

**Interactions of *Ralstonia solanacearum*, a bacterial wilt
pathogen with the xylem residing bacteria in
solanaceous vegetables**

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By

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Certificate

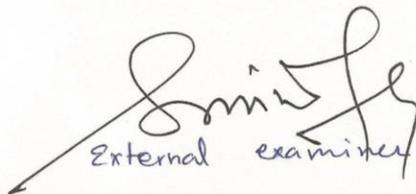
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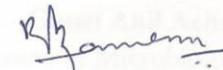
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External examiner



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Statement

I hereby state that this thesis entitled “**Interactions of *Ralstonia solanacearum*, a bacterial wilt pathogen with the xylem residing bacteria in solanaceous vegetables**” being submitted to Goa University for the award of Ph.D. degree in Microbiology is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma at any University or Institute in India or abroad. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

The literature related to the research investigation has been cited. Due acknowledgements have been made whenever facilities and material have been availed.

As suggested by the external examiners, appropriate corrections are incorporated in the relevant pages of this thesis.



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This thesis is dedicated to my beloved parents

Mr. Anil P. Achari and Mrs. Bharati Anil Achari

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Gauri A. Achari

Abbreviations

3OH-PA	3-hydroxy palmitic acid
3OH-PAME	3-hydroxy palmitic acid methyl ester
3PD-XRB	3OH-PAME degrading XRB
Acyl-HSL	Acyl-Homoserine lactone
APS	Ammonium per sulphate
BCE	Bio-control efficacy
BME	Beta mercaptoethanol
bp	Base pairs
kb	Kilo base
BSA	Bovine serum albumin
BW	Bacterial Wilt
CFU	Colony forming units
CPG	Casein peptone glucose
CTAB	Cetyltrimethyl ammonium bromide
DAI	Days after inoculation
DCM	Di chloro methane
DGGE	Denaturing gradient gel electrophoresis
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EP buffer	Electroporation buffer
EPS	Extracellular polysaccharides
Egl	Endoglucanase
GFP	Green Fluorescent Protein
GPE	Growth promotion efficacy

HCN	Hydrogen cyanide
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
HR	Homologous recombination
ISR	Induced systemic resistance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR restriction fragment length polymorphism
PNP	p-Nitrophenol
PNPA	p-Nitrophenyl acetate
PNPP	p-Nitrophenyl palmitate
QQE	Quorum quenching enzyme
rpm	Revolutions per minute
RR	Rifampicin resistant
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEFA PCR	Self-formed adapter PCR
TAE buffer	Tris Acetate EDTA buffer
TE buffer	Tris EDTA buffer
TEMED	Tetramethylenediamine
TFB	Transformation buffer
TZC	Tetrazolium chloride
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
XRb	Xylem residing bacteria

Units of measurement

μ	micron
μg	Microgram
μm	Micrometer
μL	Microlitre
$^{\circ}\text{C}$	Degree Celsius
CFU.g^{-1}	Colony forming units per gram
CFU.mL^{-1}	Colony forming units per mL
g	Gravitational force
g	Grams
g.L^{-1}	Grams per litre
h	Hour
kDa	Kilodaltons
mg	Miligrams
min	Minute(s)
mL	Milliliter
M	Molar
ng	Nanograms
nM	Nanomolar
mM	Milimolar
mm	Millimeter
nm	Nanometer
OD600	Optical density measured at 600 nm
ppm	Parts per million
rpm	Revolution per minute
U.mL^{-1}	Units per mL
V	Volts

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CHAPTER I

Introduction

INTRODUCTION

The plant family *Solanaceae* consists of 90 genera and 3000-4000 plant species. Solanaceous vegetables of economic and cultural importance cultivated throughout the world include eggplant (brinjal or aubergine), chilli (chili pepper), tomato and potato. Amongst these vegetables, eggplant (*Solanum melongena* L.) is cultivated over an area of 1.7 million hectares worldwide. Eggplant is native to Asia, currently China is the largest producer of eggplant in the world followed by India and Middle East countries, which account to 80% of the world's eggplant production (Hanson *et al.*, 2006). There are reports that document Indian origin of eggplant (Hanur, 2011; Takkar *et al.*, 2012). Major eggplant growing states in India are West Bengal, Orissa, Bihar, Gujarat, Karnataka, Maharashtra, Madhya Pradesh and Assam (Kumar *et al.*, 2004).

Chilli (*Capsicum annuum* L. and *Capsicum frutescens* L.) is native to Mexico and South American countries. It was introduced to Europe by Christopher Columbus in the 1400s after the discovery of America. Later, the Portuguese rulers introduced chilli to Goa from where it was spread across India by the Christian missionaries. Today, India is the largest producer, consumer and exporter of chilli followed by other Asian and South American countries. A wide variability in fruit size, fruit shape, fruit colour, pungency, bearing habit and crop duration of chilli is found throughout India (Yumnam *et al.*, 2012). Chilli is cultivated in all the states of India. Andhra Pradesh is the largest producer of chilli in India and other notable chilli growing states are Maharashtra, Karnataka, Orissa and Madhya Pradesh.

Eggplant and chilli are also the main solanaceous vegetables cultivated throughout the state of Goa, India. In Goa, eggplant varieties *Agassaim* and *Taleigao* are most famous. More than 23 varieties of chilli are cultivated in Goa, most notable ones

used in Goan cuisine are *Tarvati*, *Portugali*, *Kholchi*, *Moidechi*, *Mandrechi*, *Aldonechi* and others. Goan farmers have preserved the germplasm of eggplant and chilli varieties since many generations for the flavor, pungency and high sale value.

Eggplant and chilli are prone to attack by several pests including bacteria (*Ralstonia solanacearum*), fungi (*Fusarium*, *Rhizoctonia*), nematodes (root knot nematode) and insects (Shoot borer and fruit borer). The local varieties of eggplant and chilli cultivated in Goa are highly susceptible to bacterial wilt (BW) disease caused by *Ralstonia solanacearum*. In the state of Goa, BW has been reported to cause 30-100% crop loss in eggplant in major vegetable growing locales Agassaim and Taleigao (Ramesh, 2006). Recently, Ramesh *et al.* (2014) have reported that *R. solanacearum* strains from a small region as the state of Goa are genetically diverse, highly pathogenic and belong to new or unidentified sequence variants. Enormous genetic diversity of *R. solanacearum* reflects in its variability in hosts range, aggressiveness and adaptation to diverse environments (Castillo and Greenberg, 2007).

Persistence in the environment in absence of host and high genetic diversity makes management of this rapidly evolving pathogen difficult. Classically, management of BW involved use of antibiotics, crop rotation with non-host crops, application of soil fumigants (methylbromide, chlorpicrin, metam sodium), soil solarization, soil amendments and breeding plants for BW resistance (Chellemi *et al.*, 1994; Alvarez *et al.*, 2008; Chakrabarti, 2011; Fujiwara *et al.*, 2011; Lebeau *et al.*, 2011).

Biocontrol has been used as an alternative pathogen management strategy for controlling BW and an ecofriendly means towards sustainable agriculture (Yu *et al.*, 2010; Ramesh and Phadke, 2012). Several studies have shown that endophytic bacteria can be used as biocontrol agents against plant pathogens. Endophytic bacteria exhibit

several antagonistic interactions with phytopathogens mainly through production of antibiotics and induced systemic resistance. In addition capability of endophytic bacteria to colonize internal host tissues has made endophytes valuable tool in agriculture to improve crop performance (Compant *et al.*, 2005a; Rosenblueth and Martínez-Romero, 2006). Endophytic bacteria enhance plant growth by production of phytohormones, enzymes involved in growth regulatory metabolism and other growth promoting substances (Hardoim *et al.*, 2008).

Plant xylem is a common ecological niche for endophytes and vascular pathogens such as *R. solanacearum* and sharing of same niche is the basis for interactions to occur (deWeert and Bloemberg, 2006). Xylem residing bacteria (XRB) have been isolated using vacuum infiltration (Gardner *et al.*, 1982; Bell *et al.*, 1995), Scholander pressure bomb extraction (Hallmann *et al.*, 1997) and maceration techniques (Gagné *et al.*, 1987; Lampel *et al.*, 1994). Recently using green fluorescent protein (GFP) marker presence of bacteria has been demonstrated in the xylem of grapevine, poplar, switch grass and mulberry (Germaine *et al.*, 2004; Gagne-Bourgue *et al.*, 2013; Compant *et al.*, 2005b; Ji *et al.*, 2008). However, there are no reports on the identity, diversity and functionality of xylem residing bacteria from solanaceous vegetables mainly eggplant and chilli.

The disruption of quorum sensing signaling is called quorum quenching (Dong *et al.*, 2001). Quorum quenching (QQ) of acyl-Homoserine lactone (acyl-HSL) based quorum sensing in majority of plant pathogenic bacteria *viz.* *Erwinia amylovora*, *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Dickeya* sp. and *Pectobacterium caratovorum* has been shown to be effective strategy for reducing virulence by several researchers (reviewed by von Bodman *et al.*, 2003; Uroz *et al.*, 2009). Most common mediators of quorum quenching are bacterial enzymes which degrade quorum sensing

molecules (Uroz *et al.*, 2003; Dong *et al.*, 2007; Cirou *et al.*, 2012; Chen *et al.*, 2013). One of the quorum quenching based biocontrol strategies is the use of bacterial strains naturally producing or engineered to produce quorum quenching enzymes to prevent disease (Dong *et al.*, 2000; Dong *et al.*, 2001; Faure and Dessaux, 2007). Recently 3OH-PAME hydrolase from betaproteobacterium *Ideonella* sp. is reported to attenuate expression of virulence factors by *R. solanacearum* *in vitro* (Shinohara *et al.*, 2007). However, other strains able to degrade 3OH-PAME and use of 3OH-PAME degrading bacterial enzymes for biological control of *R. solanacearum* are not yet reported.

Considering literature that suggests existence of antagonistic interactions of endophytic bacteria with *R. solanacearum*, I formulated the hypothesis that **‘bacteria colonizing xylem of solanaceous vegetables may exhibit antagonistic interactions and interfere in quorum sensing signal of *R. solanacearum* and, these interactions can be a deployed for biocontrol of BW infection in solanaceous vegetables’**.

To test the hypothesis, the following objectives were proposed

OBJECTIVES:

1. To isolate and characterize the xylem residing bacteria from solanaceous vegetables and carry out their diversity analysis.
2. To study the interactions between *R. solanacearum* and xylem residing bacteria.
3. To study the quorum sensing pathway of *R. solanacearum*.
4. To screen the xylem residing antagonistic bacteria for production of quorum quenching molecules.

CHAPTER II

Review of Literature

2.1. The plant microbiome

Plants are naturally colonized by beneficial microorganisms from the root to inflorescence. Plant photosynthates act as nutrient reservoirs which attract bacteria. In individual plants, microbes inhabit tissue surfaces of organs above and below ground making unique microenvironments namely anthosphere (surface of flowers), phyllosphere (surface of leaves and stem), spermosphere (surface of a germinating seed) and rhizosphere (surface of roots). Microbes can not only colonize plant surface but also internally and systemically (Taghavi *et al.*, 2010). Microenvironments within the plants include endorhizosphere (inside roots) and endosphere (inside stems, leaves and inflorescence) (Hardoim *et al.*, 2008; Junker and Tholl, 2013; Mendes *et al.*, 2013). These ecological niches allow establishment of different communities of bacteria which survive by utilizing the plant photosynthates. Plant colonization by bacteria is a multistep process and considered a healthy interaction because bacteria are known to improve seed germination, seedling vigour, plant growth, productivity and reduce stress and diseases. Bacterial numbers in plants may exceed the number of plant cells. Hence the plant associated bacteria are known as the ‘plant microbiome’. Rhizosphere and endosphere are ‘battlegrounds’ of beneficial microbes colonizing these niches for interactions with soil borne phytopathogens (Mendes *et al.*, 2013).

2.1.1. Rhizosphere

The rhizosphere, defined as the narrow zone of soil surrounding the root and influenced by plant exudates. Rhizosphere is a hot spot for microbial activity and is considered as one of the most complex ecosystems on Earth (Mendes *et al.*, 2013). The rhizosphere can contain up to 10^{11} microbial cells per gram root (Berendsen *et al.*, 2012).

Rhizosphere harbors microbial communities which differ from those found in bulk soil. Hence, the term rhizosphere effect is used to describe the consequence of rhizosphere colonization (Curl and Truelove 1986). Root colonizing bacteria are known as rhizobacteria. Bacterial colonization of roots often starts with the recognition of chemoattractants in the root exudates. (Lugtenberg and Dekkers 1999; de Weert *et al.* 2002; Buée *et al.*, 2009). Root exudates are composed of waste products from the plants metabolic processes and are rich in sugars, acids (amino, aliphatic, aromatic and fatty acids), sterols, vitamins and proteins and act as carbon and nitrogen sources for bacteria (Faure *et al.*, 2009). It has been suggested that plants attract specific microorganisms for their own ecological benefit through their root exudates (Hardoim *et al.*, 2008). Bacteria sense the chemoattractants via the membrane bound sensor systems, well studied are the one component sensor/ response system (eg. The nod factor of Rhizobia) and the two component sensor/response systems (eg. GacS/GacA system of *Pseudomonas* sp.) (Hardoim *et al.*, 2012). Bacteria present in the soil in the vicinity of plant roots move towards the plants by chemotactically and attach to plant cells on root hair, sites of lateral root emergence or root elongation zones using pili, fimbriae, flagella, EPS, lipopolysaccharides, bacterial cellulose fibrils and proteins (Vesper, 1987;Rodriguez-Navarro *et al.*, 2007; Gorski *et al.*, 2009). Bacteria then form microcolonies or biofilms on the plant root surface (Compant *et al.*, 2010; Bogino *et al.*, 2013). Enhanced growth of microbes in response to developing plant roots and root exudates was termed as rhizosphere competence by Schmidt (1979). *Proteobacteria* is reported to be the most abundant phylum of rhizobacteria, other members reported belong to *Actinobacteria*, *Bacteroidetes*, *Acidobacteria*, *Firmicutes*, *Cyanobacteria* and *Planctomycetes* (Bulgarelli *et al.*, 2012; Mendes *et al.*, 2013). Less abundant bacteria reported include

Verrucomicrobia and *Nitrospirira* (Mirete *et al.*, 2007). *Crenarcheota* and *Euryarcheota* of the Archaeobacteria are also reported to be colonizing the rhizosphere of plants (DeAngelis *et al.*, 2005).

