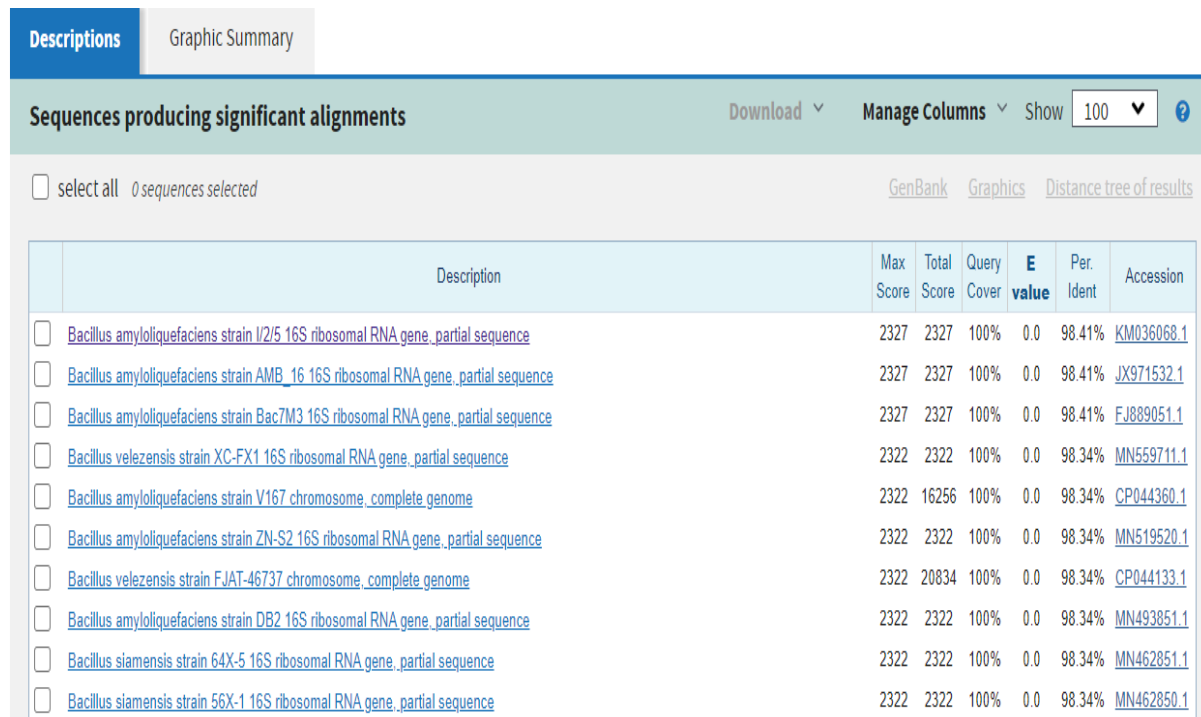
Figure 5. BLAST analysis of 13-14 (*Bacillus amyloliquefaciens*)

Figure 6. BLAST analysis of 14-33 (*Bacillus amyloliquefaciens*)

Figure 7. BLAST analysis of 14-54 (*Bacillus cereus*)

Figure 8. BLAST analysis of 14-68 (*Pseudomonas aeruginosa*)

Figure 9. BLAST analysis of 14-69 (*Pseudomonas aeruginosa*)

Figure 10. BLAST analysis of 14-70 (*Pseudomonas aeruginosa*)

Figure 11. BLAST analysis of 14-71 (*Pseudomonas aeruginosa*)

Figure 12. BLAST analysis of 14-72 (*Pseudomonas aeruginosa*)

Figure 13. BLAST analysis of RRNA2 (*Bacillus cereus*)

Figure 14. BLAST analysis of RSLB5 (*Bacillus licheniformis*)

4.5 GROWTH PROMOTION IN COWPEA

Growth promotion ability of the bacterial isolates was evaluated in cowpea. The growth parameters of cowpea seedlings *viz.*, days taken for seed germination, germination (%), number of leaves, shoot length (cm), root length (cm), leaf area were taken at 15, 30, 45 days after sowing (DAS) (Table 5, 6 & Fig. 15, Fig. 16)**.** In general, priming of cowpea seeds with bacterial isolates resulted in growth promotion (Plate 7 & Plate 8).

The number of days taken for germination varied from 3.91 (14-68) to 4.90 (13-14). There was no statistically significant difference in days required for germination among treatment. The germination percentage ranged from 56% (14-69) to 96% (RSLB5) and the isolates 14-69, 14-70, 14-71 and 14-72 found to reduce the germination of cowpea seeds. In all three observations taken on 15, 30 and 45 days after planting (DAP), the number of leaf production did not show significant difference. Number of leaf production on 15 and 30 DAP showed that except the isolate 14-69, all

other isolates showed more number of leaves compared to control. The number of leaves at 15 DAP varied from 26.8 (14-69) to 39.4 (14-68). Similarly, number of leaves at 30 DAP varied from 38.2 (14-69) to 51.4 (14-68). At 45 DAP, number of leaf production in all bacteria primed plants showed an increasing trend. It varied from 49.2 (control) to 62 (14-68).

Shoot length showed differential response on priming with different bacterial isolates. The shoot length at 15 DAP varied from 16.36 cm (14-71) to 20.85 cm (RRNA2) and except in the isolate 14-71, all other isolates showed similar growth pattern. Shoot length at 30 DAP varied from 31.01 cm (14-33) to 31.26 cm (14-68) and the length did not show significant difference due to the treatment. Three isolates, 13-14 (45.40 cm), 14-68 (45.10 cm) and 14-33 (43.10 cm) showed significantly higher shoot length compared to control (37.2 cm) on 45 DAP.

The root length varied among the treatments. Root length on 15 DAP ranged from 4.8 cm (control) to 6.5 cm (RRNA2) and all the isolates showed positive response consequent to bio-priming with bacterial isolates. At 30 DAP, the root length showed similar trend and RRNA2 (9.5 cm) and control (7.9 cm) recorded maximum and minimum root length respectively. Root lengths of cowpea plants on 45 DAP ranged from 16.68 cm (14-69) to 19.52 cm (13-14). Leaf area on 15 DAP varied from 60 cm^2 (14-70) to 75 cm^2 (13-14) and four isolates, 14-69 (63 cm^2), 14-70 (60 cm^2), 14-71 (63 cm^2) and 14-72 (62 cm^2) showed lesser leaf area than control plants (65 cm^2). At 30 DAP, only the isolate 14-70 showed lesser leaf area than control. The leaf area ranged from 70 cm^2 (14-71) to 93 cm^2 (13-14) and 87 cm^2 (RRNA2) to 120 cm^2 (13-14) on 30 DAP and 45 DAP respectively. At 30 DAP, the isolate 14-71 showed less leaf area than control, whereas at 45 DAP; only two isolates 13-14 (120 cm^2) and 14-33 (108 cm^2) showed more leaf area than control.

The isolates, 13-14, 14-33 and RRNA2 showed positive response consequent to priming with bacterial isolates. Considering different aspects, the isolate 13-14 was selected for further study.

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Table 5. Growth parameters of cowpea as influenced by bacterial isolates

Isolate	No. of days taken for germination	Germination %	Number of leaves			Shoot length (cm)		
			(15DAP)	(30DAP)	(45DAP)	(15DAP)	(30DAP)	(45DAP)
13-14	4.90	84.0[ab]	36.8	47.0	57.8	18.72[ab]	29.90[a]	45.40[a]
14-33	4.08	82.00[ab]	35.6	46.8	56.8	18.10[ab]	31.02[a]	43.10[ab]
14-68	3.94	86.00[ab]	39.4	51.4	62.0	20.34[a]	31.26[a]	45.10[a]
14-69	4.54	56.00[c]	26.8	38.2	50.2	18.62[ab]	26.84[ab]	37.00 [b]
14-70	4.48	56.00[c]	32.4	43.8	56.8	18.43[ab]	29.34[a]	40.70[ab]
14-71	4.52	56.00[c]	32.4	44.0	55.8	16.36[b]	22.50[b]	38.40[b]
14-72	4.50	60.00[c]	36.8	45.0	52.2	18.86[ab]	25.74[ab]	38.50[b]
RSLBS	4.74	96.00[a]	38.0	41.6	51.2	20.76[a]	27.84[ab]	41.90[ab]
RRNA2	4.44	90.00[a]	34.2	44.6	55.2	20.32[a]	26.92[ab]	40.90[ab]
14-54	4.46	84.00[ab]	36.0	44.4	53.0	20.86[a]	28.56[a]	42.80[ab]
Control	4.58	70.00[bc]	30.6	39.0	49.2	20.82[a]	26.20[ab]	37.20[b]
SE (d)	0.420	7.812	4.659	4.38	4.075	1.384	2.155	2.341
LSD at1%	NS	21.12	NS	NS	NS	3.74	5.828	6.3313

Table 6. Root length of cowpea plants as influenced by bacterial isolates, cm

Isolate	15 DAP	30 DAP	45 DAP
13-14	6.38[ab]	9.10[cd]	19.52
14-33	5.82[cd]	8.60[fg]	19.48
14-68	5.50[e]	8.90[de]	18.20
14-69	5.72[cde]	8.40[gh]	16.68
14-70	4.90[f]	7.94[j]	17.98
14-71	6.20[b]	9.20[c]	18.62
14-72	5.90[c]	8.10[ij]	19.86
RSLBS	5.58[de]	8.80[ef]	17.64
RRNA2	6.40[ab]	9.50[b]	18.27
14-54	4.80[f]	8.30[hi]	18.12
Control	6.50[a]	9.80[a]	20.02
SE (d)	0.093	0.105	1.253
LSD at 1%	0.2507	0.2836	NS

Plate7. Growth promotion activity in Cowpea after treated with bacterial isolates