Rhizobacteria are termed as probiotics for plants (Kim *et al.*, 2010; Fgaier and Eberl, 2011). Rhizosphere microbiota is important to plant health as much as the gut microbiota is important for animal health (Ramirez-Puebla *et al.*, 2013). Suppressive soils, induced systemic resistance (ISR), antagonism to phytopathogens, stress reduction and plant growth promotion are the traits which make rhizobacteria extremely useful in agriculture (Hariprasad and Niranjana, 2009; Yang *et al.*, 2009; Raaijmakers *et al.*, 2009).

2.1.2. Endosphere

Endosphere is the microenvironment within the plant tissues and the colonizing microorganisms collectively called endophytes. The endosphere consists of the endorhizosphere (internal root tissue) and the endophyllosphere (internal shoot and leaf tissue) (Hardoim *et al.*, 2008). The term endophytes literally means ‘within the plants’ (*Endon* Gr.: within *phyton* : plant) and was coined by de Bary (1866), a broad term which includes bacteria, fungi and algae residing within the plant (Davies and Speigel, 2011). Perotti (1926) described endophytic bacteria to be in an advanced stage of plant infection having a close mutualistic relationship with the plant. Endophytic bacteria live inside the plants at least for a part of their lifecycle, however unlike symbiotic bacteria they are not surrounded by host membranes. Hallmann *et al.* (1997) defined an endophyte as any microorganism that resides inside the plant irrespective of the tissue colonized and can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue. The term endophytic bacteria later evolved to describe bacteria that live in plant tissues

without doing substantial harm, eliciting symptoms of plant disease or gaining benefit other than residency (Kobayashi and Palumbo, 2000; Reinhold-Hurek and Hurek, 2011). However, it is now clear that though endophytic bacteria do not harm the plants they actively utilize the plant photosynthates for growth (Malfanova *et al.*, 2013). Bacteria benefit from endophytic colonization because it is a protected niche and rich source of organic and inorganic nutrients for growth of endophytic strains (Bacon and Hinton, 2011). Depending on the lifestyle of endophytes they can be distinguished as obligate and facultative. Obligate endophytes and their host plants are dependent on each other for their growth and survival. Obligate endophytes have a reduced genome size and transmitted to other plants vertically and protect their hosts from herbivores and pathogens (Carlier and Eberl, 2012).

Facultative endophytes have a stage in their life cycle in which they exist outside host plants. Facultative endophytes may alternate between plant and soil (Hardoim *et al.*, 2008). Facultative endophytic bacteria are reported to originate from the rhizosphere soil, initially entering the host plant during germination and radicle development, through wounds caused by insect feeding or by colonizing the cracks formed in lateral root junctions. Other gateways of entry into the plant tissues are wounds caused by nematodes or the stomatal opening in leaf tissue. However, root cracks are documented as the main sites for bacterial colonization (Franke *et al.*, 2000; Roseblueth and Martinez-Romero, 2006; Hardoim *et al.*, 2008). Thus endophytic bacteria infecting plants from soil need to be highly competent root colonizers to overcome the root microbiota, plant barriers and defenses. Weller (2007) described several traits namely ability to compete for resources in the root exudates, rapid growth rate, cell surface structures (lipopolysaccharides, flagella, motility and chemotaxis), ability to survive environmental stress (desiccation,

temperature fluctuations, bacteriostatic levels of putrescein), global regulators responding to environmental change (sigma factor, membrane sensory system), phenotypic/ phase variations and antibiosis, for heightened rhizosphere competence in bacteria. After their initial entry, bacteria may remain localized within the root tissues and colonize intercellular spaces of root cortex (Jacobs *et al.* 1985; Hallmann *et al.* 1997; Aravind *et al.*, 2009). Endophytically migrated bacteria have been reported to colonize plant vascular system, leaves and fruits (Gardner *et al.*, 1982; Bell *et al.* 1995; Ji *et al.*, 2008; Compant *et al.*, 2011). Fig. 2.1 depicts the process of rhizosphere and endophytic colonization by bacteria.

2.1.3. Plant vascular system as a niche for bacterial colonization

Xylem and phloem form the vascular tissues in vascular plants (tracheophytes), excluding bryophytes and algae which do not have vascular system (non-vascular plants). Xylem together with phloem, the principle water and food conducting tissue forms a continuous vascular system extending throughout the plant. Between the xylem and phloem of dicotyledenous plants, intrafascicular cambium is present which divides to produce new xylem and phloem, causing increase in diameter of roots and stems of plants. Vascular parenchyma cells store food and allow lateral movement of solutes through the vascular tissue. Fibers and sclerieids provide support to the plant organs. In the root, vascular tissue forms the central stele whereas in the leaves it forms the central midrib from which the veins arise. Vascular bundles in dicot stem are radial and internal to endodermis. Each vascular bundle is conjoint, collateral, endarch and open. Dicot stem may have medullary rays between two vascular bundle which store water and food material, and also function

in lateral conduction. The vascular bundles in dicot root are radial, conjoint, collateral, exarch and open (Evert, 2006).

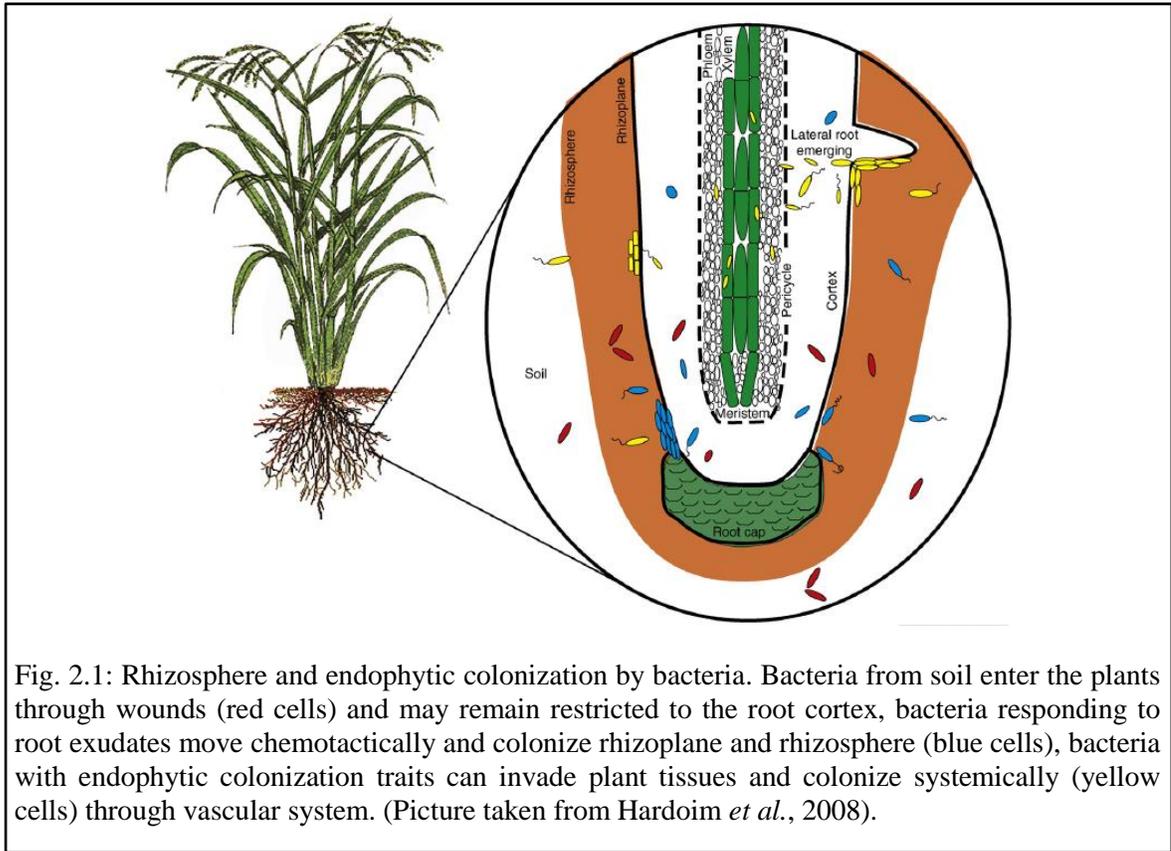


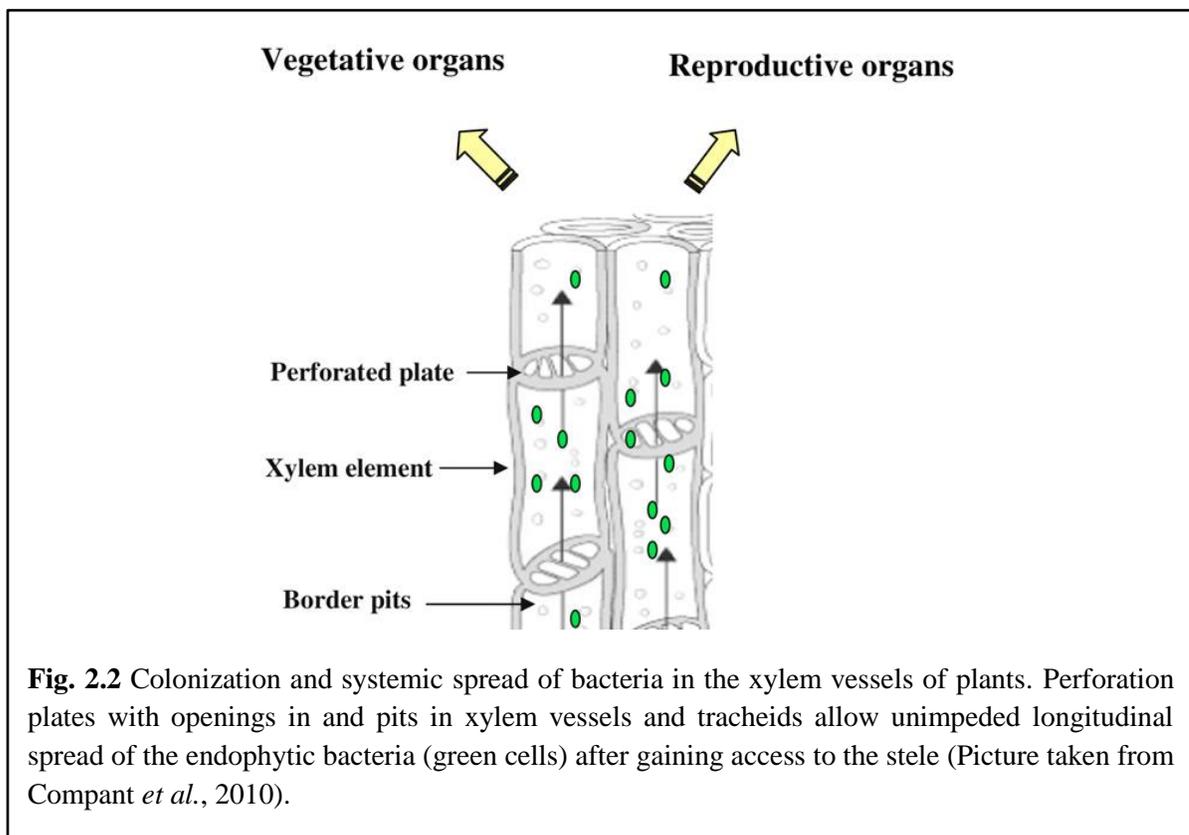
Fig. 2.1: Rhizosphere and endophytic colonization by bacteria. Bacteria from soil enter the plants through wounds (red cells) and may remain restricted to the root cortex, bacteria responding to root exudates move chemotactically and colonize rhizoplane and rhizosphere (blue cells), bacteria with endophytic colonization traits can invade plant tissues and colonize systemically (yellow cells) through vascular system. (Picture taken from Hardoim *et al.*, 2008).

Xylem is the principle water conducting tissue involved in the upward transport of solutes and support. The term xylem is derived from Greek word xylon meaning wood and was coined by Nageli in 1858. Xylem and phloem first differentiate in the young seedling from procambium in the apical meristem. As the plant grows, new xylem and phloem tissues develop forming a continuous vascular tissue (Evert, 2006). The principal conducting elements of the xylem are tracheids and vessel members which are dead cells, contain no cytoplasm, and have lignified secondary walls. The vessel elements are joined end to end into vessels. The adjoining ends of xylem vessels have perforation plates with openings. On the lateral walls of tracheids and vessel members there are pits (or apertures) having membranes separating the adjacent elements. Other xylem cells include

fiber tracheids and libriform fibers which provide support to the plant and xylem parenchyma which may store food. The principal conducting cells of the phloem are the sieve tube elements. These elements are joined end to end into sieve tubes. Companion cells are nucleated parenchymatic, nucleated cells are adjacent and load sucrose into the sieve tubes. Sieve tube elements are living cells however become enucleated at maturity. Between two adjacent sieve elements are the sieve plates. The sieve plates are clustered with pores allowing a continuous cytoplasm with the adjacent elements. The diameter of the pore ranges from 1-15 micron (Bove and Garnier, 2002).