Plate 8. Growth promotion activity in Cowpea after 1 month
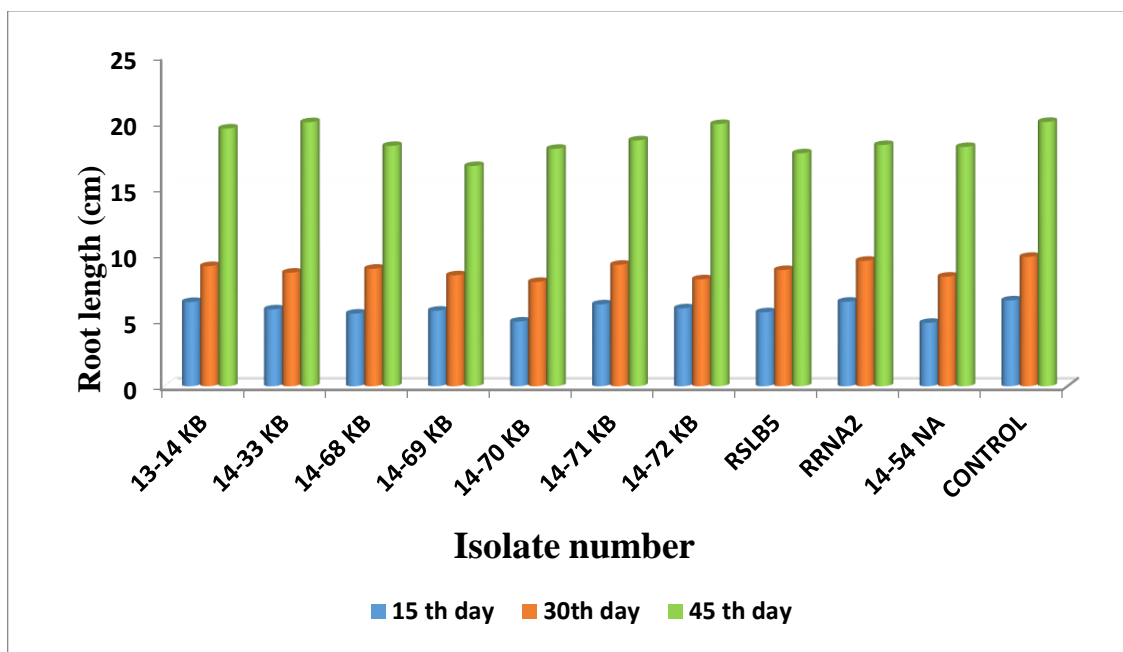
Figure 15. Root length of cowpea as influenced by bacterial isolates
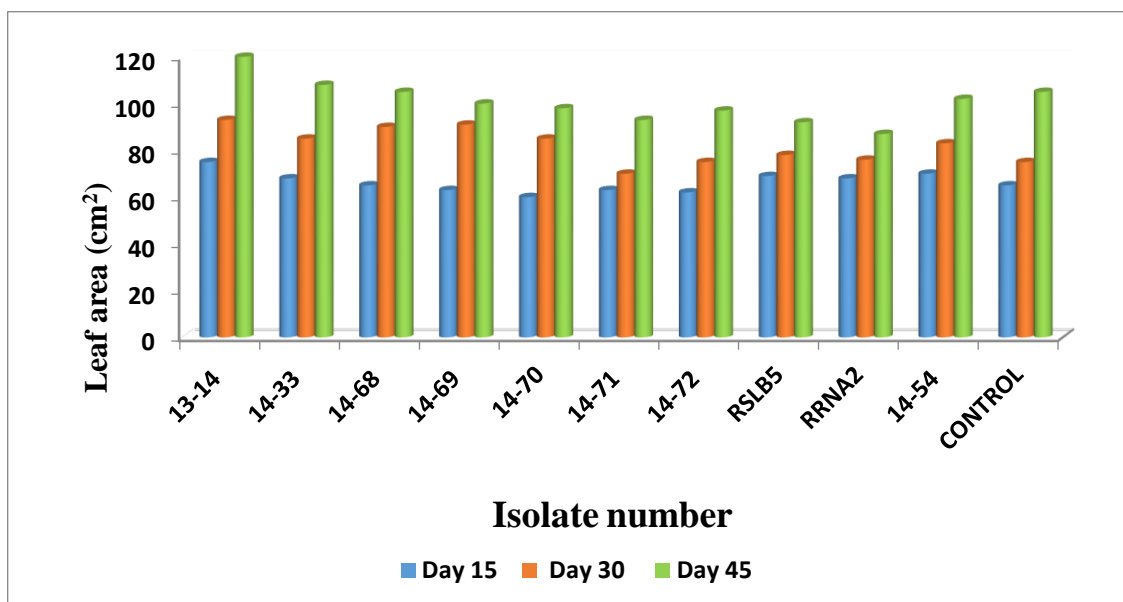
Figure 16 Leaf area of cowpea as influenced by bacterial isolates

4.6 DIFFERENTIAL EXPRESSION OF ENZYMES

Muktakeshi and Sree Kiran plants were grown in pots filled with sterile potting mixture in net house (Plate 9). After two months of planting, the plants were placed inside a moist chamber and plants were challenge inoculated with the pathogen in treatments T2, T3, T6 and T7 with the zoospore suspension of *Phytophthora colocasiae*, prepared by leaf disc method (Plate 10 & Plate 11).

The leaf samples were collected at different intervals, 0, 6, 12 and 24h, 2, 4 and 8 days for enzyme assay and differential expression of proteins. The enzymes *viz.*, peroxidase, chitinase, glucanase, phenylalanine ammonia lyase (PAL) and total phenol were assayed using standard protocols.

4.6.1 Chitinase activity

In Sree Kiran, the chitinase activity was maximum in the treatment PGPR + *P. colocasiae* (T3) (9.76 mg sugar released/ g tissue) at 12h after inoculation (hai). The increase in activity in this treatment was noted from 6 hour after inoculation and the activity showed declining trend after 12 hai. In T2 (*P. colocasiae*), the highest activity was noted at 12 hai (6.58 mg sugar released/ g tissue). However, the activity in T2 showed fluctuation in subsequent period. In T1 (PGPR alone), the chitinase activity was always in an elevated level compared to control. In control plants, the activity started increasing from 24 hai and continued till 8 days (Table 7 & Fig. 17).

In Muktakeshi, the chitinase activity was maximum in PGPR treated plants (T5-9.75 mg sugar released/ g tissue) at 12 hai. But in this treatment, variation in level of activity was noticed throughout the period. Second highest level was noticed in the treatment where plants were inoculated with the pathogen, *P. colocasiae* and elevated level of activity was noticed till 8 dai (days after inoculation). In case of the treatment PGPR + *P. colocasiae,* the activity was noted from 6 hai and the maximum activity was at 12 hai (7.27 mg sugar released/ g tissue) and the elevated level was noticed until 8 dai. In control, the increase in activity started from 12 hai and the increase level was maintained till 8 dai (Fig. 18).

4.6.2. Glucanase Activity

In Sree Kiran, the glucanase activity was maximum in T3- PGPR + *P. colocasiae* (1.83 mg sugar released/ g tissue) at 24 hai (Table 8 and Fig. 19). The increase in activity was observed from 12 hai and declined after 24 hai. In PGPR alone treated plants, the increase in activity was noted from 24 hai and the elevated production continued till 8 dai. The control plants as well as the planted challenge inoculated with *P. colocasiae* did not show a clear pattern. However, there was an increase in activity in both the cases.

In Muktakeshi, the glucanase activity was maximum in PGPR treated plants (1.93 mg sugar released/ g tissue) at 8 dai (Fig. 20). The increase in activity noted from 12 hai and remained almost same until 2 dai and increased. Increase in activity was noted 6 hai in plants which were treated with PGPR and later challenge inoculated with *P. colocasiae.* The activity in the treatment remained elevated till 2 dai.

4.6.3 Peroxidase Activity

In Sree Kiran, the peroxidase activity was maximum in T3- PGPR + *P. colocasiae* (2.48 mg of protein) at 6 hai (Table 9 & Fig. 21). The elevation in activity was found upto 12 hai and the activity started declining. In all the treatments there was an increase in activity during 12-24 hai period.

In Muktakeshi, the maximum peroxidase activity was noted in T7- PGPR + *P. colocasiae* (1.98 mg of protein) at 4 dai (Fig. 22). The activity varied highly with different time interval of assessment.

4.6.4 PAL Activity

In Sree Kiran, the maximum PAL activity was noted in T2- plants challenge inoculated with *P. colocasiae* (100.05 g of protein) at 0 hai (Table 10 & Fig. 23). The activity declined until 2 dai and then again showed an increase in production. Control plants also showed the similar trend. In PGPR and PGPR + *P. colocasiae* treatments, there was no major change in production throughout the study period.

In Muktakeshi, the maximum PAL activity was noted in control plants (105.8 g of protein) at 8 dai (Fig. 24) followed by the plants primed with PGPR at 6 hai (100.15 g of protein). In plants with PGPR + *P. colocasiae,* maximum production was noted on 4 dai (98.66 g of protein). In all other cases there was no major change in production throughout the study period.

4.6.5 Total phenol

In Sree Kiran, the maximum phenol content was recorded in control plants (2.56 mg\g tissue) at 8 dai (Table 11 & Fig. 25). In other three treatments, the total phenol content showed decreasing with increase of time. The phenol content decreased from 2.18 - 2.41 mg\g tissue at 0 h to 1.30 -1.57 mg\g tissue at 8 dai.

In Muktakeshi, the maximum phenol content was in T7- PGPR + *P. colocasiae* (4.88 mg\g tissue) at 24 hai (Fig. 26). In treatments *viz*., PGPR; *P. colocasiae* and PGPR + *P. colocasiae,* increased level of activity was noticed at 24 hai. In all cases, the phenol content showed decreasing trend after 24 hai. In control, highest quantity was recorded at 8 dai.

Table 7. Chitinase activity in different treatments over time in Sree Kiran and Muktakeshi

Treatment	Chitinase activity (mg sugar released/ g tissue)						
	Intervals						
	0	6h	12h	24h	2D	4D	8D
Sree Kiran							
PGPR	6.37	7.18	4.03	6.1	8.02	7.23	6.12
P. colocasiae	4.23	2.56	6.58	5.27	4.13	5.29	5.45
PGPR + *P. colocasiae*	5.93	7.85	9.76	5.62	5.43	4.27	5.79
Control	2.5	4.31	5.85	8.3	7.02	7.38	7.46
Muktakeshi							
PGPR	5.81	4.06	9.75	6.15	7.53	6.52	6.38
P. colocasiae	4.03	4.18	9.71	7.28	7.17	7.05	6.54
PGPR + *P. colocasiae*	3.83	7.27	8.83	6.53	5.38	6.59	6.32
Control	2.18	3.22	5.49	6.23	7.18	6.59	6.31

Table 8. Glucanase activity in different treatments over time in Sree Kiran and Muktakeshi

Treatment	Glucanase activity (mg sugar released/ g tissue)						
	Intervals						
	0	6h	12h	24h	2D	4D	8D
Sree Kiran							
PGPR	0.34	0.48	0.531	0.95	1.42	1.25	1.02
P. colocasiae	0.80	0.91	0.991	1.508	0.326	0.95	1.13
PGPR + *P. colocasiae*	0.753	0.85	1.34	1.83	0.84	0.735	0.72
Control	0.051	1.105	1.3	0.92	1.75	0.68	0.531
Muktakeshi							
PGPR	0.023	0.92	1.058	0.935	1.37	1.28	1.93
P. colocasiae	0.435	0.83	1.38	1.13	0.892	0.749	0.63
PGPR + *P. colocasiae*	0.698	1.4	1.35	1.28	1.18	0.83	0.708
Control	0.438	0.48	0.85	0.924	0.6	0.73	0.7

Table 9. Peroxidase activity in different treatments over time in Sree Kiran and Muktakeshi

Treatment	Peroxidase activity (mg of protein)						
	Intervals						
	0	6h	12h	24h	2D	4D	8D
Sree Kiran							
PGPR	1.13	1.85	1.784	1.35	0.897	0.49	0.328
P. colocasiae	1.28	1.39	1.73	0.593	1.497	1.38	1.257
PGPR + *P. colocasiae*	1.58	2.48	2.286	1.98	1.85	1.738	1.72
Control	1.34	1.27	1.39	1.95	0.957	0.35	0.22
Muktakeshi							
PGPR	0.45	0.596	1.85	0.28	0.83	1.07	0.93
P. colocasiae	1.2	0.953	1.67	0.79	1.25	1.08	0.97
PGPR + *P. colocasiae*	1.35	1.43	1.49	0.48	0.76	1.98	1.83
Control	1.48	1.89	1.46	0.325	0.851	0.235	0.15

Table 10. PAL activity in different treatments over time in Sree Kiran and Muktakeshi

Treatment	Phenylalanine ammonia lyase activity (nM/Min/g)						
	Intervals						
	0	6h	12h	24h	2D	4D	8D
Sree Kiran							
PGPR	50.8	40.53	53.68	58.72	57.3	57.18	56.18
P. colocasiae	100.05	38.71	49.863	66.37	39.49	90.05	92.38
PGPR + *P. colocasiae*	57.35	69.82	46.27	39.74	49.62	52.8	53.09
Control	82.15	65.28	28.6	33.29	55.6	61.02	62.125
Muktakeshi							
PGPR	75.62	100.15	52.59	51.95	50.76	51.72	52.38
P. colocasiae	49.1	69.17	55.38	63.29	53.64	56.73	56.59
PGPR + *P. colocasiae*	57.89	48.5	53.4	60.35	67.2	98.66	88.35
Control	52.8	47.39	55.27	57.85	65.73	105.8	99.8

Table 11. Total phenol content in different treatments over time in Sree Kiran and Muktakeshi