Openings in perforation plates and pits in the xylem may allow relatively unhindered longitudinal spread of the endophytic bacteria which gain access to the stele (Fig. 2.2) (Compant *et al.*, 2010). Wounds caused by nematodes and sites of lateral root emergence and elongation may also allow direct access of bacteria to the stele (Reinhold-Hurek *et al.*, 2006). Endophytic bacteria are reported to migrate to aerial plant parts through the vascular system passively with the transpirational flow and/or through assistance of cell wall degrading enzymes (Compant *et al.*, 2005b; Thorne *et al.*, 2006; Chatelet *et al.*, 2006). Cell wall degrading enzymes of endophytic bacteria cause less damage to the plant host because of lower levels of expression and are cell surface associated causing only mild localized damage to slowly facilitate bacterial migration (Gardner *et al.*, 1982; Gagné *et al.*, 1987; Bell *et al.*, 1995; Rosenblueth & Martinez-Romero 2006; Hardoim *et al.*, 2008). Bacteria living in the xylem sap known as xylem residing bacteria (XRB) are dependent on limited nutrient sources in sap which includes sugars (glucose, fructose, saccharose, maltose, raffinose, trehalose, and ribose). Xylem sap of healthy tomato plant contains approximately 300 μM sucrose (Jacobs *et al.*, 2012). Xylem sap also contains various proteins, amino acids, and organic acids and

tricarboxylic acid cycle intermediates. Nitrate, sulfate, calcium, potassium, magnesium, manganese and phosphate are among the most abundant inorganic ions in the xylem sap. Bacteria colonizing the xylem are efficient in acquiring the scarce nutrients available in the xylem sap for their growth. Certain endophytic bacteria from xylem vessels may degrade xylem vessel walls to colonize adjacent parenchyma cells (Jacobs *et al.*, 2012; Yadeta and Thoma, 2013). Xylem is considered low oxygen environment but anaerobic bacteria are rarely found in the plant xylem. Oxygen tension in the xylem has been reported to be sufficient to support growth of aerobic and facultative anaerobic bacteria (Gardner *et al.* 1982; Jacobs *et al.* 2012).



Presence of XRB in the xylem of various plants have been previously reported. Using tetrazolium staining and electron microscopy, bacteria have been demonstrated in the

xylem of maize (Patriquin and Döbereiner, 1978) and xylem elements and xylem parenchyma of sugar beets (Jacobs *et al.*, 1985). Bacteria that have adapted to life in xylem can usually also survive well outside the xylem. Gardner *et al.*, (1982) isolated XRB from citrus roots by vacuum infiltration and included the majorly *Pseudomonas* sp., *Enterobacter* sp., *Bacillus* sp., *Corynebacterium* sp. and *Serratia* sp. By employing maceration technique, Gagné *et al.* (1987) isolated fluorescent and non-fluorescent *Pseudomonas* sp., *Erwinia* sp. and *Bacillus* sp. from xylem of alfalfa seedlings. Bazzi *et al.* (1987) isolated *Agrobacterium tumefaciens* from grapevine xylem by using vacuum extraction method. A combination of vacuum infiltration and maceration was deployed by Bell *et al.* (1995) to extract bacteria from xylem of grapevine and reported *Enterobacter* sp. and *Pseudomonas* sp. as dominant genera. Hallmann *et al.* (1997) reported use of Scholander pressure bomb (an instrument used for measuring the water potential of plants), to extract xylem sap residents of herbaceous plants with soft stems. XRB isolated by Scholander pressure bomb were less frequently encountered on isolation by maceration. *Agrobacterium*, *Pseudomonas* and *Phyllobacter* were isolated using maceration and Scholander pressure bomb (Hallmann *et al.*, 1997). Recently molecular markers are used for detection and studies on re-colonization of XRB. *Azorhizobium caulinodans* tagged with *lacZ* reporter was visualized in the xylem of *Sesbania rostrata*, *Oryza sativa* and *Arabidopsis thaliana* by microscopic studies and it was proposed that xylem could be a suitable low oxygen niche for nitrogen fixation by endophytic diazotrophs (O'Callaghan *et al.*, 1997; Gopaldaswamy *et al.*, 2000; Stone *et al.*, 2001). Recently using Green fluorescent protein (GFP) tagging strains of *Pseudomonas* sp. and *Bacillus* sp. have been confirmed to colonize grapevine xylem (Compant *et al.*, 2008; Compant *et al.*, 2011).

In addition *Clavibacter xyli* subsp. *cyanodontis*, a xylem resident of Bermuda grass when genetically engineered to express *cry* gene, effectively control insect infestation in *Zea mays* (Lampel *et al.*, 1994). Recently xylem colonist *Leifsonia xyli* subsp. *cyanodontis* was reported to be a suitable vector for expression of foreign genes in rice (Li *et al.*, 2007).

Vascular colonists can easily move to aerial parts of plants and colonize other microenvironments of plants. Systematically migrated endophytes have been isolated from stems of several plants (Kuklinsky-Sobral *et al.*, 2004; Scortichini and Loretti, 2007; Procopio *et al.*, 2009), inflorescence of grapevine (Compant *et al.*, 2008), seeds of *Phaseolus vulgaris* (Lopez-Lopez *et al.*, 2010), fruits of *Coffea canephora* (Miguel *et al.*, 2013), radish and mulberry leaves (Seo *et al.*, 2010; Ji *et al.*, 2008). In contrast to the xylem, phloem is richer in sugar and nutrients. High osmotic pressure in the phloem makes it difficult for mutualistic bacteria to penetrate (Yadeta and Thomma, 2013). No bacteria were detected in the phloem of healthy potato tubers when observed by using Scanning electron microscopy (Liu *et al.*, 2013).

However, xylem and phloem of several plants are colonized by fastidious pathogenic bacteria known as xylem or sieve tube restricted bacteria. These are indigenous bacteria unlike the other transient XRB and are transmitted during grafting or by phloem feeding insects (leafhoppers and physillids) and xylem sap feeding insects (sharpshooters). However, *in vitro* cultivation of these indigenous bacteria from the xylem and phloem has proved difficult on standard bacteriological media (Bove and Garnier, 2002; Jacobs *et al.*, 2012; Vinatzer, 2012).

2.2. Diversity of endophytic bacteria

Classically endophytic bacteria have been characterized and identified based on colony morphology and biochemical traits. Since the last few decades polyphasic approach for identification of bacteria has emerged which used a combination of biochemical tests, DNA and protein based methods. DNA based methods mainly whole genome fingerprinting by rep-PCR or PCR-RFLP are rapid tools for determining genetic diversity of bacteria (de Bruijn, 1992; Poussier *et al.*, 2000).

2.2.1. Amplified rDNA Restriction Analysis (ARDRA)

Amplified rDNA Restriction Analysis (ARDRA) a type of PCR based RFLP method has been used extensively in estimating genetic diversity endophytic bacterial populations. Several researchers have used ARDRA for determining genetic diversity of endophytic bacteria. Lagacé *et al.*, (2004), used enzymes *RsaI*, *HaeIII*, and *AluI* and identified representative from each ARDRA haplotype as members of *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* from xylem sap of maple. Based on ARDRA profiles, it was confirmed that endosphere colonists of tomato are a subset of rhizobacteria (Marquez-Santacruz *et al.*, 2010). *Pseudomonaceae*, *Burkholderiaceae* and *Enterobacteriaceae* families were predominant endophytic colonists in soybean when analyzed by ARDRA (Kuklinsky-Sobral *et al.*, 2004). Diversity of ACC deaminase producing bacteria from *Nicotiana attenuata* was determined by using *HinfI* and *MspI* (Long *et al.*, 2010). Dominant plant growth promoting bacteria from poplar were identified members of *Gammaproteobacteria* by ARDRA followed by 16S rRNA gene based identification (Taghavi *et al.*, 2009). Diversity of endophytic siderophore producing bacteria isolated from *Oryza sativa* was determined by ARDRA and the

members of the largest haplotype were identified as *Pantoea* sp., and other siderophore producers included *Sphingomonas* sp., *Pseudomonas* sp., *Burkholderia* sp. and *Enterobacter* sp. (Loaces *et al.*, 2010). Recently, diversity analysis by ARDRA followed by identification revealed presence of phyla *Proteobacteria* and *Firmicutes* as endophytes in banana (Souza *et al.*, 2013). Tiwari and Thakur (2014) have characterized endophytic bacteria from neem from different locations by ARDRA.

2.2.2. rep-PCR

PCR based whole genome fingerprinting method for analysis of diversity of plant associated bacteria is the amplification of bacterial repetitive sequences which are interspersed throughout the genome of bacteria. Three families of repetitive sequences studied in detail include repetitive extragenic palindromic (REP) which are 38-40 bp in length. REP elements also known as Palindromic units contain 5 bp variable loop in the proposed stem loop structure. Enterobacterial intergenic consensus sequence (ERIC) which are 124-127 bp possess central conserved palindromic structures. BOX elements consist of differentially conserved subsequences and three BOX subunits were originally identified include boxA (57 bp), boxB (43 bp) and boxC (50 bp). However, only boxA subunit sequences are highly conserved amongst diverse bacteria. boxB and boxC are only found in *Streptococcus pneumoniae*. Interestingly Gram negative bacteria totally unrelated to *S. pneumoniae* have boxA elements dispersed throughout their genome. Primers reading outward from the repetitive sequences are used for amplification of intervening sequences. Agarose gel electrophoresis of the different sized intervening sequences leads to a distinct fingerprint of each species of bacteria. The corresponding PCR for amplification based on each repetitive element are called REP-PCR, ERIC-PCR,

BOX-PCR and collectively as rep-PCR (Versalovic *et al.*, 1994; Louws *et al.*, 1994). Diversity of cultured plant associated bacteria has been widely studied using rep-PCR (Vinuesa *et al.*, 1998; Louws *et al.*, 1998; Souza *et al.*, 2013). Recently Amaresan *et al.* (2014) have studied the genetic diversity of endophytic bacteria from chilli and identified 19 different clusters amongst the endophytic bacteria by BOX-PCR fingerprinting. REP-PCR was found superior than ERIC-PCR, BOX-PCR and 16S rRNA gene sequencing for discriminating diverse *Bacillus* sp. associated with apple (Kumar *et al.*, 2014). Rep-PCR is also used for confirming the identity of introduced and re-isolated bacteria from plants (Barac *et al.*, 2004; Andreote *et al.*, 2010; Ghirardi *et al.*, 2012).

2.2.3. Culture independent techniques to study uncultured endophytic bacteria

It is now known that only a fraction of bacteria are cultured *in vitro* and several cultured bacteria can enter viable but non culturable (VNBC) state (Oliver, 2005). Culture independent techniques used for the analysis of largely uncultured bacteria include construction of 16S rRNA gene based metagenomic libraries and sequencing of similar clones after performing ARDRA, fluorescent *in situ* hybridization (FISH) and Denaturing Gradient Gel Electrophoresis of PCR amplicons (PCR-DGGE). In PCR-DGGE, separation of DNA fragments of same size is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a gradient of denaturants (urea and formamide). A partially melted DNA molecule stops migrating once it has reached the denaturant concentration that completely melts it. Variation in G+C content of sequence causes the denaturant concentrations to differ, and molecules with different sequences will stop migrating at different positions in the gel. Separation efficiency of DGGE is increased by

adding a 30-50 nucleotide GC rich sequence (GC clamp), to one side of the DNA fragment. The GC rich sequence acts as a high melting domain preventing the complete dissociation of the two DNA strands (Muyzer and Smalla, 1998). Separation of PCR products by DGGE gives rise to a fingerprint which can be used to estimate the diversity and identity of plant associated bacterial community (Duineveld *et al.*, 2001; Araújo *et al.*, 2002). PCR-DGGE of *nifH* gene was useful for determining the functional diversity of diazotrophic endophytic bacteria (Rosado *et al.*, 1998). PCR-DGGE has been used to study endophytic colonization by bacteria and its effect on indigenous endophytic bacterial communities (Garbeva *et al.*, 2001; Götz *et al.*, 2006; Andreote *et al.*, 2010). PCR-DGGE was also useful in studying shifts in endophytic communities in soybean in response environmental factors, plant type and nutrient status and herbicides (Yang and Crowley, 2000; Smalla *et al.*, 2001; Sehgers *et al.*, 2004; Kuklinsky-Sobral *et al.*, 2005). Population dynamics of *Crysanthemum* microbiome has also been studied by PCR-DGGE (Duineveld *et al.*, 1998). Interactions between endophytic bacteria and pathogens have been studied by PCR-DGGE (Araújo *et al.*, 2002; van Overbeek *et al.*, 2002).

2.3. Endosphere: a niche of Bacterial Wilt pathogen *Ralstonia solanacearum*

2.3.1. Introduction to the BW pathogen

R. solanacearum is a soil borne betaproteobacterium. It has a broad host range and infects around 54 plant families and 450 plant species (Wicker *et al.*, 2007). *R. solanacearum* has been phenotypically classified into 6 biovars based on the metabolic profiles related to the ability to reduce three disaccharides (cellobiose, lactose, maltose) and three hexose alcohols (dulcitol, mannitol, sorbitol) and into five races based on the host range namely Race 1 (strains infecting solanaceous plants), Race 2 (strains pathogenic banana), Race 3

(strains from potato and tomato from temperate regions), Race 4 (strains infecting ginger), Race 5 (strains infecting mulberry) (Wicker *et al.*, 2007).

R. solanacearum strains across the world have a very high genetic diversity and considered as a species complex of genetically diverse related strains (Genin and Denny, 2012). Based on the ITS region, Fegan and Prior (2005) proposed classification of the *R. solanacearum* species complex in phlotypes. Phylotype I consists of strains isolated from Asia, Phylotype II consists of strains from America, Phylotype III includes strains isolated from Africa, Reunion Island and Madagascar islands in the Indian Ocean whereas Phylotype IV includes strains from Indonesia, Australia and Japan and two other species *R. syzygii* and *Ralstonia celebensis*.

The expression of virulence factors in *R. solanacearum* mainly EPS and endoglucanase (Egl) in *R. solanacearum* is governed by quorum sensing system called the phenotype conversion (Phc) system, which comprises of *phcA* and *phcBSRQ* operon. The quorum sensing molecule of *R. solanacearum* is 3-hydroxy palmitic acid methyl ester (3OH-PAME) (a non-acyl-Homoserine lactone) which is produced by SAM dependent methyl transferase, a product of *phcB* gene (Flavier *et al.*, 1997a; Schell, 2000).

2.3.2. Disease cycle of *R. solanacearum*

2.3.2.1. Persistence in the environment

R. solanacearum grows well at temperatures between 25-37°C, survives in deep soils, seeds, weeds and irrigation water (Grey and Steck, 2001; Denny 2006; Hong *et al.*, 2008; Champoiseau *et al.*, 2009; Stevens and Elsas, 2010; Ramesh *et al.*, 2011). Pathogen is shed in large numbers from infected and dead plants to soil. Survival and dispersal of *R. solanacearum* through planting material of potato and ginger has also been reported

(Wullings *et al.*, 1998). Spread of BW has been is also known to occur through agricultural tools and insects (Denny, 2006). Root knot nematode *Meloidogyne incognita* causes wounds on plant roots that aids in entry of *R. solanacearum* (Deberdt *et al.*, 1999). Starvation in soil, river water and exposure to copper ions induces the transition of *R. solanacearum* to VNBC state; however the pathogen still remains infectious (Denny, 2006; Alvarez *et al.*, 2008). Latent infection of weeds and other hosts which remain asymptomatic is considered an efficient survival strategy of the pathogen until it encounters a suitable host (Pradhanang *et al.*, 2000; Ramesh *et al.*, 2011).

2.3.2.2. Plant colonization and wilting

R. solanacearum utilizes its flagellar swimming motility and chemotaxis to move towards plants in response to their root exudates (Tans-Kersten *et al.*, 2001; Yao and Allen, 2006). The bacterium infects the plants through wounds or root cracks at the site of root emergence and presence of just one exposed site is sufficient for colonization. Before the appearance of wilt symptoms, the intercellular spaces of the root cortex are rapidly colonized. Cell wall degrading exoenzymes mainly endoglucanase (Egl) disrupt the plant cell walls and facilitate its entry in the vascular cylinder by breaching the endodermis in about 2- 6 days after entry (Vasse *et al.*, 1995). Plants produce tyloses along the length of xylem vessels to block longitudinal spread of pathogens and colonization in vascular system. However, in susceptible varieties the rate and frequency of xylem tylose formation is low and the pathogen can migrate into the tyloses that form within the xylem vessels. In certain circumstances tyloses itself can cause vascular dysfunction aiding in wilt (Denny, 2006).

Once inside the xylem vessels *R. solanacearum* cells normally encounter few barriers as the end walls of vessels are completely degenerated allowing axial spread. Inside the xylem vessels, the bacterial populations rapidly reach very high levels of 10^{10} cells.cm⁻¹ of stem (Genin and Boucher, 2002). High cell density and large amounts of acidic EPS of *R. solanacearum* leads to clogging of xylem vessels, wilting and eventually plant death occurs (Saile *et al.*, 1997).

Cells of *R. solanacearum* aggregate near the pit membranes of vessels causing the parenchyma cells to die. The pit membranes break and allow radial colonization of pathogen in the pith as well as cortical cells through the intercellular spaces. Wilting occurs due to vascular dysfunction which prevents sufficient water transport to leaves and also as there is no evidence of toxins of the pathogen which may cause excessive transpiration through the stomata. Crucial factors responsible for wilting is plugging of pit membranes with high molecular mass EPS, high bacterial cell densities, by-products of plant cell wall degradation, and plant-produced tyloses and gums (Grimault *et al.*, 1994; Ishihara *et al.*, 2012).

2.4. Interactions of endophytes with pathogens

Several antagonistic interactions have been reported to be occurring between the beneficial microbes and plant pathogens. Production of diffusible and volatile antimicrobials, competition for iron, competition for endophytic niche and induced resistance are considered as possible antagonistic activities of endophytic bacteria against phytopathogens (Keel *et al.*, 1992; Whipps, 2001; Ran *et al.*, 2005b).

2.4.1. Antibiosis by diffusible antagonistic compounds

Production of inhibitory diffusible and volatile antimicrobials increases the survival of bacteria in soil by reducing the population of potential competitors and phytopathogens (Dilantha-Fernando *et al.*, 2006).

2.4.1.1. Antibiotics

Antibiotics are diffusible bacterial secondary metabolites having bactericidal and bacteriostatic properties. Plant associated bacteria are known to produce a wide variety of antibiotics including beta lactams, macrolides, peptides etc. These antibiotics have structural diversity and broad-spectrum activity against phytopathogens. One type of antibiotic could be produced by several bacterial strains, and one bacterial strain can produce more than one kind of antibiotic against plant pathogens (Wang and Raaijmakers, 2004). Plant associated fluorescent and non-fluorescent pseudomonads, *Burkholderia* sp., *Streptomyces* sp. are well known for production of antibiotics such as phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol, pyrrolnitrin, rhamnolipids, ecomycin, butyrolactones, sulphonamides, pyoluteorin, pseudomonic acid (murporicin) azomycin (Dilantha-Fernando *et al.*, 2006; Weller, 2007). Plant associated *Actinobacteria* of genera *Streptomyces*, *Microbispora* and *Nocardioides* are known for production of a wide variety of secondary metabolites with antimicrobial properties (Hardoim *et al.*, 2012). *Bacillus* sp. are well known for production of zwittermicin A (aminopolyol), and kanosamine (aminoglycoside) (Emmert and Handelsman, 1999). Non-pathogenic *Agrobacterium radiobacter* produces agrocin which inhibits pathogenic *A. tumefaciens* (Whipps, 2001). Agrocin is a substituted nucleotide with adenine in its core. Agrocin inhibits DNA synthesis at low concentrations and both RNA, and protein synthesis at higher concentration (Hendson *et al.*, 1983). *Enterobacter* sp. is known to

produce 2-phenylethanol having antimicrobial properties which also imparts a pleasant floral odor to some strains. *Enterobacter* sp. also produces 4- hydroxybenzoate, a precursor of ubiquinone and has antimicrobial effects (Taghavi *et al.*, 2010).

2.4.1.2. Bacteriocins

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by several Gram positive and Gram negative bacteria. They are classified into lanthibiotics (containing the modified amino acid lanthionine), cystibiotics (containing one or more disulfide bonds essential for their activity), and thiolbiotics (containing an active -SH group) (Oscariz *et al.*, 1999). Bacteriocins produced by *Bacillus cereus* include cerein 8A which has activity against Gram positive as well as Gram negative bacteria (Bizani *et al.*, 2005). *Pseudomonas aeruginosa* produces pyocins which are similar to the colicins of *E. coli*. Bacteriocins may enable invasion of a strain into an established microbial communities such as in the rhizosphere. They may also prevent the invasion of other strains or species into an occupied niche. Certain bacteriocins of Gram positive bacteria are reported to mediate quorum sensing (Riley and Wertz, 2002). Bacteriocin production by plant associated bacteria is considered to be an added advantage for plant colonization by bacteria (Muñoz-Rojas *et al.*, 2005).

2.4.1.3. Siderophores

Microbes need iron for incorporation and functioning of several proteins including nitrogenase, ferredoxins, cytochromes, and leghemoglobin. Siderophores produced by *Pseudomonas* can chelate iron, making it unavailable to the pathogen and consequently arrest their growth. Siderophore producing ability also contributes to root-colonizing

ability of *Pseudomonas* strains and thus playing a role in niche exclusion. Microbes can use their own siderophore molecules and also those produced by other microbes (Bouizgarne, 2013). Antagonistic rhizosphere and endophytic bacteria are known to sequester heterologous siderophore-iron complexes of pathogens, thereby limiting their growth (Compant *et al.*, 2010). Interestingly, competition for iron tends to decrease with increasing iron concentration.

2.4.2. Antibiosis by volatile antagonistic compounds

2.4.2.1. Hydrogen cyanide

Hydrogen cyanide (HCN) is a volatile secondary metabolite of *P. fluorescens* and *P. aeruginosa* and produced under reduced oxygen levels (Castric, 1975; Castric, 1983). HCN and CO₂ are formed from glycine catalyzed by HCN synthase (Dilantha-Fernando *et al.*, 2006). *P. aeruginosa* produces upto 300µM cyanide. This concentration inhibits haem copper oxidases (Cooper *et al.*, 2003). HCN produced by *Pseudomonas* has been reported to be toxic to plant pathogenic bacteria with cytochrome cbb (3) type oxidase of the haem-copper oxidase family (Ray and Williams, 1997; Lanteigne *et al.*, 2012). Haem-copper oxidases are sensitive to micromolar concentrations of HCN whereas cyanide insensitive oxidase (cio) are sensitive to millimolar concentrations of cyanide or not at all. HCN producing *P. aeruginosa* has cio type oxidase thus under cyanogenic conditions it is not inhibited until the cyanide concentrations reach millimolar levels. Plants themselves are cyanogenic are unaffected by HCN produced by bacteria. Rhizosphere colonizing fluorescent *Pseudomonas* sp. are known to control fungal and bacterial root borne diseases through HCN production (Rust *et al.*, 1980; Ramette *et al.*, 2003; Noori and Saud, 2012; Lanteigne *et al.*, 2012).

2.4.2.2. Ammonia

Sensitivity to ammonium is a universal phenomenon and occurs in animals, plants, and microorganisms, although toxicity levels depend on the type of organism. In bacteria, ammonia causes dissipation of proton gradients across membranes, the acidification of the external medium in response to ammonium uptake, or disequilibrium in the acid/base balance (von Wiren and Merrick, 2004). Free ammonia diffuses inside the bacterial cells and dissolves in the membrane lipids. Uncharged lipid soluble ammonia inhibits enzyme activity and causes proton imbalance that can shut down the respiratory ETC (Aktan *et al.*, 2012). Ammonia has been shown to be toxic and reduced growth of *R. solanacearum* when amended in soil for biological soil disinfestation (Michel and Mew, 1998; Yadessa *et al.*, 2010). In fungi ammonia has been known to inhibit hyphal elongation, cessation of hyphal growth and senescence in *Pythium*, *Verticillium dahliae* and *Rhizopus* (Conn *et al.*, 2005; Howell and Stiphanovic 1988; Sparringa and Owens, 1999). Ammonia is also a plant growth promoting substance easily taken up by plants.

2.5. Induced Systemic Resistance

The term Induced systemic resistance (ISR) is described as the activation of the plant's physical or chemical defenses by an inducing agent. ISR is phenotypically similar to systemic acquired resistance (SAR), which develops when plants activate their defense mechanism in response to infection by a pathogen and hypersensitive reaction that it induces. ISR is effective against different types of pathogens but differs from SAR in that the inducing beneficial endophyte does not cause visible symptoms on the plant. Ability to induce ISR has been reported for rhizobacteria as well as endophytic bacteria. ISR has been shown to suppress plant diseases in the greenhouse and field conditions against

virtually all types of plant pathogens, including viruses, fungi, bacteria and nematodes. Endophytes are known to elicit stronger ISR since they are in close association with plants. ISR strengthens plant cell wall by increased callose deposition, increases accumulation of phenolics and enhances synthesis of pathogenesis-related proteins (PR proteins such as PR-1, PR-2), chitinases and peroxidases (Compant *et al.*, 2010; Yi *et al.*, 2013).

Bacterial surface structures (flagella and lipopolysaccharides) and products of pyruvate metabolism (acetoin and 2, 3 butanediol) have been reported to trigger ISR (Compant *et al.*, 2010). Majority of endophytic bacteria activate ISR via a salicylic acid (SA)-independent pathway involving jasmonate (JA) and ethylene (ET) signals. However, acetoin elicited ISR is dependent on SA and ET signaling pathways. Acetoin producing *Bacillus* sp. and *Enterobacter* sp. are used as consortia for induction of ISR in plants against phytopathogens (Jetiyanon and Kloepper, 2002; Choudhary and Johri, 2009; Yi *et al.*, 2013). Acetoin and 2-3 butanediol are water soluble, inexpensive, function at low concentrations ($\text{ng}\cdot\text{ml}^{-1}$) and are safe to animals and humans. Thus, these volatiles can be applied to plants for control of pathogens. Bacterial volatiles also confer resistance to salt and drought (Ryu *et al.*, 2004; Taghavi *et al.*, 2010; Kang *et al.*, 2011, Kim *et al.*, 2011; Hao *et al.*, 2012). Fluorescent *Pseudomonas* sp. actively secretes SA in rhizosphere in nanogram quantities and is also converted to siderophore pyochelin. The pyochelin and pyocyanin act synergistically to produce reactive oxygen species that cause cell damage and induced resistance (Dilantha-Fernando *et al.*, 2006). In addition bacterial siderophores are known elicitors of induced systemic resistance (ISR) in plants (Choudhary *et al.*, 2007).

Recently role of antagonistic endophytic bacteria in BW resistance in tomato is proposed (Feng *et al.*, 2013). It is known that plants treated with non-pathogenic endophytic bacteria are primed to ward off infection due to induction of ISR. It is proposed that endophytic bacteria may play a role in plant disease resistance by acting mutualistically with the plant host and interacting with the invading pathogen, this phenomenon is termed defensive mutualism. Protection against pathogen attack can occur through endophyte mediated activation of the host's immune response or by production of antagonistic compounds (Hussa and Goodrich-Blair, 2013). It is speculated that plants have co-opted bacteria as a part of their disease suppressive mechanism against phytopathogens, and is mirrored in the antagonistic and biocontrol activities of endophytic bacteria against pathogens (Sturz *et al.*, 1999).

2.6. Niche exclusion and competition for nutrients

Competition for colonization sites in plants is called *niche exclusion* (Beattie, 2006). Competition for nutrients and niche exclusion are amongst the mechanisms by which rhizosphere and endophytic bacteria protect plants from phytopathogens. In order to protect plants against pathogens microbes must be able to establish themselves in the rhizosphere and endophytic tissues. Rhizosphere and endophytic niches of plants are nutrient rich and attract beneficial microbes as well as pathogens. Beneficial antagonistic microbes should be able to compete with the indigenous microflora, including the pathogen and establish in the plant tissues rapidly. An overlap of niches for colonization of endophytes and plant pathogen is the basis for interaction and competition *in planta* (de Weert and Bloemberg, 2006).