Treatment	Total phenol content (mg phenol/g tissue)						
	Intervals						
	0	6h	12h	24h	2D	4D	8D
Sree Kiran							
PGPR	2.34	1.635	2.187	1.97	1.853	1.605	1.437
P. colocasiae	2.41	1.628	2.12	2.193	1.326	1.59	1.302
PGPR + *P. colocasiae*	2.18	2.26	1.85	2.28	1.749	1.68	1.572
Control	1.87	2.45	1.49	1.35	2.138	2.48	2.56
Muktakeshi							
PGPR	2.85	2.1	2.42	3.218	1.76	2.39	2.48
P. colocasiae	2.21	2.19	1.872	3.82	2.57	2.24	2.09
PGPR + *P. colocasiae*	2.15	2.19	2.285	4.88	2.52	2.83	2.75
Control	1.58	2.23	1.75	1.92	1.917	2.75	2.62
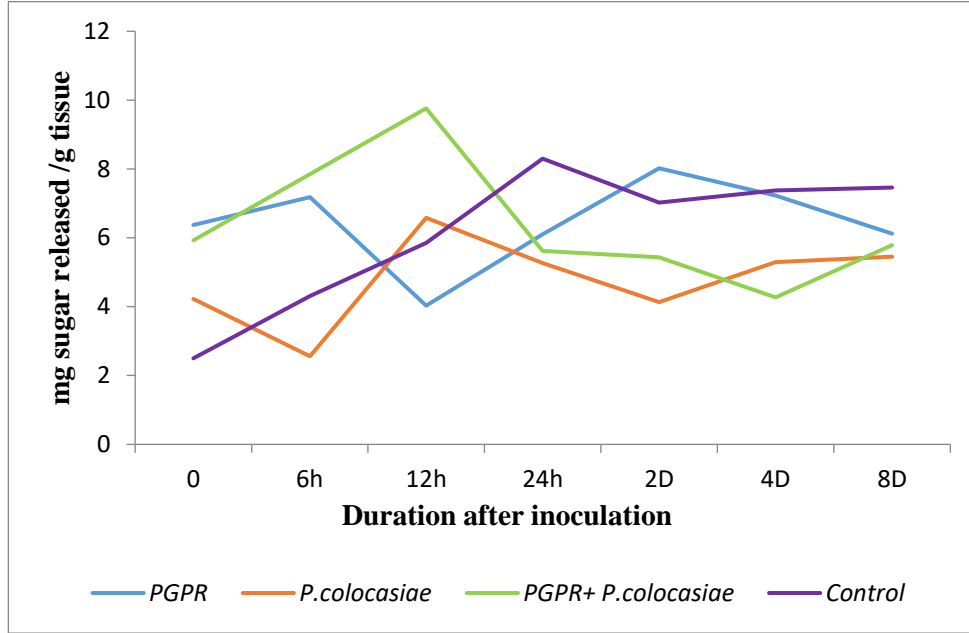
Figure 17. Chitinase activity in different treatments over time (Sree Kiran)
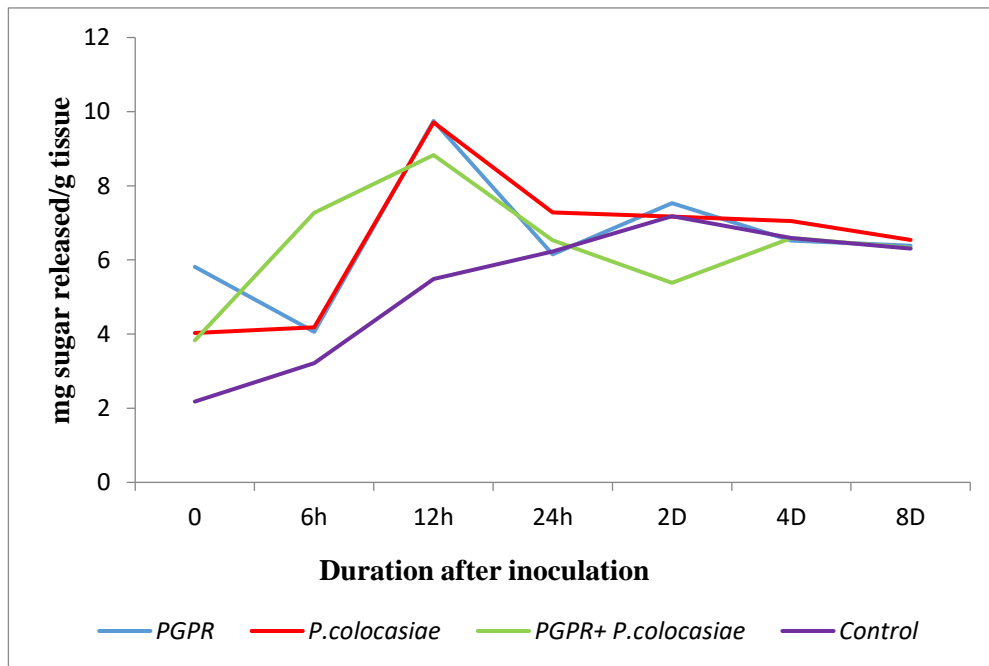
Figure 18. Chitinase activity in different treatments over time (Muktakeshi)
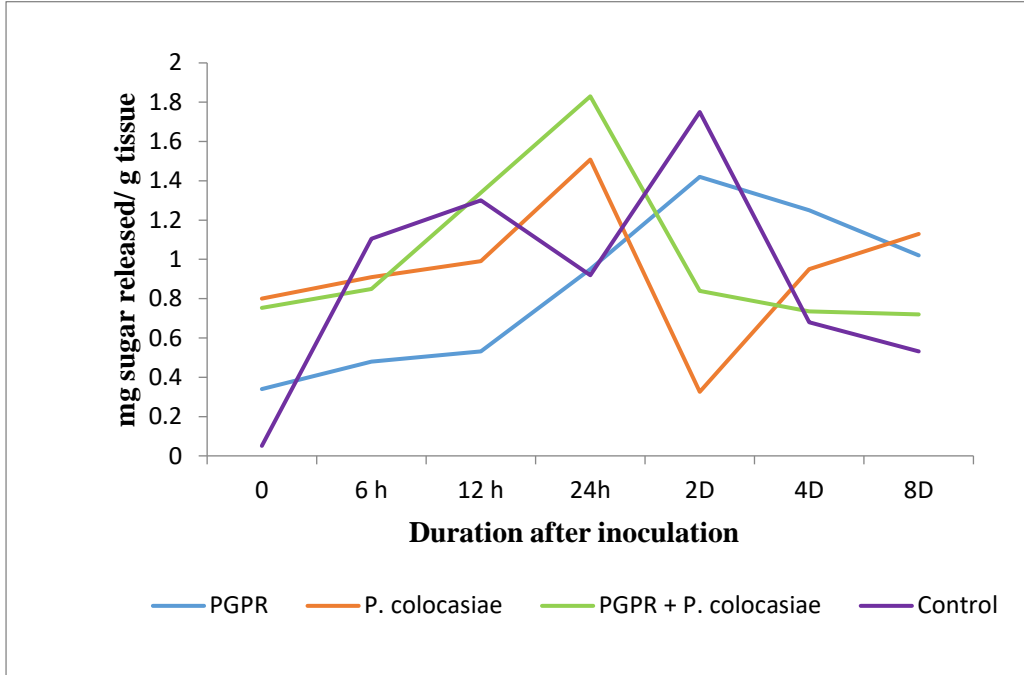
Figure 19. Glucanase activity in different treatments over time (Sree Kiran)
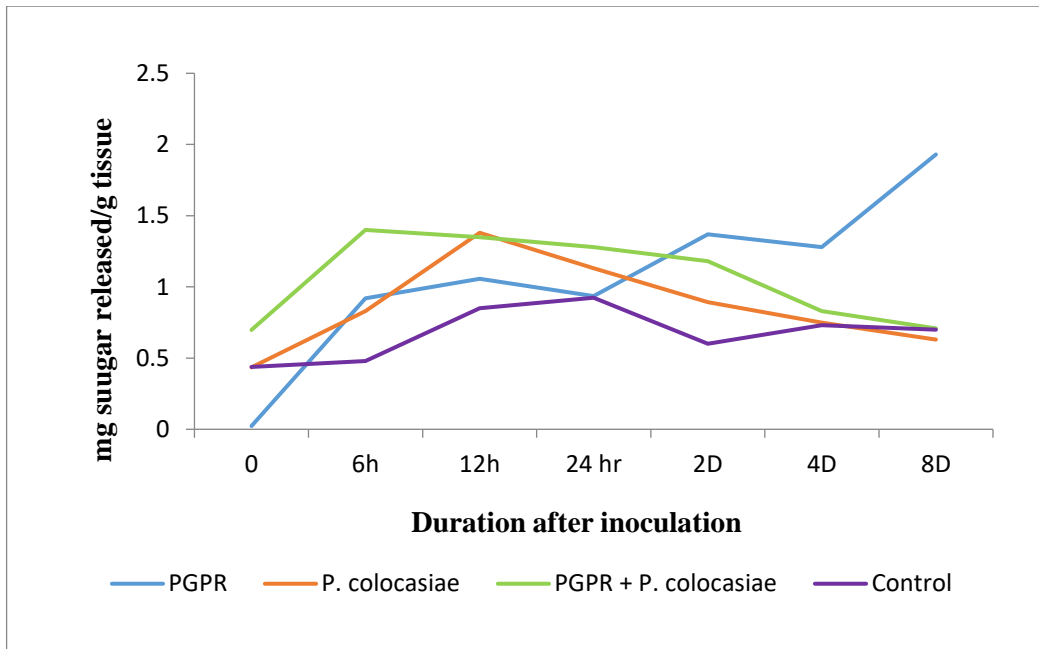
Figure 20. Glucanase activity in different treatments over time (Muktakeshi)
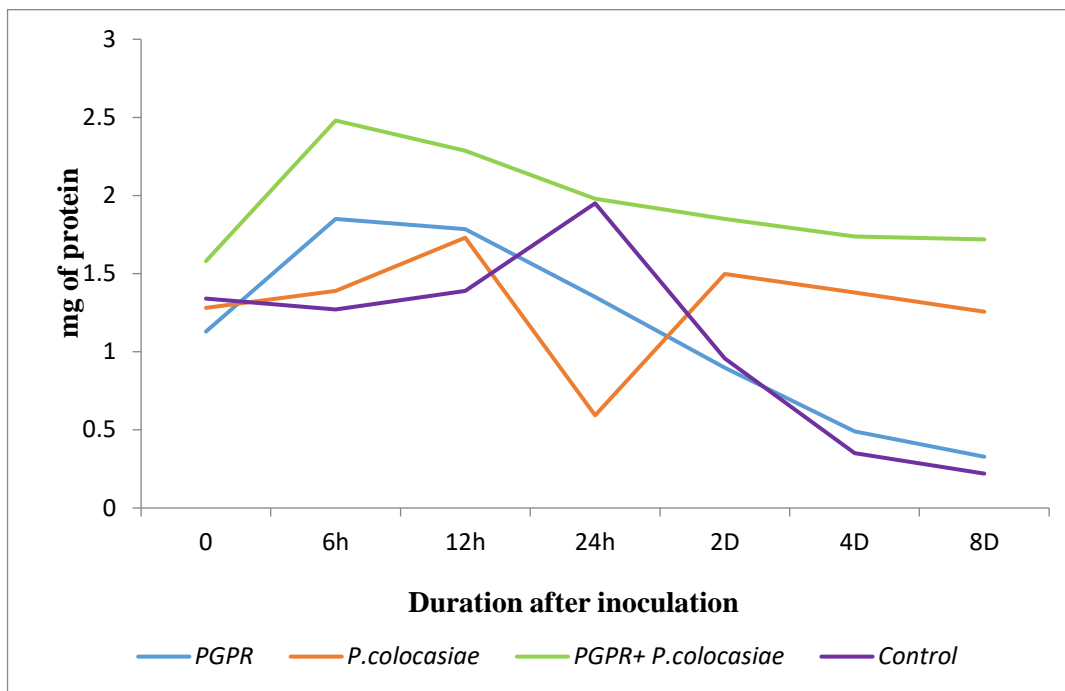
Figure 21. Peroxidase activity in different treatments over time (Sree Kiran)
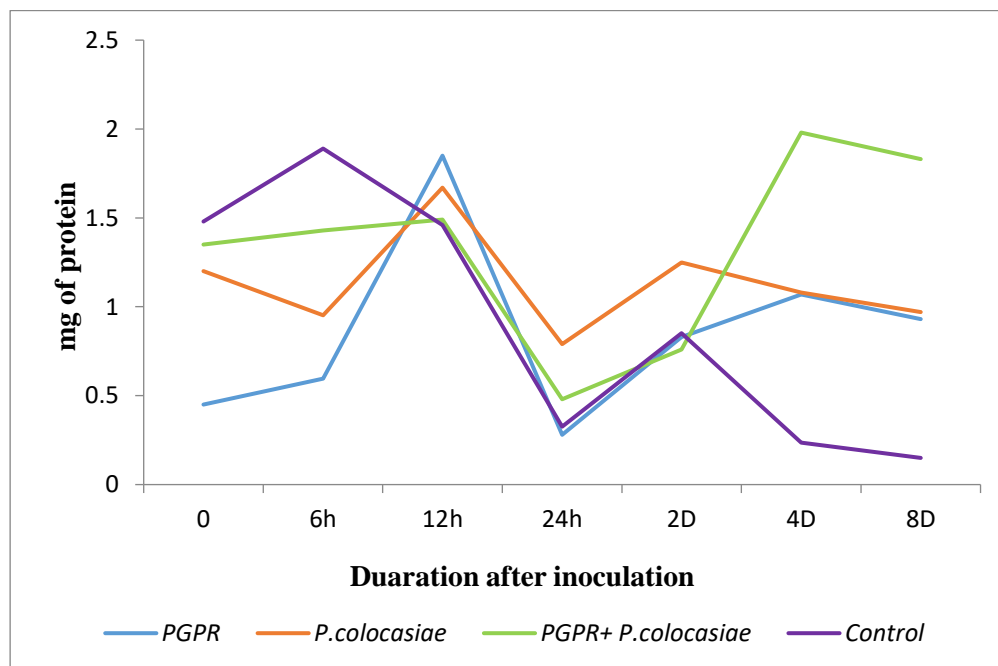
Figure 22. Peroxidase activity in different treatments over time (Muktakeshi)
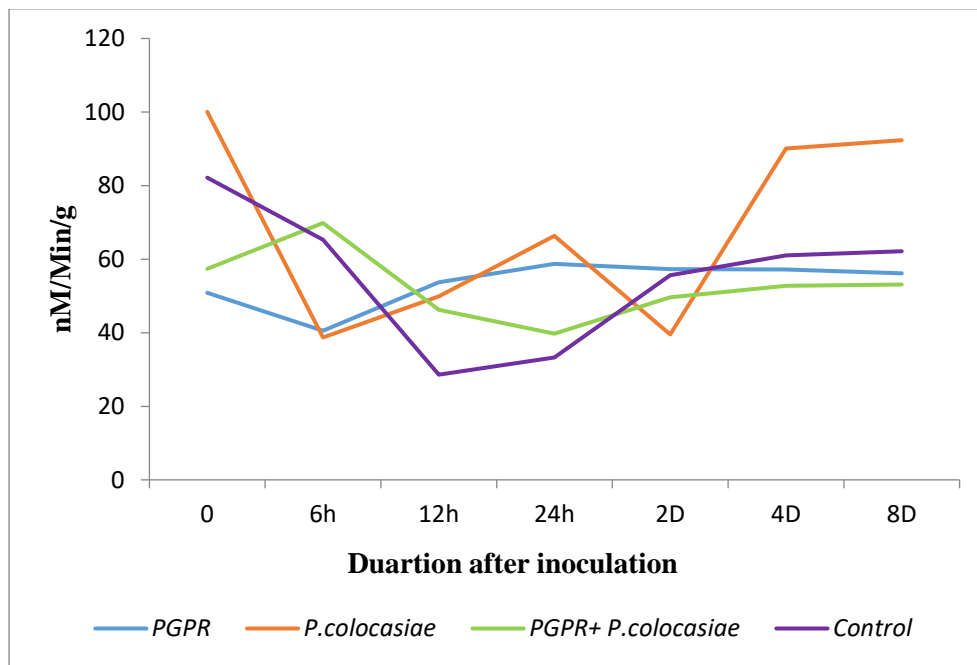
Figure 23. PAL activity in different treatments over time (Sree Kiran)
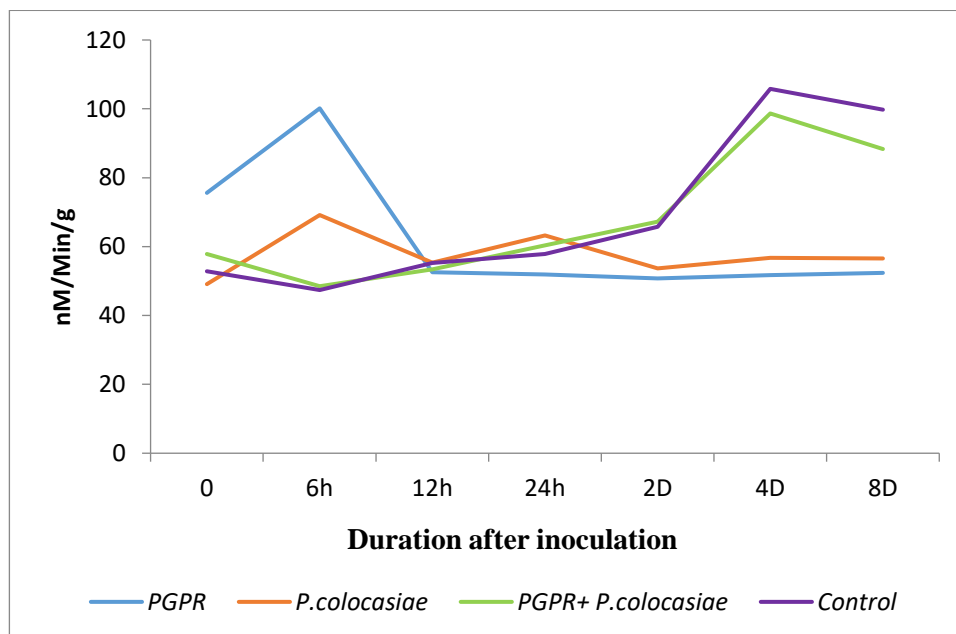
Figure 24. PAL activity in different treatments over time (Muktakeshi)
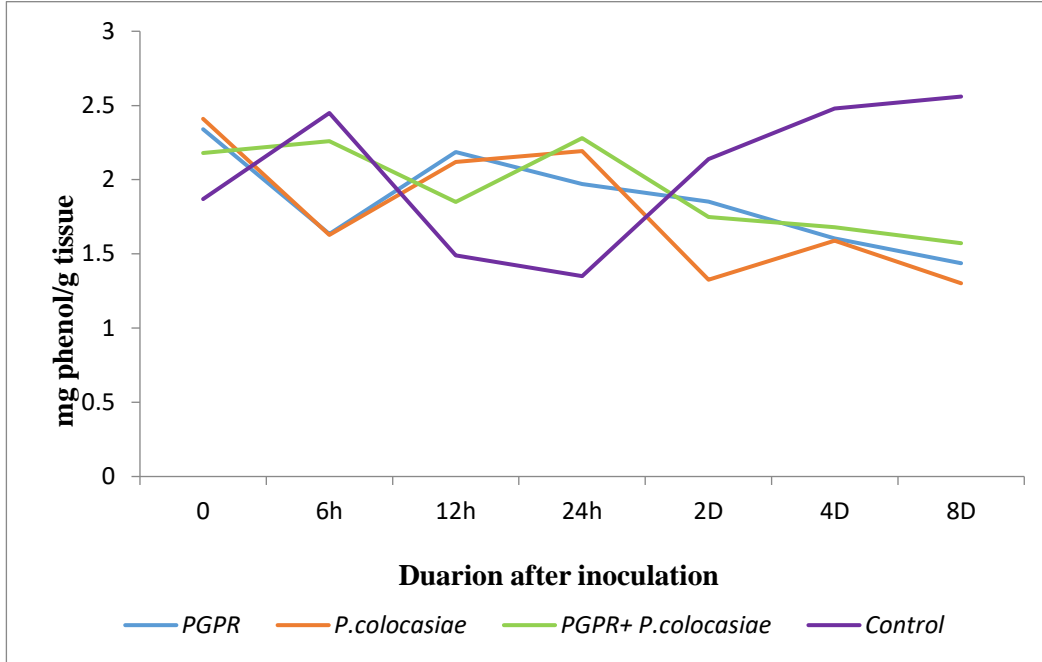
Figure 25. Total phenol production in different treatments over time (Sree Kiran)
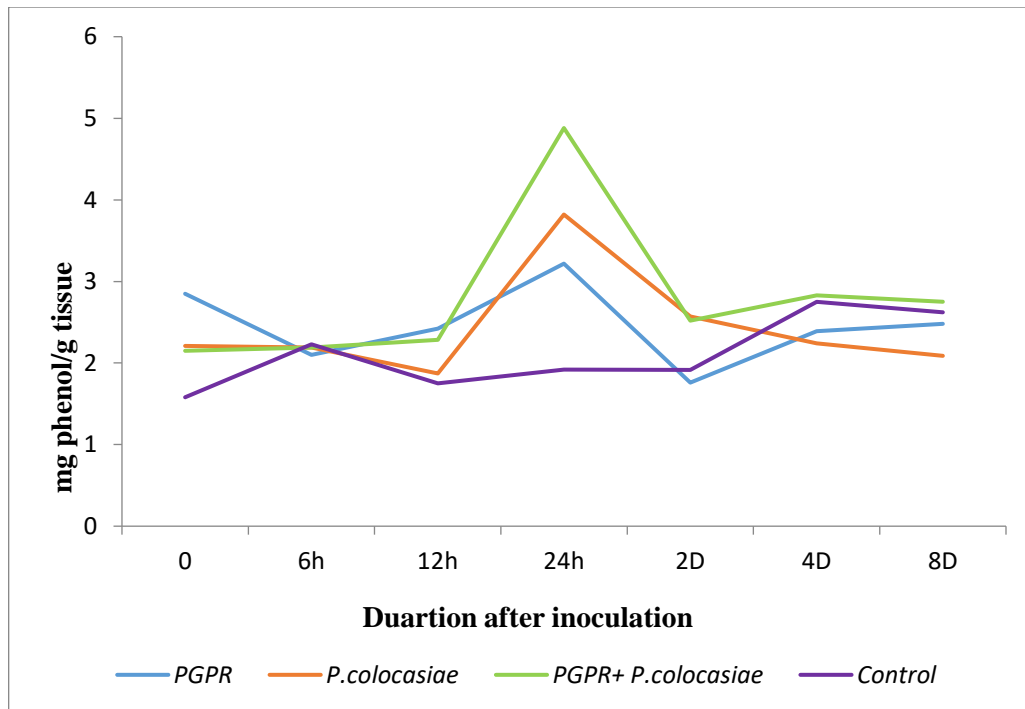
Figure 26. Total phenol production in different treatments over time (Muktakeshi)