An efficient and rapid colonization is necessary to abolish establishment of the pathogen on plants during its entry (de Weert and Bloemberg, 2006). Plant exudates may have antimicrobial properties leading to selection of distinct microbial population. Competence of rhizosphere or endophytic colonization is also determined by several factors. Chemotaxis, lipopolysaccharides, type IV pili, high growth rate, ability to synthesize vitamin B₂, exudation of NADH dehydrogenase, site specific recombinases are involved in making a bacterium competent to colonization of plant microenvironment (Compant *et al.*, 2005a). Using in vivo expression technology (IVET) and microarray analysis additional traits important for plant colonization by *Pseudomonas* sp. is reported to be O-antigenic side chain of lipopolysaccharides, nitrate reductase, surfactants, amino-acids transport and metabolism, and oxidative stress resistance (Ghirardi and Dessaux, 2012). One of the mechanism involved in biocontrol of several plant diseases by *Bacillus* sp. has been reported to be niche exclusion (Bacon and Hinton, 2002; Timmusk *et al.*, 2005; Choudhary and Johri, 2009; Bacon and Hinton, 2011). Non-pathogenic *Agrobacterium tumefaciens* could out-compete pathogenic ones for colonization of tumours (Belanger *et al.*, 1995; Llop *et al.*, 2009). *Enterobacter* sp. are well adapted to endophytic niches and deploy carbenapem and phenazines to outcompete indigenous inhabitants of endophytic niche (Toth *et al.*, 2006; Taghavi *et al.*, 2010). Ice nucleation negative mutants of *Pseudomonas syringae* were also able to compete with the pathogenic *Pseudomonas syringae* for epiphytic niche on *Citrus* (Lindow, 1987). Ability to utilize plant xylem sugars by *Pseudomonas* sp. is also of importance for bacteria to be able colonize xylem (Malfanova *et al.*, 2013).

2.7. Interactions of endophytic bacteria with host plants

Beneficial interactions of endophytes with their hosts involve plant growth promotion and disease prevention. Beneficial effects of endophytic bacteria are greater than those of many rhizosphere-colonizing bacteria (Hardoim *et al.*, 2008). Endophytic bacteria produce an array of metabolites involved in phytostimulation, defense, competition and plant signaling which results in enhanced plant growth and disease suppression (Brader *et al.*, 2014). Well known plant growth promoting bacteria belong to genera *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Herbaspirillum* (Bouizgarne, 2013). Beneficial effect of these rhizobacteria and endophytic bacteria involve the following mechanisms:

2.7.1. Phytostimulation

Phytostimulation is the direct promotion of plant growth through the production of phytohormones (Bloemberg and Lugtenberg, 2001). Plant hormones are defined as naturally occurring substances operating at low concentrations and are able to translocate within the plant body and bind to a specific receptor protein. Based on chemical structure, the phytohormone groups are auxins (indole acetic acid, phenyl acetic acid, chloroindole acetic acid), cytokinins (isopentenyladenine and its hydroxylated derivative zeatin), gibberellins (Gibberelin A1, Gibberallane, Kaurane), ethylene, abscisic acid, polyamines, brassinosteroids, jasmonates, SA and strigolactones. Microbe mediated plant growth promotion is largely reported via production of indole acetic acid (IAA), ACC deaminase, cytokinins and gibberellins. Plant IAA is involved in rapid root establishment by stimulating elongation of primary roots and by proliferation of lateral and adventitious

roots (Patten and Glick, 2002). However, plants can perceive microbial IAA which is identical to the indigenous plant IAA. Microbial IAA is associated with plant growth stimulation by endophytes or pathogenesis of phytopathogens. Diverse genera of epiphytic, endophytic and rhizosphere colonizing bacteria are known to produce IAA. Plant associated bacteria belonging to genera *Agrobacterium* sp., *Enterobacter* sp., *Pseudomonas* sp., *Rhizobium* sp., *Bradyrhizobium* sp., *Acetobacter* sp. and *Alcaligenes* sp. produce IAA (Patten and Glick, 1996). However, plant pathogenic *Agrobacterium tumefaciens*, *Erwinia herbicola* and *Pseudomonas syringae* produce IAA for enhancing pathogenesis and is reported as tumorigenic (Patten and Glick, 1996).

At higher levels, IAA can induce the synthesis of the enzyme 1-aminoacylcopropane, 1-carboxylic acid synthase in the plants that produces 1-aminoacylcopropane, 1-carboxylic acid (ACC). ACC is a substrate for ACC oxidase that forms ethylene, CO₂ and HCN in plants. Ethylene is a plant hormone when applied exogenously, causes formation of adventitious root and root hair. Endogenously it regulates xylem formation, flowering, and flower wilting and fruit ripening. However, during stress, it inhibits root elongation, speeds aging and senescence and abscission (Glick *et al.*, 2007b). During stress, rhizosphere and endophytic bacteria can lower levels of plant hormone ethylene by producing enzyme ACC deaminase. ACC synthesized by the plant, is also exuded through the plant roots. Rhizosphere colonists and root endophytes possessing the enzyme ACC deaminase can take up the ACC and cleave it to ammonia and α -ketobutyrate, which are readily metabolized by the bacteria. As a consequence of lowering the level of ACC within a plant, the amount of ethylene that can form is reduced. Thus, ACC deaminase-producing bacteria act as a sink for ACC, leading to increased plant root and shoot length, an increase in biomass, and protection of plants

from inhibitory effects of ethylene synthesized due to biotic and abiotic stresses (Glick *et al.* 2007a; Glick *et al.*, 2007b).

2.7.2. Biofertilization

2.7.2.1. Nitrogen fixation

Biologically available nitrogen, also called fixed nitrogen, is essential for plant life. All known nitrogen-fixing organisms (diazotrophs) are prokaryotes, and the ability to fix nitrogen is widely distributed in the bacterial and archaeal domains. The nitrogen fixation is one of the most metabolically expensive processes. Symbiotic and mutualistic endophytes and freeliving bacteria which fix atmospheric nitrogen are known as diazotrophs. Nitrogenase enzyme which catalyzes nitrogen fixation is composed of the iron (Fe) protein encoded by the *nifH* gene and the molybdenum iron (MoFe) protein encoded by the *nifDK* genes. Conserved amino acid sequences in the *nifH* gene have been used to design PCR primers to amplify nitrogen fixing genes by diazotrophic plant associated bacteria (Ding *et al.*, 2005). Putative diazotrophs can be identified by their growth in medium devoid of nitrogen sources (Eckert *et al.*, 2001). However, the most reliable method to assess nitrogen fixation is by acetylene reduction assay (Suarez-Moreno *et al.*, 2008). Nodule symbionts of leguminous plants of genera *Rhizobium*, *Bradyrhizobium*, *Allorhizobium* and others are long known as diazotrophs. Genera of mutualistic endophytic and rhizosphere bacteria involved in nitrogen fixation include *Pseudomonas* sp., *Bacillus* sp., *Sphingomonas azotifigens*, *Agrobacterium tumefaciens*, *Enterobacter sacchari*, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* (Kanvinde and Sastry, 1990; Triplett, 1996; Ding *et al.*, 2005; Xie and Yokota, 2006; Suarez-Moreno *et al.*, 2008; Zhu *et al.*, 2013). Xylem is identified to be a non-nodular

low oxygen niche ideal for nitrogen fixation by mutualistic endophytic diazotrophs in non-leguminous plants (Cocking, 2003). Ability to provide fixed nitrogen to the plants may be a part of endophyte-plant symbiosis, where in return the, bacteria may be utilizing plant photosynthate for growth in xylem (James *et al.*, 2001).

2.7.2.2. Phosphate solubilization

Phosphorus is the second important macronutrient after nitrogen necessary for plant growth. Phosphorus is largely present as insoluble organic and inorganic forms and its bioavailability is restricted. The phosphorus content in average soil is about 0.05% (w/w) but only 0.1% of the total phosphorus is available to plant because of poor solubility and its fixation in soil. Soils are supplemented inorganic phosphate fertilizers to enhance productivity. However, inorganic mineral phosphates and phosphate fertilizers added to agricultural soils get converted to insoluble precipitates of calcium in high pH soils or of ferric iron or aluminium in low pH soils (Goldstein, 1986). Plants are unable to utilize these forms of phosphorous. Solubilization of insoluble phosphate by microorganisms was first reported by Pikovskaya (1948). Phosphate solubilizing bacteria (PSB) can convert the insoluble forms of phosphorous to soluble orthophosphates, monobasic (H_2PO_4) and dibasic (HPO_2^{-4}) ions. Citrate, lactate, succinate and other organic acids secreted by PSB dissolve the mineral phosphates by anion exchange or chelate both Fe and Al ions associated with phosphate (Bajpai and Rao, 1971; Hariprasad and Niranjana, 2009). Phosphate solubilizing bacteria identified include *Bacillus* sp., *Pseudomonas* sp., *Burkholderia* sp., *Enterobacter* sp., *Serratia* sp., *Arthrobacter* sp., *Chryseobacterium* sp., *Rhodococcus* sp., *Azotobacter* sp., *Pantoea* sp., *Klebsiella* sp., and symbiotic diazotroph *Rhizobium leguminosarum* (Sharma *et al.*, 2013a). In addition to bacteria,

other phosphate solubilizing microbes include fungi (*Aspergillus* sp. and *Penicillium* sp.), *Actinobacteria* (*Actinomyces* sp., *Streptomyces* sp.), Cyanobacteria (*Anabena* sp., *Calothrix braunii*, *Nostoc* sp., *Scytonema* sp.) and vesicular arbuscular mycorrhiza (*Glomus fasciculatum*) (Gupta *et al.*, 2012; Lopez *et al.*, 2012; Sharma *et al.*, 2013a).

2.7.2.3. Iron uptake

Iron is the fourth most abundant element in the earth's crust. In aqueous solutions, iron exists in two states namely ferrous (Fe^{2+}) and ferric (Fe^{3+}). Fe^{3+} forms exist as insoluble oxides or hydroxides which are not readily utilizable by plants. Plants can naturally acquire iron by acidification of the rhizosphere followed by reduction of Fe^{3+} ions by membrane-bound Fe (III) - chelate reductase to more soluble Fe^{2+} or by using low molecular weight phytosiderophores to mobilize iron in root cells. However, the process reported to be less efficient in calcareous and alkaline soils. Iron fertility of soils can be improved by applying inorganic FeSO_4 and synthetic Fe-EDTA chelates. However, these practices may have negative environmental impacts (Radzki *et al.*, 2013). Scarcity of bioavailable iron in the rhizosphere is efficiently scavenged by microbial low molecular weight iron scavenging siderophores (Greek, 'iron carriers'). Siderophores are produced under iron limiting conditions, have high-affinity for Ferric ion (Fe^{3+}) are secreted outside by the cell. Hydroxamate or catechol type siderophores are involved in microbial mediated iron chelation. The iron-siderophore complex is captured back by the microbial cell as well as plants for their nutritional requirements (Bar-Ness *et al.*, 1992; Neilands, 1995; Loaces *et al.*, 2010). Siderophore molecules produced by plant associated bacteria include pyoverdine and pseudobactin (pyochelin) of fluorescent *Pseudomonas* sp., catechol type siderophores namely agrobactin, enterobactin and chrysobactin of

Agrobacterium sp., *Enterobacter* sp. and *Erwinia* sp. respectively and hydroxymate type aerobactin of *Enterobacter* sp. (Loper and Buyer, 1991).

2.8 Biocontrol of bacterial wilt using endophytic bacteria

According to Cook and Baker (1983) biological control is defined as ‘the reduction of the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man. The terms biological control and its shortened synonym biocontrol are used interchangeably in entomology and plant pathology (Pal and McSpadden Gardener, 2006). In plant pathology, the term is defined as the use of microbial antagonists to suppress diseases and the organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA). Pal and McSpadden Gardener (2006) defined biological control as utilization of introduced or resident living organisms other than disease resistant host plants to suppress the activities and populations of one or more plant pathogens. Bale *et al.* (2008) defined biological control as the use of an organism to reduce the population density of animals, weeds and diseases. Eilenberg *et al.* (2001) suggested use of microbial control as a useful term to describe the use of micro-organisms as biological control agents and as a sub-set of biological control. Eilenberg *et al.* (2001) also suggested the term ‘biological control’ should be limited to the use of living organisms and not their metabolites. Based on these broad definitions of biological control, organisms and procedures considered as biocontrol include use of antagonistic microorganisms, avirulent or hypo-virulent populations of the pathogenic species and induction of the plant defense to resist the pathogen (Alabouvette *et al.*, 2006).

In recent years biological control is emerging and is widely used for management of phytopathogens. Microbiological control of plant diseases has narrow spectrum of activity and does not affect non-target plant associated bacteria (Alabouvette *et al.*, 2006). Bacterial and fungal biocontrol agents can be combined with traditional management practices for controlling BW and reducing the environmental pollution due to use of chemical pesticides (Lwin and Ranamukhaarachchi, 2006). Biocontrol agents are also known to improve plant growth, prevent crop loss and reduce drug resistance of phytopathogens (Yu *et al.*, 2010). Plant associated antagonistic microbes ubiquitously colonize plants and share an econiche with phytopathogens and are a rich source for varied antimicrobials and inhibitory molecules (Emmert and Handelsman, 1999; Rosenblueth and Martinez-Romero, 2006). Bacteria of phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* are reported to be highly antagonistic to several phytopathogens including *R. solanacearum*.

Wilt suppression by endophytic bacteria is attributed to production of volatile and diffusible antimicrobial compounds, niche exclusion and induced systemic resistance (Compant *et al.*, 2005a). These properties of endophytes make them valuable for biocontrol of BW. 2,4-diacetylphloroglucinol (DAPG), HCN and siderophores are diffusible and volatile antimicrobials produced by endophytes and reported to inhibit pathogenic *R. solanacearum* strains (Zhou *et al.*, 2012). Use of volatile compound ammonia as soil amendments has been reported to control BW (Michel and Mew, 1998; Yadessa *et al.*, 2010). Bacteria of the genera *Pseudomonas*, *Bacillus*, *Paenibacillus* and *Sphingomonas* isolated from BW resistant plants were reported to be highly antagonistic to *R. solanacearum* (Feng *et al.*, 2013). Very high biocontrol effect against BW and growth promotion in tomato was exhibited by *P. fluorescens* (Seleim *et al.*, 2011).

Endophytic strains of *Burkholderia* sp., *Bacillus* sp. and DAPG producing *Pseudomonas* sp. from eggplant were found inhibitory to *R. solanacearum* (Ramesh *et al.*, 2009). Endophytic *Bacillus* sp. and *Serratia* sp. isolated from tomato and chilli inhibited *R. solanacearum* by production of siderophores (Amaresan *et al.*, 2012). Rasche *et al.* (2006) found antagonistic effects of *Agrobacterium*, *Ochrobacterium*, *Microbacterium*, *Klebsiella* and *Arthrobacter* isolated from potato towards *R. solanacearum*.

Similarly, Lemessa and Zeller (2007) have reported BW prevention under field conditions using endophytic bacteria having inhibitory effects against *R. solanacearum*. *Streptomyces* sp. has been shown to be effective in preventing BW (El Albyad *et al.*, 1996). Endophytic *Staphylococcus* sp. and *Bacillus amyloliquefaciens* were found to prevent BW in tomato under green-house conditions (Nawangsih *et al.*, 2011). Xue *et al.* (2009) reported soil drenching with antagonistic strains of *Acinetobacter* sp. and *Enterobacter* sp. reduced BW incidence in tomato plants. Xylem colonizing strain of *Bacillus subtilis* reduced BW incidence in mulberry through production of antimicrobials and niche exclusion as seen from its high endophytic and rhizosphere population densities (Ji *et al.*, 2008). Chitosan is a known inducer of ISR, and a combination of chitosan and chitisanolytic bacteria have been reported to reduce BW incidence in tomato (Prevost *et al.*, 2006; Algam *et al.*, 2010). McLaughlin *et al.* (1990) have reported dual control of BW and root knot nematode by using biocontrol agents.

Avirulent *R. solanacearum* strains and *Pseudomonas fluorescens* induced ISR in tomato plants and prevented BW (Kempe and Sequeira, 1983). Hara and Ono (1991) have reported bacteriocin producing avirulent *R. solanacearum* mutant protected tobacco plants from BW, possibly by antagonism and niche exclusion. In addition, Frey *et al.* (1994) have reported bacteriocin is involved in BW prevention by *Hrp*⁻ mutants of *R.*

solanacearum. Interestingly *hrpB* mutants of *R. solanacearum* prevented colonization of virulent *R. solanacearum* by niche exclusion and expression of abscisic acid related genes in *Arabidopsis thaliana* and controlled wilting symptoms (Feng *et al.*, 2012).

Messiha *et al.* (2007) have reported that antagonistic *Stenotrophomonas maltophilia* isolated from Egypt could control brown rot in potato caused by *R. solanacearum* in Egyptian soils and not in the Netherlands. This indicates that biocontrol agents may fail under certain conditions. Biocontrol agents may not have same efficacy against diverse members of same pathogen. Great variation was observed in the biocontrol activities of antagonistic bacteria against several strains of *R. solanacearum* and it is reported that genetic diversity of *R. solanacearum* is one cause of variable responses of biocontrol agents (Xue *et al.*, 2013).

2.9. Quorum sensing system in phytopathogens

2.9.1. Quorum sensing by acyl-homoserine lactones

The term Quorum sensing was coined by E. P. Greenberg and colleagues (Fuqua, Parsek and Greenberg, 2001) and a widely accepted and simple definition is ‘bacterial cell to cell communication system’ (von Bodman *et al.*, 2003; Chen *et al.*, 2013). Quorum sensing (QS) is mediated by low molecular mass, diffusible signal molecules called autoinducers (AI) or ‘quormones’ whose external concentration increases as a function of increasing cell population density (Whitehead *et al.*, 2001; Zhang and Dong, 2004). Bacteria detect the accumulation of a minimal threshold stimulatory concentration of AI and alter/regulate gene expression in its response. The phenomenon is named quorum sensing because initiation of the strong population response depends on the population reaching a minimal population unit called ‘quorum’ (Fray, 2002). The quorum contains a sufficient

number of bacteria to carry out processes that require cooperation of a large number of cells to be effective (Winnans and Bassler, 2002).

QS signals are involved in regulation of a variety of biological functions, including luminescence, plasmid transfer, motility, virulence, pigment and siderophore production, epiphytic fitness and biofilm formation. Plant associated bacteria frequently employ this signaling mechanism to modulate and coordinate their interactions with plants, including control of tissue maceration, antibiotic production, toxin release and horizontal gene transfer (von Bodman *et al.*, 2003). A deficiency in QS leads to reduction or loss of virulence in phytopathogenic bacteria (Soto *et al.*, 2006).

Microbial quorumones can be divided into two main categories the amino acids and short peptides, commonly utilized by Gram-positive bacteria and fatty acid derivatives acyl homoserine lactones (acyl-HSLs), often utilized by Gram negative bacteria. In Gram negative bacteria QS is also mediated by non-acyl-HSL signals, namely bradyoxetin in *Bradyrhizobium japonicum*, Pseudomonas quinolone signal (PQS) in *Pseudomonas aeruginosa*, 3-hydroxypalmitic acid methyl ester (3OH-PAME) in *Ralstonia solanacearum*, diffusible signal factor (DSF) chemically cis-11-methyl-2-dodecenoic acid in *Xanthomonas campestris* pv. *Campestris* and *Xylella fastidiosa* (Whitehead *et al.*, 2001; Uroz *et al.*, 2009).

2.9.2. Quorum Sensing in *Ralstonia solanacearum*

R. solanacearum possesses non-acyl-HSL based autoinducer known as 3OH-PAME. Expression of virulence determinants mainly EPS and Egl is governed by QS system involving 3OH-PAME as autoinducer, and not the alternate acyl-HSL based QS system. Expression of the QS system in *R. solanacearum* is cell density dependent, with maximal

expression at cell densities (quorum) of 10^7 CFU.ml⁻¹ (Flavier *et al.*, 1997b). The Phc (phenotype conversion) system of *R. solanacearum* forms the main network that regulates expression of virulence and pathogenicity factors. The Phc system is composed of *phcA* and the products of *phcBSRQ* operon (Schell, 2000).

Acyl-HSL QS system of *R. solanacearum* is not involved in the expression of virulence factors. The LuxI and LuxR homologues identified in *R. solanacearum* are named SolI and SolR. SolI encodes an enzyme for synthesis N-hexanoyl- and N-octanoyl-homoserine lactones (C6 and C8 HSLs). The SolR/SolI-regulated gene *aidA* has been identified in *R. solanacearum* however its role in expression of virulence are unclear (Flavier *et al.*, 1998).

2.9.2.1. PhcA

PhcA is a LysR-type transcriptional regulator (Brumbley *et al.*, 1993) encoded by the *phcA* gene. Cells with high levels of PhcA produce large amounts of EPS, exoenzymes mainly Egl and are highly virulent. When PhcA is inactivated, *R. solanacearum* becomes avirulent producing no EPS and very levels of Egl. However upregulated traits include production of polygalacturonase, siderophore, Hrp secretion apparatus and motility. Thus, PhcA acts like a switch in upregulating and downregulating gene expression (Schell, 2000). *PhcA* mutants can arise spontaneously in a still broth culture or culture on agar plates incubated for long periods. The spontaneous mutants are unable to wilt host plants, but can colonize plants and cause stunting, stem necrosis and formation of adventitious roots (Poussier *et al.*, 2003). Interestingly, PhcA activity is controlled by a two-component system made up of PhcR and PhcS, which are responsive to 3OH-PAME (Whitehead *et al.*, 2001). PhcA does not require any co-inducer (as

normally the case of LysR type transcriptional regulators) and directly binds to the Egl promoter to activate its transcription. Expression of EPS via PhcA involves the intermediate transcriptional regulator xpsR (P_{xpsR}) and the VsrA/VsrD system and VsrB/VsrC systems (Schell, 2000).

2.9.2.2. *PhcB* and 3OH PAME

3OH-PAME is active both in solution and in the vapour phase (Flavier *et al.*, 1997a). 3OH-PAME is synthesized from 3-Hydroxy-Palmitoyl-acyl carrier protein an intermediate in the biosynthesis of palmitic acid by the action of the product of *phcB* gene that is S adenosyl methionine (SAM) dependent methyltransferase. This enzyme displaces the acyl carrier protein and links methyl group from SAM to form 3OH-PAME. Instead of the action of dehydrase to produce palmitic acid by removal of the hydroxyl group, the catalytic action of the product of *phcB* (SAM dependent methyltransferase) might be involved in displacement of the acyl carrier protein due to the donation of the methyl group from SAM. SAM dependent methyl transferase is a membrane bound enzyme. Inactivation of *phcB* gives rise to mutants with a phenotype similar to that of *phcA* mutants. However, *phcB* mutants are restored to wild type by exposure to culture supernatants of from wild-type cells containing 3OH-PAME (Schell, 2000).

In confined environments such as plant xylem or culture flask, when cell densities of *R. solanacearum* reach 10^7 cells.ml⁻¹, the external concentration of 3OH-PAME increases to 1 nM. In open environments the 3OH-PAME diffuses away before reaching the threshold concentration. At this concentration the expression of phc regulon is also low. As cell density increases beyond 10^7 cells.ml⁻¹, the external 3OH-PAME concentration rises above 5 nM. At this point, the *phcA* expression increases for

expression of QS regulated traits. At concentrations of 3OH-PAME below 5 nM cells are motile, produce siderophore, pili and polygalacturonase (factors repressed at high levels of phcA). The traits are essential when the pathogen is a saprophyte in soil in search of a host. Inside plant xylem where 3OH-PAME diffusion is minimal and rises above 5 nM virulence traits mainly production of EPS and Egl facilitating dispersal of pathogen are activated. Recognition of 3OH-PAME by *R. solanacearum* is highly specific. Compounds with altered acyl chain or substitutions in methyl group do not have QS activity in *R. solanacearum* (Clough *et al.*, 1994; Schell, 2000).

2.9.2.3. PhcS and PhcR

PhcS encodes a sensor kinase PhcS, while *phcR* encodes an unusual response regulator PhcR with a C-terminal kinase domain instead of the usual DNA binding domain. Together PhcS and PhcR repress production of PhcA in absence 3OH-PAME. During the QS signal transduction, 3OH-PAME stimulates PhcS to phosphorylate PhcR reducing its ability to inhibit production of active PhcA posttranscriptionally (Fig. 2.3). Involvement of additional proteins most likely encoded by *phcQ* gene in inhibition of PhcA expression by phosphorylated PhcR is likely. However, inactivation of *phcQ* had no effect on wild types or any *phc* mutant tested (Clough *et al.*, 1997; Schell, 2000).

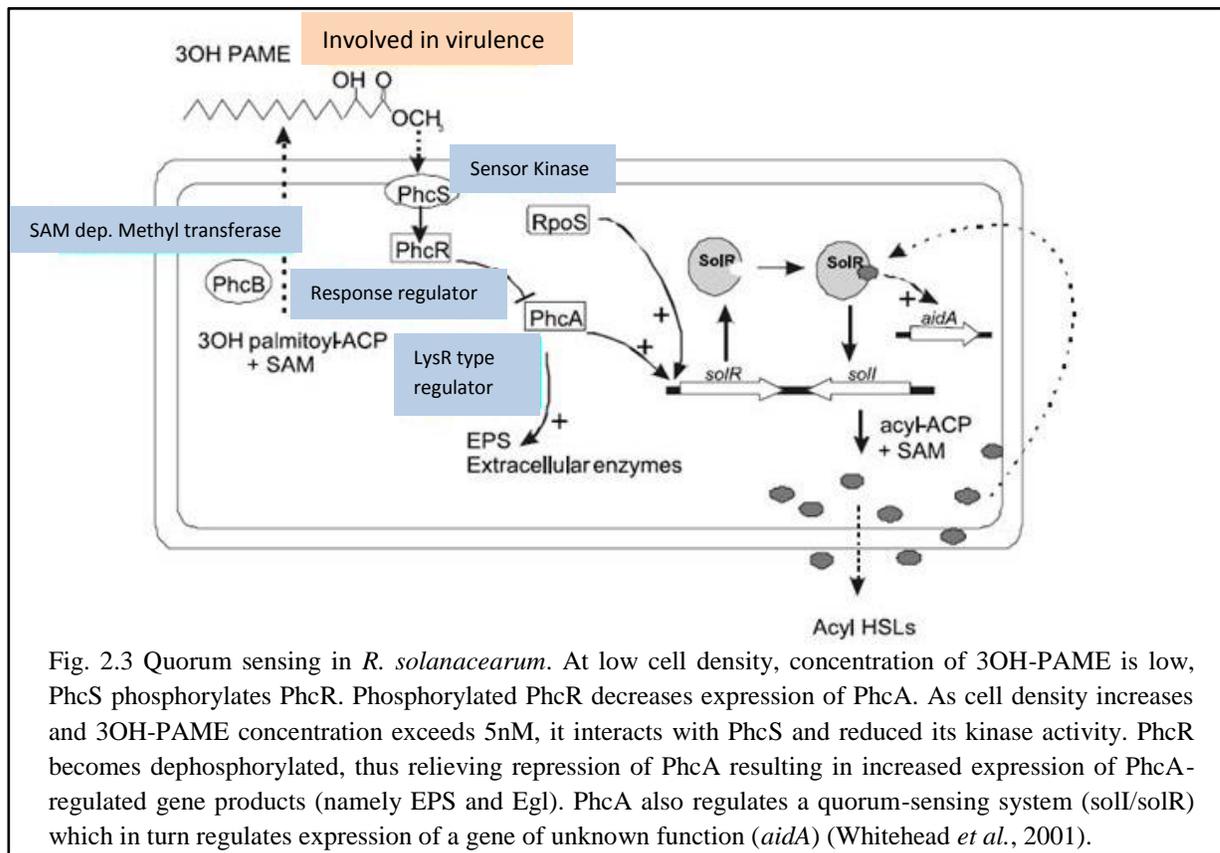


Fig. 2.3 Quorum sensing in *R. solanacearum*. At low cell density, concentration of 3OH-PAME is low, PhcS phosphorylates PhcR. Phosphorylated PhcR decreases expression of PhcA. As cell density increases and 3OH-PAME concentration exceeds 5nM, it interacts with PhcS and reduced its kinase activity. PhcR becomes dephosphorylated, thus relieving repression of PhcA resulting in increased expression of PhcA-regulated gene products (namely EPS and Egl). PhcA also regulates a quorum-sensing system (solI/solR) which in turn regulates expression of a gene of unknown function (*aidA*) (Whitehead *et al.*, 2001).