Plate 9. Taro plants grown in pot for studying expression of enzymes

Plate 10. Taro plants were challenge inoculated and kept inside the chamber

Sree Kiran plant with *Phytophthora colocasiae* infection 3 days after inoculation in T2

Sree Kiran plant with PGPR+ *Phytophthora colocasiae* infection 3 days after inoculation in T3

Muktakeshi plant with *Phytophthora colocasiae* infection 4 days after inoculation in T6

Muktakeshi plant with PGPR+ *Phytophthora colocasiae* infection 4 days after inoculation in T7

Plate 11. Infection in taro plants after challenge inoculation with *P. colocasiae*

4.7 GROWTH PARAMETERS OF COLOCASIA

The growth parameters such as number of days taken for sprouting, height of the plant, number of leaves, leaf length, leaf breadth of colocasia were recorded at regular intervals (Table 12a, 12b, 12c & 12d).The number of days taken for sprouting (Fig. 27) ranged from 16-21 days in Sree Kiran and 21-22 days in Muktakeshi plants consequent to incorporation of PGPR.

In Sree Kiran, the maximum number of leaves was in T1 (PGPR alone) followed by PGPR + *P. colocasiae* (T3) plants. The maximum height of the plant was noted with T1 (PGPR alone) followed by PGPR + *P. colocasiae* (T3) (Table 12a). In Muktakeshi, the maximum number of leaves were noted in T5 (PGPR alone) plants. Maximum plant height was noted in T5 (PGPR alone) followed by PGPR + *P. colocasiae* (T7) (Table 12b).

In Sree Kiran, the leaf length and leaf breadth was higher in T1 (PGPR alone) plants followed by PGPR + *P. colocasiae* (T3) plants (Table 12c). In Muktakeshi, leaf length and leaf breadth was higher in T5 (PGPR alone) plants followed by PGPR + *P. colocasiae* (T7) plants (Table 12d).
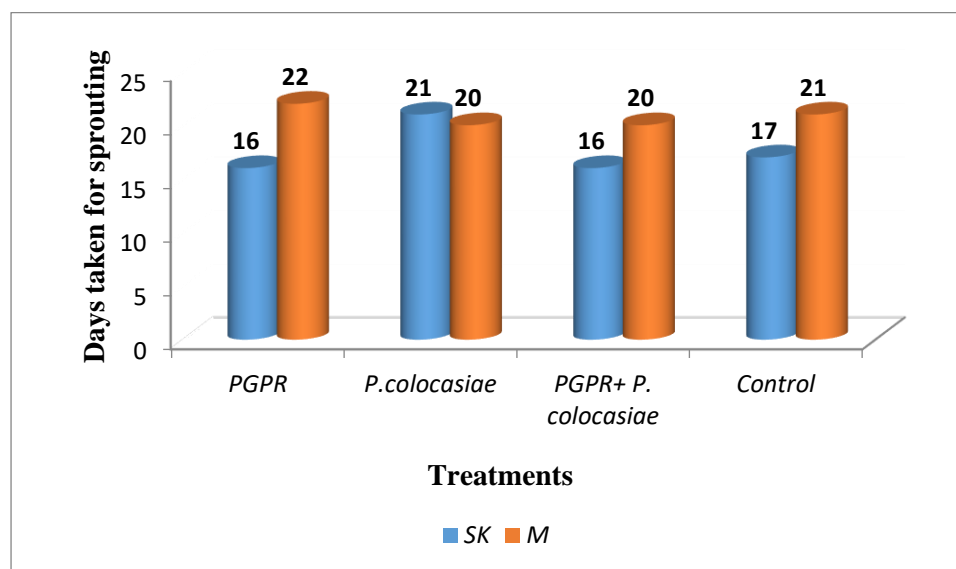
Figure 27. Growth parameter of taro in Sree Kiran and Muktakeshi

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Table 12a Growth parameters of taro (Sree Kiran) consequent to PGPR incorporation

Treatment	Number of leaves			Height of the plant (cm)		
	1st MAP	2nd MAP	3rd MAP	1st MAP	2nd MAP	3rd MAP
PGPR alone	4.3	3.8	5.0	42.17	87.2	91.0
P. colocasiae alone	3.7	4.3	4.3	35.3	84.0	86.8
PGPR + *P. colocasiae*	4.3	4.0	4.8	38.0	82.7	90.3
Control	3.8	4.3	4.3	32.7	82.2	87.7
LSD at 1%	NS	NS	NS	NS	NS	NS

Table 12b Growth parameters of taro (Muktakeshi) consequent to PGPR incorporation

Treatment	Number of leaves			Height of the plant (cm)		
	1st MAP	2nd MAP	3rd MAP	1st MAP	2nd MAP	3rd MAP
PGPR alone	4.3	15.0	14.7	27.3	58.5	61.5
P. colocasiae alone	4.3	12.3	14.2	21.2	52.3	57.3
PGPR + *P. colocasiae*	4.2	13.2	13.7	23.0	54.0	60.7
Control	4.7	11.8	13.5	17.8	52.3	57.3
LSD at 1%	NS	NS	NS	NS	NS	NS

Table 12c Growth parameters of taro (Sree Kiran) consequent to PGPR incorporation

Treatment	Leaf length (cm)			Leaf breadth (cm)		
	1st MAP	2nd MAP	3rd MAP	1st MAP	2nd MAP	3rd MAP
PGPR alone	27.8	41.3	42.3	24.2	36.3	37.8
P. colocasiae alone	23.6	37.7	41.3	20.3	32.7	36.8
PGPR + *P. colocasiae*	24.6	40.5	42.0	21.7	35.7	37.3
Control	19.3	35.8	40.7	17.3	31.8	35.8
LSD at 1%	NS	NS	NS	NS	NS	NS

Table 12d Growth parameters of taro (Muktakeshi) consequent to PGPR incorporation

Treatment	Leaf length (cm)			Leaf breadth (cm)		
	1st MAP	2nd MAP	3rd MAP	1st MAP	2nd MAP	3rd MAP
PGPR alone	16.5	29.3	31.7	12.0	20.7	26.7
P. colocasiae alone	15.4	27.5	29.2	10.0	18.0	24.2
PGPR +*P. colocasiae*	15.8	28.0	30.2	11.5	19.0	24.3
Control	15.0	27.5	29.5	11.0	18.2	22.8
LSD at 1%	NS	NS	NS	NS	NS	NS

In Sree Kiran and Muktakeshi consequent to PGPR incorporation (Table 13a, 13b), the number of mother corms, number of cormels, weight of mother corms and weight of cormels were higher in PGPR + *P. colocasiae* plants.

In Sree Kiran, the growth parameters *viz*., number of mother corms and cormels, weight of mother corms and cormels did not show significant variation due to the treatments (Table 13a). The maximum number of mother corms, number of cormels, weight of mother corms and weight of cormels were noted with the treatment T1 (PGPR alone) followed by PGPR + *P. colocasiae* (T3).

In Muktakeshi, the growth parameters *viz*., number of mother corms and cormels, weight of mother corms and cormels did not show significant variation due to the treatments (Table 13b). The maximum number of mother corms, number of cormels, weight of mother corms and weight of cormels were noted with the treatment T1 (PGPR alone) followed by PGPR + *P. colocasiae* (T3).