2.10. Quorum quenching by bacteria

The term quorum-quenching (QQ) was coined by Dong *et al.* (2001) and is defined as interference in quorum-sensing systems of bacterial pathogens through inactivation of quorum-sensing signals. Recently Faure and Dessaux (2007) described QQ to include natural phenomena or engineered procedures causing perturbation and the attenuation of the expression of QS-regulated functions in bacteria. QQ strategies do not kill bacteria or limit their growth because QS is not necessary for growth. However, QQ strategies do affect the expression of a virulence factors of pathogenic bacteria and thus they are now called as anti-virulence/ anti-QS strategies. In addition, QQ does not exert selective pressure on bacteria and is valuable for the current rising bacterial drug resistance (Tay and Yew, 2013). Targets for QQ include the important steps in the QS signaling pathway mainly the synthesis of signal molecules, the signal molecule itself and sensing of the

signal molecule by the receptor protein. Widely studied QQ mechanisms include inhibition of QS sensing and inhibition of signal accumulation due to degradation.

2.10.1. Inhibition of synthesis of QS signal

Synthesis of acyl-HSL and 3OH-PAME is mediated by S-adenosyl-methionine (SAM) and a fatty acid, linked to an acyl carrier protein. SAM analogues namely L-S-adenosylhomocysteine and sinefugin (an S-adenosyl-methionine-like antibiotic) are reported to inhibit synthesis. Other inhibitors include the bactericidal molecule triclosan which targets the enoyl-acyl carrier protein reductase involved in synthesis of acyl-HLs (Faure and Dessaux, 2007). However, there are no reports in inhibition of synthesis of 3OH-PAME of *R. solanacearum* till date.

2.10.2. Inhibition of sensing of QS signal

Halogenated furanones are well known QS sensing inhibitors. Furanones produced by algae *Delisea pulchra* binds to the LuxR receptor of bacteria and displaces acyl-HSLs. Extracts of pea, fruit extracts of grape and strawberry, soybean, vanilla, Gernaium, lily, *Medicago* sp., garlic and pepper inhibit bacterial QS sensing. Fungal lactones namely patulin and penicillic acid are also known to inhibit bacterial QS. Structural analogues of acyl-HSLs namely phenyl-acyl- and chlorophenyl-acyl-homoserine lactone can act as QS inhibitors (Faure and Dessaux, 2007). However, there are no reports on inhibitors or structural analogues of 3OH-PAME of *R. solanacearum* till date.

2.10.3. Degradation of QS molecules

Biological degradation of QS is mainly occurs by QS molecule degrading and modifying enzymes. Enzymatic degradation of QS Acyl-HSLs was first observed in *Variovorax* sp. (Leadbetter and Greenberg, 2000) and later in *Bacillus* sp. (Dong *et al.*, 2000). Recently, enzymatic degradation of 3OH-PAME is reported to occur by hydrolysis of the ester bond between the methyl group and the 3-hydroxy fatty acid by an esterase produced by *Ideonella* sp. (Shinohara *et al.*, 2007).

Acyl-HSL lactonase hydrolyses the homoserine lactone ring of molecule acyl-HSL reversibly to open the homoserine lactone ring and reduces its effectiveness as QS molecule. This type of degradation is called lactone hydrolysis. Bacteria known to have acyl-HSL lactonases are *Rhodococcus* sp., *Pseudomonas* sp. and *Klebsiella* sp. QsdA of *Rhodococcus erythropolis* belongs to the phosphotriesterase family that harbors phosphotriesterase, lactonase or amidohydrolase activities (Uroz *et al.*, 2009; Chen *et al.*, 2013).

Acyl-HSL acylase irreversibly hydrolyzes the amide bond between the acyl chain and homoserine of acyl-HSL molecules releasing homoserine lactone and fatty acid, both of which do not act as QS molecules. This type of degradation is called amidohydrolysis. Acyl-HSL acylases from various groups of bacteria namely *Ralstonia* sp., *Streptomyces* sp., *P. aeruginosa* and *Anabaena* sp have been reported (Uroz *et al.*, 2009). Recently, acylase has been identified in *R. solanacearum* GMI1000. Other acyl-HSL acylase producers include *Comamonas* sp., *Pseudomonas* sp., *Shewanella* sp., *Streptomyces* sp. and *Variovorax* sp. (Uroz *et al.*, 2009). Oxidoreductase from *Rhodococcus erythropolis* reduces the keto group of 3-oxo-HSLs (C-8 to C-14 acyl chain) to the corresponding 3-

hydroxy derivative, without degrading the acyl-HSL (Chen *et al.*, 2013). Fig. 2.4 depicts mechanisms of degradation of quorum sensing molecules of phytopathogenic bacteria.

2.10.4. Bacteria for quorum quenching against plant pathogens

Bacterial populations both endophytic and rhizosphere colonist can be screened for degradation by randomly assaying their activities against of QS molecules. Another approach widely followed is enrichment of QS degraders by screening using QS molecules as sole source of carbon and/or nitrogen. (Leadbetter and Greenberg 2000; Faure and Dessax, 2007). *Ideonella* sp. isolated by enrichment using 3OH-PAME as sole carbon source from rhizosphere of tomato degraded 3OH-PAME of *R. solanacearum* and reduced expression of virulence factors namely EPS (Shinohara *et al.*, 2007). Dong *et al.* (2002) isolated *Bacillus* sp. with high activity towards acyl-HSL degradation.

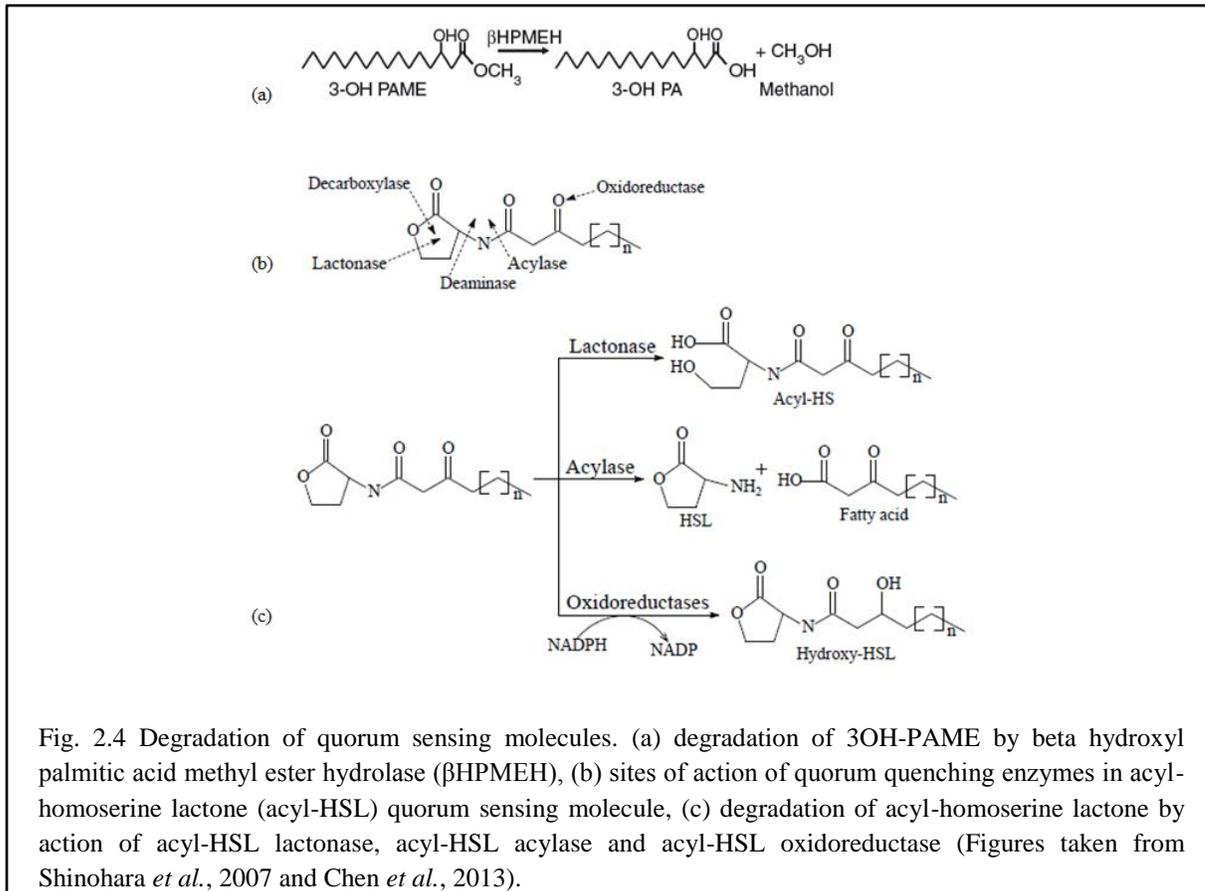


Fig. 2.4 Degradation of quorum sensing molecules. (a) degradation of 3OH-PAME by beta hydroxyl palmitic acid methyl ester hydrolase (β HPMEH), (b) sites of action of quorum quenching enzymes in acyl-homoserine lactone (acyl-HSL) quorum sensing molecule, (c) degradation of acyl-homoserine lactone by action of acyl-HSL lactonase, acyl-HSL acylase and acyl-HSL oxidoreductase (Figures taken from Shinohara *et al.*, 2007 and Chen *et al.*, 2013).

Dong *et al.* (2004) have shown that *in planta*, *Bacillus thuringiensis* decreased the incidence of *E. carotovora* infection and development of soft rot symptoms in potato. The biocontrol efficiency of *B. thuringiensis* was correlated with the ability of bacterial strains to produce acyl-HSL lactonase. Endophytic *Bacillus firmus* and *Enterobacter* sp. degraded acyl-HSL and reduced biofilm forming ability of *Pseudomonas aeruginosa* PAO1 and PAO1-JP2 (Rajesh and Ravishankar, 2013). Phyllosphere bacteria from tobacco leaves were quorum quenchers as they degraded acyl-HSL molecules (Ma *et al.*, 2013).

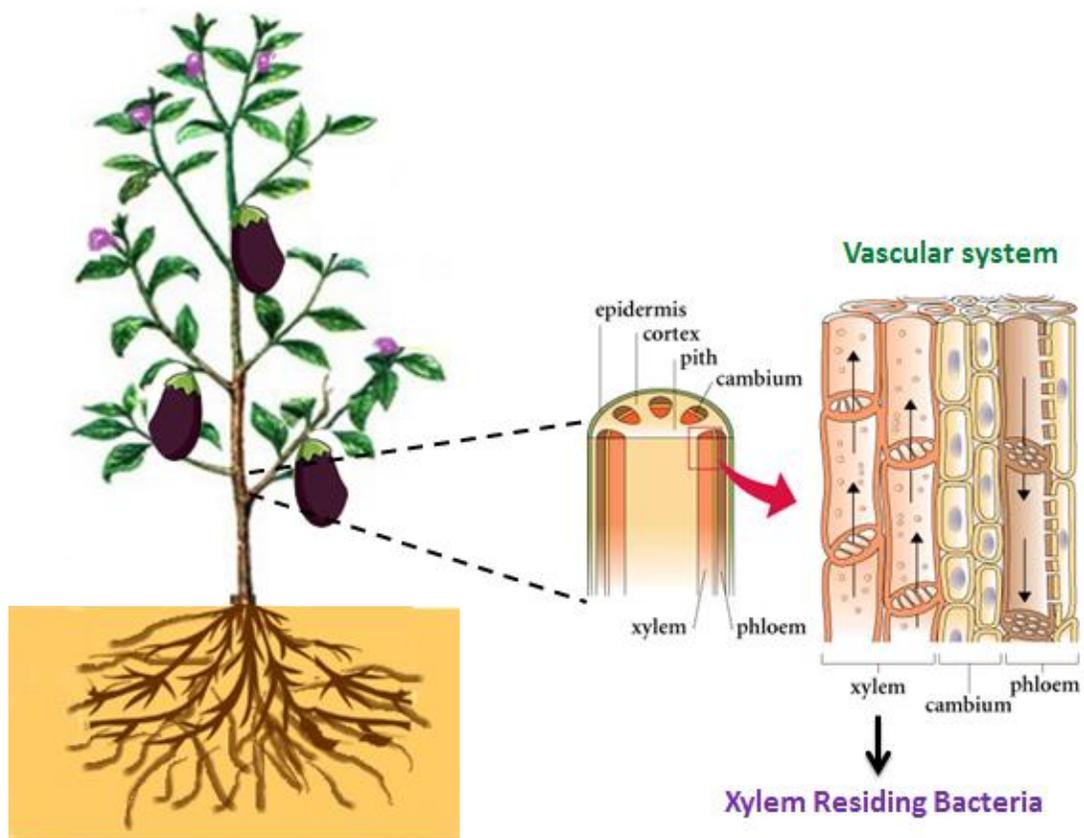
Using structural analogues of acyl-HSL namely gamma caprolactone, growth of rhizosphere populations of *Rhodococcus erythropolis* were stimulated which can efficiently degrade acyl-HSLs of *Pectobacterium atrosepticum* and were efficient biocontrol agents (Crépin *et al.*, 2012; Cirou *et al.*, 2012; Barbey *et al.*, 2013). Diffusible signal factor (DSF) is a fatty acid signal molecule involved in virulence in *Xanthomonas* species as well as *Xylella fastidiosa*. *Bacillus*, *Paenibacillus*, *Microbacterium*, *Staphylococcus*, and *Pseudomonas* were identified that were capable of rapid degradation of DSF. Co-inoculation of *X. campestris* with DSF-degrading bacteria into mustard and cabbage leaves reduced disease severity similarly disease incidence and severity in grape stems co-inoculated with *Xylella fastidiosa* and DSF-degrading strains were also reduced (Newman *et al.*, 2008). Acyl-HSL lactonase producing *Acinetobacter* sp., *Klebsiella* sp. and *Burkholderia* sp. enriched from ginger rhizosphere could quench QS and prevented *Erwinia caratovoa* infection potato (Chan *et al.*, 2011). Strains of *Pseudomonas* sp., *Variovorax* sp., *Comamonas* sp. and *Rhodococcus* sp. isolated from tobacco rhizosphere possessed QQ activities (Uroz *et al.*, 2003).