Table 13a Yield parameters in Sree Kiran consequent to PGPR incorporation

Treatment	No. of mother corms	No. of cormels	Wt. of mother corms (g)	Wt. of cormels (g)
PGPR alone	3.67^a	14.33^a	243.33^a	158.33
P. colocasiae alone	2.33^b	8.67^b	158.33^{ab}	141.67
PGPR + *P. colocasiae*	2.67^b	14.0^a	208.33^{ab}	153.33
Control	1.33^c	12.0^{ab}	136.67^b	150.08

Table 13b Yield parameters in Muktakeshi consequent to PGPR incorporation

Treatment	No. of mother corms	No. of cormels	Wt. of mother corms (g)	Wt. of cormels (g)
PGPR alone	5.67	23.33^a	240.67^a	425.0^a
P. colocasiae alone	4.67	14.0^c	225.0^{ab}	258.3^b
PGPR + *P. colocasiae*	5.0	19.33^{ab}	225.0^{ab}	325.0^{ab}
Control	5.67	15.33^{bc}	115.0^b	296.67^{ab}
LSD at 1%	NS	5.3154		163.1

In Sree Kiran, the PDI was lower in T1 (PGPR alone) plants and highest PDI was in T2 (*P. colocasiae* alone) plants (Table 14a). In Muktakeshi, least PDI was in T5 (PGPR) plants and highest PDI was noted in T6 (*P. colocasiae* alone) plants (Table 14b). The disease incidence was very less in Muktakeshi compared to Sree Kiran (Plate 12).

Table 14a TLB incidence in Sree Kiran consequent to PGPR incorporation

Treatment	PDI 1	PDI 2	PDI 3
PGPR alone	6.2	9.7^b	24.8
P. colocasiae alone	8.6	25.0^a	32.2
PGPR + *P. colocasiae*	8.5	22.8^a	31.7
Control	6.9	19.2^{ab}	30.3
LSD at 1%	NS	13.1	NS

Table 14b TLB incidence in Muktakeshi consequent to PGPR incorporation

Treatment	PDI 1	PDI 2	PDI 3
PGPR alone	3.06^b	7.50^b	9.0^b
P. colocasiae alone	10.17^a	18.22^a	18.2^a
PGPR + *P. colocasiae*	7.50^{ab}	10.22^b	14.9^{ab}
Control	6.33^{ab}	8.33^b	9.17^b
LSD at 1%	6.13	6.60	8.50

Plate 12. Taro plants grown in grow bag

DISCUSSION

5. DISCUSSION

Taro (*Colocasia esculenta*) is a member of Araceae family. Tropical root and tuber crops regarded as third most important food crops after cereals and pulses (Mohan *et al.*, 2016). Taro leaf blight is the most destructive and prevalent disease of taro caused by *Phytophthora colocasiae*. In India, taro is cultivated in the states *viz.*, Assam, Andhra Pradesh, Bihar, Gujarat, Himachal Pradesh, Kerala, Maharashtra, Manipur, Orissa, Tamilnadu, Telanagana, Uttarakhand, and West Bengal (Choudhary *et al.*, 2011). Leaf blight in taro occurs during rainy season and the symptom starts as small water soaked spots, which enlarges in the size and number causing fully destruction of leaf lamina and petiole of the plant. Under cloudy weather conditions with intermittent rains and temperature around 28°C, the disease spreads at tremendous speed and the entire field gives a blighted appearance (Misra *et al.,* 2007; Lokesh *et al.*, 2014). The use of beneficial microorganisms is considered as one of the most capable method for safe crop management (Ongena and Jacques, 2008).

The present study, entitled the "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight" aimed to identify efficient PGPR isolates with high antagonistic activity against *P. colocasiae*, the pathogen responsible for TLB in colocasia; to characterize the isolates using biotechnological tools (16SrRNA gene primers) and to study the differential expression of PR proteins in susceptible and tolerant varieties of taro on application of PGPR. Application of *Trichoderma* spp. is being recommended for TLB management. PGPR application induces plant defense genes and priming the plant against pathogen attack. However, no serious attempt has been made with bacterial isolates to control TLB.

Eighty four bacterial isolates representing different parts of the country and *P. colocasiae* isolates received from microbial repository, ICAR-CTCRI were used for the study. Different methods of screening *viz.*, dual culture/direct confrontation (Skidmore and Dickinson, 1976), diffusible (Dennis and Webster, 1971a) and volatile metabolites production methods (Dennis and Webster, 1971b) were adopted for identifying the most potent isolate (PGPR) against the taro leaf blight pathogen.

In dual culture, 42 isolates showed more than 50% inhibition to *P. colocasiae.* Whereas in diffusible method, only 12 isolates showed more than 50% inhibition to *P. colocasiae.* In volatile metabolites production method, 30 isolates showed more than 50% inhibition to *P. colocasiae.* While studying the effect of vermicompost and vermiwash in controlling the taro leaf blight and collar rot of elephant foot yam disease, a total of 309 culture dependant bacterial isolates of vermicompost origin were screened against the target pathogens adopting these techniques and found 18.9% and 36.4% of these organisms showed >50% inhibition against *S. rolfsii* and *P. colocasiae* respectively (Veena *et al*., 2013). Similar study was done by Sujina *et al*. (2017) to evaluate antagonism of potent endophytes against the pathogen using dual culture method. The results showed that out of 65 bacterial isolates, 10 bacterial isolates showed more than 70% inhibition, 14 isolates showed more than 60%. Compared to these studies, the organisms used in this study showed more inhibition indicating its scope to be utilized as a bio-agent to manage the disease. It is well-known that bacteria can commonly produce cell wall-degrading enzymes and secondary metabolites to hinder the growth of other microorganisms (Shoda, 2000; Kohl *et al*., 2019).

Based on the present knowledge about the morphological and biochemical characteristics, it is insufficient to identify the bacterial isolates at species level, so it is important to check the identity of bacteria with oligonucleotide probes. During the last few years, there are many reports about the sequences of the high molecular weight of rRNAs of bacteria 16S and 23S rRNAs which provides ideal target for specific hybridization probes at different taxonomical levels from species to domain (Schleiffer *et al*., 1993; Yang *et al*., 2007). Previously, O' Callaghan *et al*., (1994) indicated that PCR amplification of DNA sequences using oligonucleotides permitted for identification of different bacteria directly from colonies. Molecular techniques exhibit high sensitivity and specificity for identifying the microbes. In the present study, the bacteria were identified by using 16SrRNA gene primers. Using the software, Bio Edit v7.0.9 the obtained sequences were searched for sequence similarity with BLAST (NCBI) tool. The 10 bacterial isolates were identified using NCBI database

as *Bacillus amyloliquefaciens* (13-14, 14-33), *Pseudomonas aeruginosa* (14-68, 14-69, 14-70, 14-71, 14-72), *B. cereus* (14-54, RRNA2) and *B. licheniformis* (RSLB5).

The bacterial isolates belonging to the genera *Bacillus* and *Pseudomonas* were found to control *Phytophthora* spp. under *in vitro* and *in vivo* (Syed-Ab-Rahman *et al*., 2018). It has been reported that *Bacillus* can produce some of the secondary metabolites, lipopeptides, and antibacterial proteins, such as amino peptidase and chitinase, which are the main antibacterial components (Chowdhury *et al*., 2015; Syed-Ab-Rahman *et al*., 2018). *B. amyloliquefaciens* and *B. cereus* have been widely reported as plant growth promoters and biocontrol agents against a broad range of soil-borne pathogens including Fusarium wilt of banana and potato dry rot caused by *Fusarium* sp. (Recep *et al*., 2009; Wang *et al*., 2013; Xu *et al*., 2013; Meng *et al*., 2016; Adibi *et al*., 2017). When *B. amyloidosis* is induced by methyl salicylate secreted by plants, it produces antibiotic *Bacillus* peptide and mycin to help plants to resist fungal infection of *Fusarium oxysporum* (Hou *et al*., 2012; Liu *et al*., 2019). All the organisms identified in the study are known to possess excellent pathogen suppression as well as growth promotion activities. Since, reports show that *Pseudomonas aeruginosa* can serve as a human pathogen, those isolates were not considered for further studies despite their excellent pathogen suppression and growth promotion activities.

Based on IAA production study, 14 isolates produced more than 10 μgml^{-1} of IAA. The IAA production ranged from 0.30 μgml^{-1} to 16.80 μgml^{-1}. The elevated level of IAA would cause plant growth promotion. These results were supported by many studies stating that the *Bacillus* spp. are the micro-factories of IAA production (Waqi and Ahmed, 2019). Tryptophan being the precursor of IAA, bacteria produce increased amount of IAA. There have been reports on significant increase in IAA production by various bacteria on application of exogenous tryptophan (Patten and Glick, 2002; Jasim *et al*., 2014; Raut *et al*., 2017). IAA producing bacteria from different soils were identified to promote plant growth (Hariharan *et al*., 2014, Monita *et al*., 2014, Farah *et al*., 2005, Ashrafuzzaman *et al*., 2009).

Growth promotion ability of the bacterial isolates was evaluated using cowpea. The growth parameters of cowpea seedlings *viz.*, days taken for seed germination, germination (%), number of leaves, shoot length (cm), root length (cm), leaf area were taken at 15, 30, 45 days after sowing (DAS). None of the isolates could significantly fasten the germination of cowpea seeds. Six isolates showed improved germination percentage compared to control. Bacterial priming could not increase the production of leaves or root length. However, some of the isolates could promote shoot length. Plants showed an increasing trend which was primed with bacteria.

Similar studies were carried out by Prashanth and Mathivanan (2008) and recorded IAA production (23 mg/ml) under optimised conditions. Seed treatment of *B. licheniformis* MML2501 in groundnut showed a significant increase in seed germination, other growth parameters and yield parameters under potted plant experiments. *Bacillus* has been reported as predominating bacterial genera within non-rhizobial endophytes isolated from nodules of leguminous plants like common bean, soybean, mung bean (Bai *et al*., 2002; Zakhia *et al*., 2006; Selvakumar *et al*., 2008; Tariq *et al*., 2012; De Meyer *et al*., 2015). Sasirekha *et al.* (2012) reported that inoculation with *Pseudomonas aeruginosa* culture filtrate enhanced seed germination (82.4%) and increase in root length and shoot length of cowpea seeds over the control treatment under pot culture conditions. The plant growth promotion properties were assesssed in cow pea (*Vigna unguiculata*) under greenhouse conditions (Deepa *et al*., 2010). The bacterial inoculation resulted in significant increment in root, shoot and biomass production.

In this study, priming of cowpea seeds with bacterial isolates resulted in growth promotion. The isolates, 13-14, 14-33 and RRNA2 showed positive response on priming with bacterial isolates. Considering different aspects, the isolate 13-14 (*Bacillus amyloliquefaciens*) was selected for further study.

The selected bacterial isolate (13-14) was used for further study in *Colocasia*. The differential expression of PR proteins in susceptible and tolerant varieties of taro (on priming with PGPR) was estimated. After two months of planting, the plants were

placed inside a moist chamber. The plants were challenge inoculated with the pathogen in treatments T2, T3, T6 and T7 with the zoospore suspension of *Phytophthora colocasiae*, prepared by leaf disc method. The leaf samples were collected at different intervals, 0, 6, 12 and 24h, 2, 4 and 8 days for enzyme assay and differential expression of proteins. The enzymes *viz.*, peroxidase, chitinase, glucanase, phenylalanine ammonia lyase (PAL) and total phenol were assayed using standard protocols.

The chitinase activity in Sree Kiran, was maximum in the treatment PGPR + *P. colocasiae* (T3) at 12 hours after inoculation (hai). The increase in activity in this treatment was noted from 6 hour after inoculation and the activity showed declining trend after 12 hai. In Muktakeshi, the chitinase activity was maximum in PGPR treated plants at 12 hai. In both cases PGPR incorporation helped in elevating chitinase activity. Even though, the increase in activity was noted with all treatments, the early elevation of chitinase activity in PGPR + *P. colocasiae* might have helped the plant to mitigate the damage.

The glucanase activity in Sree Kiran, was maximum in T3 (PGPR + *P. colocasiae*) at 24 hai. The increase in activity in the treatment was noticed from 12 hai and the activity declined after 24 hai. Whereas in PGPR alone treated plants, the increase in activity was noted from 24 hai and the elevated production continued till 8 dai. In Muktakeshi, the glucanase activity was maximum in PGPR treated plants at 8 dai. Increase in activity was noted 6 hai in plants which were treated with PGPR and later challenge inoculated with *P. colocasiae.*

Misra *et al*. (2008) studied the biochemical alterations in taro plants infected by the leaf blight pathogen *Phytophthora colocasiae* in resistant (Muktakeshi) and susceptible (Telia) cultivars. They have found that β-1,3-glucanase markedly induced in *P. colocasiae* infected taro leaf of resistant cv. Muktakeshi over its uninfected counterparts. The maximum increase in infected taro leaves of resistant and susceptible cvs. was found to be 4 and 2.0 fold respectively at 5 day after the appearance of symptom. Plants respond to the presence of microbial pathogens by *de novo* synthesis of certain proteins often referred to as pathogenesis-related (PR) proteins. β-1,3-

glucanase and phenyl alanine lyase (PAL) are the representatives of the PR proteins. The induction of such enzymes occur in diverse plant species in response to fungal attack (Hanselle and Barz, 2001; Kapoor *et al.* 2003; Zhao *et al.*, 2005). The result of this study showed that β-1,3-glucanase markedly induced in *P. colocasiae* infected taro leaf of Sree Kiran over its uninfected counterparts.

Similarly, β-1,3-glucanase has been induced in celery plants infected with *Fusarium oxysporum* (Krebs and Grumet, 1993) and chickpea infected with *Ascochyta rabiei* (Hanselle and Barz, 2001) with 2.0 and 5.5 fold increase over control respectively. The enzyme β-1,3-glucanase has been reported to have a role in defense against invading fungal pathogen because of their potential to hydrolyze fungal cell wall polysaccharides, β-1,3-glucan (Kang and Buchenouer, 2002). The higher induction of β-1,3-glucanase in Sree Kiran is suggests its role in disease resistance against *P. colocasiae*.

The peroxidase activity in Sree Kiran was maximum in T3 (PGPR + *P. colocasiae*) at 6 hai. The increasing trend continued upto 12 hai and then started declining. In all the treatments there was an increase in activity during 12-24 hai. In Muktakeshi, peroxidase activity was maximum in T7 (PGPR + *P. colocasiae*) at 4 dai. Misra *et al.* (2008) found that peroxidase activity was highest at the fifth day after the inoculation of *P. colocasiae*, where approximately 2-fold increase over uninfected counterparts was observed in resistant variety of taro. Some studies showed that the induction of peroxidase activity has been repeatedly reported in several plant species in response to pathogen infection (Mlickova *et al.*, 2004; Mohamed and Hasabo, 2005; Bindschedler *et al.*, 2006). The peroxidase activity, in general increases under different stress conditions like wounds, fungi infections, salinity, water stress and nutritional disorders, inducing also the lignin increment and production of ethylene and induce the increase of the production of phenols oxidized at the cell wall (Van Huystee, 1987; Schallenberger, 1994). This activity, suggests a cell effort for the establishment of a physiochemical barrier, able to isolate the infected area (Urs and Dunleavy, 1975). The enhancement of peroxidase in taro leaf upon *P. colocasiae* infection herein was in

agreement with that reported for muskmelon *Cucumis melo* upon infection with *Pseudoperonospora cubensis* (Reuveni *et al*., 1992), in sugarcane upon *Colletotrichum falcatum* invasion (Sundar *et al*., 1998), *Cucumis sativus* upon inoculation with cucumber downy mildew *P. cubensis* (Lebeda and Dolezal, 1995; Lebeda *et al*., 2001), green bean upon infection with *Uromyces appendiculatus* (Siegrist *et al*., 1997), where a positive correlation between the enhancement of peroxidase activity and the degree of plant resistance towards the pathogen was recorded.

The maximum PAL activity in Muktakeshi, was noted in control plants 8 dai followed by the plants primed with PGPR at 6 hai. In plants with PGPR + *P. colocasiae,* maximum production was noted on 4 dai. In Sree Kiran, there was not any significant change in production. Misra *et al*. (2008) stated that PAL induced in *P. colocasiae* infected taro leaf over its uninfected counterparts. The potential of the PGPR + *P. colocasiae* inoculation in taro leaves presenting increase in the activity of PR-proteins in applied inoculation of leaf blight pathogen and it seems to be the most responsive genotype against *P. colocasiae.* PAL is an enzyme of the general phenyl propanoid metabolism and controls a key branch point in the biosynthetic pathways of flavonoid phytoalexins, which are antimicrobial compound (Bowles *et al*., 1990). Some studies (Hahlbrock and Schell, 1989; Campbell and Ellis, 1992) point the enzyme PAL as the precursor of the lignin biosynthesis, phenols, flavonoids and phytoalexins by plant tissues, related to the plant response system against microorganisms, insects and other stress factors.

In Muktakeshi, in all treatments, maximum phenol content was noticed at 24 hai. The maximum phenol content was noted in T7 (PGPR + *P. colocasiae*). The total phenol content decreased with increase of time in Sree Kiran plants with treatments, PGPR alone; *P. colocasiae* and PGPR + *P. colocasiae*. Similar study was done by using *Piriformospora indica,* for growth promotion and disease management in taro (Lakshmipriya *et al*., 2016). Increase in the activity of defense enzymes like chitinase, β-1,3 glucanase and total phenol was observed in *P. indica* colonized plants compared to un-inoculated plants during initial hours of infection. Misra *et al*. (2008) reported

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that the phenol content was highest (1.8 fold) in the resistant variety than the susceptible variety.

Even though, the enzyme did not show a clear cut trend, the priming of cormels with the isolate induced enzyme production and the induction was noticed at earlier period in case of primed plants upon challenge inoculation. It may not be the just quantity, but the early induction must be preventing the pathogen spread.

In pot culture study with taro plants and the bacterial isolate, the growth parameters, such as number of days taken for sprouting, height of the plant, number of leaves, leaf length, leaf breadth of colocasia were recorded at regular intervals in colocasia.

In Sree Kiran and Muktakeshi, the maximum number of leaves; height of the plant; leaf length and leaf breadth; number of mother corms and cormels, weight of mother corms and cormels were recorded in T1 (PGPR alone) followed by PGPR + *P. colocasiae* (T3) plants. In Sree Kiran as well as in Muktakeshi, the PDI was lower in T1 (PGPR alone) plants and highest PDI was in T2 (*P. colocasiae* alone) plants. The disease incidence was very less in Muktakeshi compared to Sree Kiran. Effect of soil application, seed treatment, and foliar spray of rhizobacterial cultures that were isolated from *C. esculenta* on Phytophthora blight reduced the disease incidence and severity and increased the yield, compared to untreated pathogen-inoculated control plants (Sriram *et al*., 2003).

The present study could identify few PGPRs (13-14) which could be tapped for their potential to suppress the taro leaf blight pathogen, *Phytophthora colocasiae.* Apart from identification of the potent isolates, the study also revealed differential expression of enzymes in Sree Kiran and Muktakeshi plants consequent to usage of PGPR primed cormels. The major threat in organic cultivation is the management of pests and diseases. Identification and utilization of microbes with multipronged abilities such as growth promotion and pathogen suppression may be a stepping stone for organic way of plant health management.

SUMMARY

6. SUMMARY

The present study entitled the "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight" was conducted in the Division of Crop Protection, ICAR-Central Tuber Crops Research Institute (CTCRI), Sreekariyam, Thiruvananthapuram during the period 2014-2016. The objective of this study was to select efficient plant growth promoting rhizobacteria (PGPR) from the bacterial cultures maintained at microbial repository at ICAR-CTCRI for taro leaf blight (TLB) management in colocasia to characterize the most potent bacterial isolates using biotechnological tools and to study the differential expression of PR proteins in susceptible and tolerant varieties of taro, consequent to application of PGPR.

Eighty four bacterial isolates were screened for identifying the most potent bacterial isolates (PGPR) against the taro leaf blight pathogen *P. colocasiae* by adopting different methods *viz.*, dual culture/direct confrontation, diffusible and volatile metabolites production method. In dual culture method, 42 isolates showed more than 50% inhibition to *P. colocasiae.* Whereas in diffusible method, 12 isolates showed more than 50% inhibition to *P. colocasiae* and in volatile metabolites production method, 30 isolates showed more than 50% inhibition to *P. colocasiae.*

The IAA production of 84 bacterial isolates was evaluated. Fourteen isolates produced more than 10 μgml^{-1} of IAA. The IAA production ranged from 0.30 μgml^{-1} to 16.80 μgml^{-1}. The highest IAA production was recorded with the isolate (13-14). Based on the mycelial growth inhibition as well as the IAA production of the bacterial isolates, 10 isolates were selected for further study, 13-14, 14-33, 14-54, 14-68, 14-69, 14-70, 14-71, 14-72, RRNA2 and RSLB5 to check the growth promotion activity in cowpea seedlings.

Molecular characterization of the ten potent isolates was done by using 16SrRNA gene primers which resulted in 1500 bp amplified product. The eluted PCR products were purified using QIA quick gel extraction kit, and were sequenced at

RGCB (Rajiv Gandhi Centre for Biotechnology), DNA Finger printing Lab, Thiruvananthapuram and sequence similarity was done using NCBI BLAST. The isolates were identified as *Bacillus amyloliquefaciens* (13-14, 14-33), *Pseudomonas aeruginosa* (14-68, 14-69, 14-70, 14-71, 14-72), *Bacillus cereus* (14-54, RRNA2) and *Bacillus licheniformis* (RSLB5).

The growth promotion activity of the selected ten bacterial isolates was studied using cowpea seeds by pot trial method. The seeds were treated with bacterial suspension. Each bacterial isolates contains five replication of cowpea seedlings/pot. Ten pots were kept for each isolate and growth parameters of cowpea seedlings *viz*., days taken for seed germination, germination (%), number of leaves, shoot length (cm), root length (cm), and leaf area were taken at 15, 30, 45 days after sowing. Based on the growth promotion study, the isolates, 13-14, 14-33 and RRNA2 showed positive response consequent to priming with bacterial isolates. Considering disease suppression, IAA production, growth promotion in cowpea seedlings and identity of the pathogen, the isolate (13-14) was selected for further study.

The selected bacterial isolate (13-14) was used for further study in colocasia. The differential expression of PR proteins in susceptible and tolerant varieties of taro (on priming with PGPR) was estimated. Muktakeshi and Sree Kiran plants were grown in net house. There were eight treatments, T1- Sree Kiran with bacterial isolate (13-14), T2 - Sree Kiran with *P. colocasiae*, T3- Sree Kiran with bacterial isolate (13-14) + *P. colocasiae*, T4- control; T5 - Muktakeshi with bacterial isolate (13-14), T6- Muktakeshi with *P. colocasiae,* T7- Muktakeshi with bacterial isolate (13-14) + *P. colocasiae,* T8- control. After two months of planting, the plants were challenge inoculated with the pathogen in treatments T2, T3, T6 and T7 with the zoospore suspension of *Phytophthora colocasiae*, prepared by leaf disc method. The leaf samples were collected at different intervals, 0, 6, 12 and 24 hours, 2, 4 and 8 days for doing enzyme assay and differential expression of proteins. The enzymes *viz*., peroxidase, chitinase, glucanase, phenylalanine ammonia lyase (PAL) and total phenol were assayed using standard protocols.

The chitinase activity in Sree Kiran and Muktakeshi, was maximum in PGPR treated plants (T3 and T5) at 12 hours after inoculation (hai). The glucanase activity in Sree Kiran, was maximum in T3 (PGPR + *P. colocasiae*) at 24 hai whereas, in Muktakeshi, highest activity was noted in PGPR treated plants (T5) at 8 dai. The peroxidase activity in Sree Kiran and Muktakeshi was maximum in PGPR + *P. colocasiae* inoculated plants (T3 and T7) at 6 hai and 4 dai respectively. The PAL activity in Sree Kiran and Muktakeshi, there was not any significant increase in the production. The total phenol content in Sree Kiran, was noted in control plants and in Muktakeshi, the maximum phenol content was in the treatment PGPR + *P. colocasiae* (T7) at 24 hai.

Further, the effect of bacterial isolate (13-14) on plant growth and disease suppression was studied in *Colocasia* using Sree Kiran and Muktakeshi in grow bags. Six plants were kept for each treatment. The disease incidence (PDI) was also noted at weekly basis. The growth parameters such as number of days taken for sprouting, height of the plant (cm), number of leaves, leaf length (cm), leaf breadth (cm) of *Colocasia* were recorded at regular intervals of time. During harvest, the number of mother corms, number of cormels, weight of mother corms and weight of cormels also recorded and showed an increasing trend as a result of bio-priming with the bacterial isolate 13-14.

The present study could identify few PGPRs which could be utilized for their potential to suppress the taro leaf blight pathogen, *Phytophthora colocasiae*. Apart from identification of the potent isolates, the study also revealed differential expression of enzymes in Sree Kiran and Muktakeshi plants consequent to usage of PGPR primed cormels. The major threat in organic cultivation is the management of pests and diseases. Identification and utilization of microbes with multipronged abilities such as growth promotion and pathogen suppression may be a stepping stone for organic way of plant health management.

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APPENDICES

8. APPENDIX

APPENDIX I

Medium preparation

NUTRIENT AGAR (pH - 7.2 ± 0.2)

Peptone	5.0g
Beef extract	3.0g
NaCl	5.0g
Agar	20.0g
Distilled water	1000ml

KING'S B BASE MEDIUM (pH- 7.2 ± 0.2)

Peptone	20.0g
$MgSO_4$	1.5g
K_2HPO_4	1.5g
Glycerol	10ml
Agar	20.0g
Distilled water	1000ml

CARROT AGAR

Carrot	250.0g
Agar	20.0g
Distilled water	1000ml

POTATO DEXTROSE AGAR

Potato	200.0g
Dextrose	20.0g
Agar	15.0g
Distilled water	1000 ml

LURIA BERTANI BROTH (pH 7.5 ± 0.2)

Tryptone	1.0g
Yeast extracts	0.5g
NaCl	1.0g
Distilled water	1000ml

KING'S B BASE MEDIUM BROTH (pH 7.2±0.2)

Proteose peptone	20.0 g
Dipotassium hydrogen phosphate	1.5 g
Magnesium sulphate.heptahydrate	1.5 g
Distilled water	1000ml

APPENDIX II

DNA Isolation reagents

1. TE buffer

10mMTris - 1.21 g

1mM EDTA - 0.37 g

Distilled water - 1000 ml

2. 10% Sodium Dodecyl Sulfate (SDS)

SDS - 10 g

Distilled water - 1000 ml

3. Proteinase K (20 mg ml^{-1})

Proteinase K - 20 mg

Protenase Nuclease free water - 1 ml

4. TAE buffer, 50 X (pH 8.0)

Tris - base - 242 g

Glacial acetic acid - 57.1

0.5 M EDTA – 100 ml

Dissolve in 600 ml of distilled water, adjust the pH to 8.0 and make up the volume to 1L with distilled water.

5. TAE buffer, 1X

2 ml 50 X TAE + 98 ml distilled water.

6. Ethidium Bromide (10 mg ml^{-1})

Add 1g of Ethidium bromide to 100 ml of water. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminium foil or transfer the solution to a dark bottle and store at room temperature.

7. Gel loading dye (6X)

Bromophenol blue (w/v) - 0.25%

Xylene cyanol FF (w/v) - 0.25%

Glycerol   - 50%

EDTA (pH 8.0)   -10 mM

Dissolve all the components in nuclease free water and store at -20ºC.

8. Agarose gel (1.5%)

Agarose   - 1.5 g

1X TAE   - 100 ml

Weigh the agarose and dissolve in 1X TAE buffer and boil it.

APPENDIX III

POTASSIUM PHOSPHATE BUFFER (0.1M, pH 6.0)

Prepare 800 mL of distilled water in a suitable container. Add 2.405 g of K_2HPO_4 to the solution. Add 11.73 g of KH_2PO_4 to the solution. Add distilled water until volume is 1 L.

APPENDIX IV

SODIUM BORATE BUFFER (0.1M, PH 7.0)

Dissolve 8g NaOH and 47g Boric acid in 900ml water. Make up to 1 L using distilled water. Adjust pH to 7 using NaOH.

APPENDIX V

SODIUM ACETATE BUFFER (50MM, PH 5.0)

Prepare 50 ml 0.2M acetic acid (A) and 50 ml 0.2M Sodium acetate solution (B). Mix 8.8 ml solution A with 41.2 ml solution B and make up to 200 ml using distilled water. Adjust the pH to 5 using HCl.

APPENDIX VI

NCBI SUBMISSION

MN589742.1*Bacillusamyloliquefaciens* strain 13.14 16S ribosomal RNA gene, partial sequence

MN589743.1*Bacillusamyloliquefaciens* strain 14.33 16S ribosomal RNA gene, partial sequence

MN588090.1*Bacilluscereus* strain 14.54 16S ribosomal RNA gene, partial sequence

MN555434.1*Pseudomonasaeruginosa* strain 14.68 16S ribosomal RNA gene, partialsequence

MN555441.1 *Pseudomonasaeruginosa* strain 14.69 16S ribosomal RNA gene, partialsequence

MN555445.1*Pseudomonasaeruginosa* strain 14.70 16S ribosomal RNA gene, partial sequence

MN555450.1*Pseudomonasaeruginosa* strain 14.71 16S ribosomal RNA gene, partialsequence

MN555461.1*Pseudomonasaeruginosa* strain 14.72 16S ribosomal RNA gene, partialsequence

MN588083.1 *Bacilluscereus* strain RRNA2 16S ribosomal RNA gene, partialsequence

MN598652.1*Bacilluslicheniformis* strain RSLB5 16S ribosomal RNA gene, partialsequence

ABSTRACT

EXPRESSION OF PATHOGENESIS RELATED PROTEINS BY PLANT GROWTH PROMOTING RHIZOBACTERIA IN CONTROLLING TARO LEAF BLIGHT

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Abstract of thesis

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9. ABSTRACT

The work was conducted at ICAR- Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram with an aim to study the "Expression of pathogenesis related proteins by plant growth promoting rhizobacteria in controlling taro leaf blight". Taro is an oldest cultivated crop grown for its edible corms, leaves and also for its broad medicinal properties. Taro leaf blight (TLB) is caused by the pathogen *Phytophthora colocasiae* and is the most destructive disease which causes about 50% yield loss. The use of resistant cultivars, cultural methods, employing chemical and biological control measures and integrated disease management are different methods for the control of TLB. Muktakeshi and Sree Kiran are TLB tolerant and susceptible varieties of taro respectively. A total of 84 bacterial isolates were taken for screening the most potent bacterial isolates (PGPR) against *P. colocasiae* by different methods namely, dual culture/direct confrontation, diffusible and volatile metabolites production method. The isolates were also assessed for IAA production. Ten potential bacterial isolates were selected for further study and these isolates were precisely identified by adopting biotechnological tools. The molecular identification of the bacteria revealed that the organisms belong to the genus of *Bacillus* and *Pseudomonas*. With an objective to identify the isolates with abilities for pathogen suppression as well as growth promotion, the isolates with good antagonistic potential were assessed for growth promotion in cow pea seedlings. Various growth parameters were observed during specific intervals (at 15, 30, 45 days) after sowing. The results showed that isolate *Bacillus amyloliquefaciens* (13-14) promotes growth parameters in cowpea plants. This organism was used to carry out experiments for the quantification of defense enzymes. The differential expression of enzymes such as chitinase, glucanase, peroxidase, PAL and total phenol were studied in different treatments using Sree Kiran (susceptible) and Muktakeshi (tolerant) varieties of taro. The results indicated the differential expression of various enzymes such as glucanase, chitinase, PAL etc during different period of pathogenesis. Further growth promotion

and disease suppression potential of isolate, 13-14 (*B. amyloliquefaciens*) was tested on taro plants. The reduced disease incidence and growth parameters *viz.*, height of the plants, number of leaves, leaf length etc indicated the multipronged potential of the isolate and its suitability to be used as an bio-agent in organic cultivation of taro.