Zwitterimycin producing strain of *Bacillus cereus* was genetically modified to express acyl-HSL lactonase, and found to reduce incidence of *Erwinia caratovora* infection (Zhao *et al.*, 2008). *Burkholderia* sp. an endophyte from rice was engineered to produce QQ lactonase for use as biocontrol agent (Cho *et al.*, 2007). Gene encoding *aiiA* (autoinducer inactivation) from *Bacillus* sp. was cloned into pathogenic *Erwinia caratovora* and reduction in release of its own acyl-HSL was observed. In addition, decreased extracellular pectolytic enzymes, attenuation in pathogenicity of the engineered pathogen on potato, eggplant, Chinese cabbage, carrot, celery, cauliflower, and tobacco were reported (Dong *et al.*, 2000). The current literature thus suggests the enormous potential of plant associated bacteria for quorum quenching in phytopathogens.

However it is recently reported that bacteria can gain resistance to QQ agents. Mutants of *P. aeruginosa* gained resistance to QQ by increasing efflux of the QQ compound furanone. Using computational biology researchers have discovered that bacteria may evolve resistance by reaching the QS quorum at low cell densities (Beckmann *et al.*, 2012). In addition, biofilms may be resistant to QQ just as they are to antibiotics due to penetration barrier. It has been suggested that there may be additional mechanisms by which bacteria may gain resistance to QQ which include an increase in autoinducer production, synthesis of modified autoinducers (less susceptible against the attack of the degrading enzymes), mutations in the LuxR-like receptors that increase their affinity to the AI (so the necessary threshold of AI concentration decrease) (Maeda *et al.*, 2012; García-Contreras *et al.*, 2014). However such resistance mechanisms are not identified in *R. solanacearum*.

CHAPTER III

Isolation, identification, characterization and diversity of xylem residing bacteria from solanaceous vegetables



3.1. INTRODUCTION

Solanaceous vegetables mainly eggplant and chilli are not only are of economic and cultural importance but are common ingredients in the Indian cuisine. In the state of Goa, Eggplant cv. *Agassaim* and cv. *Taleigao* are preferred by farmers because of large fruit size and high sale value. The Goan cuisine would be unauthentic without the use of local chilli varieties *Tarvatti*, *Kholchi*, *Moirechi* and others. However, the major constraint in the cultivation of the local eggplant and chilli is the crop loss due to infection by the BW pathogen *R. solanacearum*. The disease is severe in humid climate and prevalent throughout the year in the coastal state Goa (Ramesh, 2006).

Earlier studies have shown that endophytes from local eggplant and chilli varieties inhibited *R. solanacearum* (Ramesh *et al.*, 2009). Therefore, antagonistic interactions between XRB and BW pathogen which share the same ecological niche are anticipated. However identity and diversity of cultured and non-culturable XRB from solanaceous vegetables grown in Goa is unknown and their potential interactions with *R. solanacearum* have not been studied.

Since endophytic XRB are in close association with plants, ability to enhance eggplant growth through production of phytohormones, mineral nutrition and other mechanisms may contribute to their fitness as biocontrol agents. Identification of diverse antagonistic XRB with multiple plant beneficial traits is the first and critical step in selecting efficient biocontrol agent for management of BW. This chapter presents the work pertaining to the first objective of the present study **‘to isolate and characterize the xylem residing bacteria from solanaceous vegetables and carry out their diversity analysis’**.

3.2. MATERIALS AND METHODS

3.2.1. Isolation of xylem residing bacteria

3.2.1.1. Sample collection

Sampling of eggplant (*Solanum melongena* L.) and chilli (*Capsicum annuum* L.) was carried out in the growing season in May 2009 and from March 2010 to May 2010 in the state of Goa, from farmer's fields in the major vegetable growing locales in North Goa and South Goa districts of the coastal state of Goa, India (Fig. 3.1 and Table 3.1). Eggplant samples were from two known BW susceptible varieties *Agassaim* and *Taleigao* and other undescribed susceptible varieties which are cultivated throughout Goa. BW resistant varieties were sampled from experimental plots of ICAR RC for Goa, Old Goa. Wild relative of eggplant *Solanum torvum* Swartz which is naturally resistant to BW was included in the study for isolation of XRB as an out-group and sampled from ICAR RC for Goa plots. Chilli samples were from the locally grown undescribed cultivar, which is susceptible to BW (Fig. 3.2 and Table 3.2). Apparently healthy plants were uprooted, placed in clean polypropylene bags and brought to the laboratory. Coordinates of the location were determined using a Garmin GPS 12 device (Garmin International, USA). Isolation of xylem residing bacteria (XRB) was carried out as described below within six hours from the time of sampling.

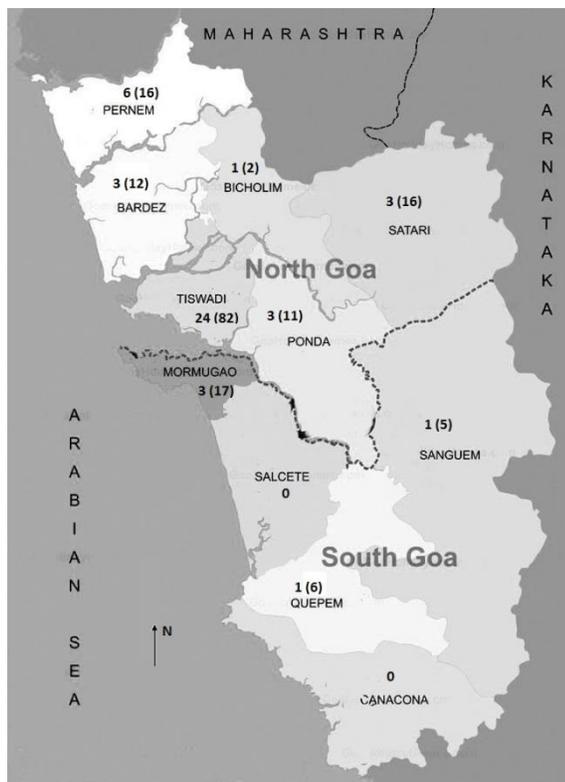


Fig. 3.1 Map of the state of Goa, India showing the districts and talukas from where the eggplant, chilli and *S. torvum* samples were collected. Numbers indicate no. of plants collected from each taluka and numbers in parenthesis indicate the number of isolates obtained.

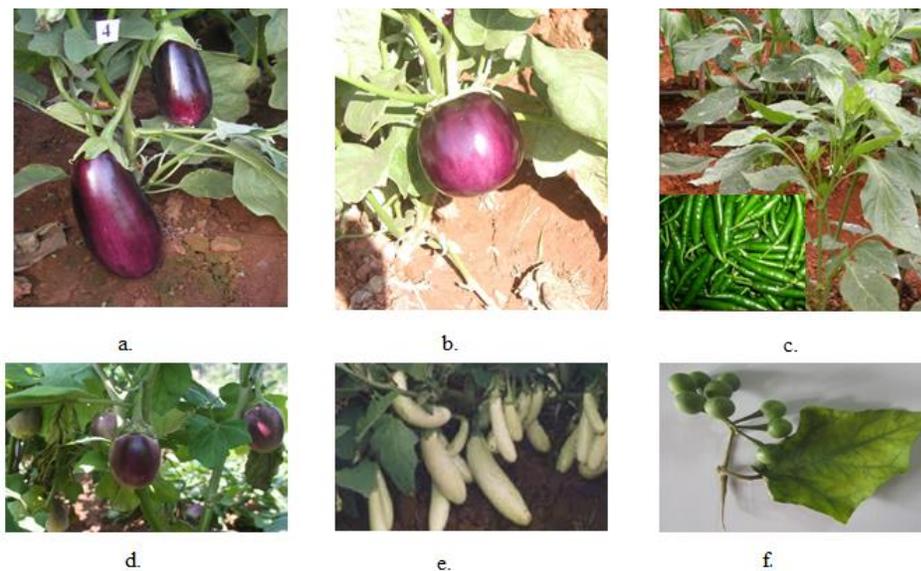


Fig. 3.2 Different varieties of eggplant, chilli and *S. torvum* sampled for isolation of XRB. a. Eggplant cv. *Agassaim*, b. Eggplant cv. *Taleigao*, c. Chilli, d. Eggplant cv. *Surya*, e. Eggplant cv. *Swetha*, f. *Solanum torvum* (wild eggplant).

Table 3.1 Location of sampling sites for collection of solanaceous vegetables in Goa.

Village/ place	Taluka	District	No. of samples	GPS coordinates
Sulabhat	Tiswadi	North Goa	2	15.4380°N, 73.8996°E
Taleigao	Tiswadi	North Goa	1	15.4730°N, 73.8213°E
Pilar	Tiswadi	North Goa	1	15.4400°N, 73.8933°E
Mandur	Tiswadi	North Goa	1	15.4507°N, 73.9227°E
Old Goa (ICAR)	Tiswadi	North Goa	19	15.5027°N, 73.9154°E
Amona	Bicholim	North Goa	1	15.5329°N, 73.9771°E
Dhargal	Pernem	North Goa	1	15.7079°N, 73.8167°E
Mandrem	Pernem	North Goa	4	15.6631°N, 73.7419°E
Parcem	Pernem	North Goa	2	15.6667°N, 73.7667°E
Guirim	Bardez	North Goa	3	15.5635°N, 73.8154°E
Sakhli	Sattari	South Goa	1	15.5606°N, 74.0256°E
Pastawada	Sattari	South Goa	1	15.2993°N, 74.1239°E
Keri	Sattari	South Goa	2	15.4568°N, 74.0050°E
Chicalim	Mormugao	South Goa	1	15.3909°N, 73.8624°E
Vasco-da-Gama	Mormugao	South Goa	2	15.3860°N, 73.8440°E
Dhavali	Ponda	South Goa	2	15.3874°N, 73.9992°E
Shiroda	Ponda	South Goa	1	15.3292°N, 74.0271°E
Costi	Sanguem	South Goa	1	15.2599°N, 74.1680°E
Caurem	Quepem	South Goa	1	15.2993°N, 74.1239°E

Table 3.2 Different varieties of solanaceous hosts sampled for isolation of XRB and their reaction to BW infection.

Host Plant	Reaction to BW	No. of samples
Eggplant cv. <i>Agassaim</i>	Susceptible	5
Eggplant cv. <i>Taleigao</i>	Susceptible	1
Eggplant cv. <i>Arka Nidhi</i>	Resistant	1
Eggplant cv. <i>Utkal Tarini</i>	Resistant	1
Eggplant cv. <i>Arka Keshav</i>	Resistant	1
Eggplant cv. <i>Arka Anand</i>	Resistant	1
Eggplant cv. <i>Swetha</i>	Resistant	2
Eggplant cv. <i>Surya</i>	Resistant	2
Eggplant cv. <i>Agassaim x Surya</i>	Resistant	6
Eggplant cv. <i>Arka Kusumakar</i>	Susceptible	1
Local eggplant	Susceptible	15
Local chilli	Susceptible	7
<i>Solanum torvum</i>	Resistant	2

3.2.1.2. Isolation of XRB from eggplant, chilli and *S. torvum*

3.2.1.2.1. Vacuum infiltration

Xylem sap from eggplant and *S. torvum* was extracted as described by Gardner *et al.* (1982). Stem pieces (approx. 25 cm length) of samples were surface sterilized by dipping in 80% ethanol and flaming and rinsing thrice in sterile water. Five cm pieces from the end of the stem were cut with a sterile blade. The epidermis and cortex layers were removed aseptically from each ends. The de-corticated ends were again dipped in 80% ethanol, flamed and washed thrice with sterile water. The stem was inserted through the flamed glass tubing in the rubber cork in such a way that one end remains out. To the other end, a sterile plastic tubing was attached to hold 500 μ L of 1 X PBS (Appendix A). The cork with plant sample attached was then fitted onto a Buchner flask (Fig. 3.3). For extraction of PBS through the xylem vessels a suction pressure 8 mbar was applied using a diaphragm pump MPC101Z (Ilmvac GmbH, Germany). A total of four successive infiltrations using 500 μ L of 1 X PBS were performed for each sample. The sap was collected directly in a sterile test tube placed inside the Buchner flask.

3.2.1.2.2. Maceration

Bacteria from the xylem of chilli and young eggplant samples with thin and soft stems were isolated by maceration of decorticated stem pieces (Gagné *et al.*, 1987). Briefly, stem pieces of 6- 8 cm length were surface sterilized by dipping in 0.1% mercuric chloride (w/v in distilled water) for 1 min and rinsed thrice in sterile water. One cm piece from each end was discarded. The epidermis and cortex from the surface sterilized stem piece were removed aseptically to expose the vascular bundles. The decorticated pieces were macerated in a sterile mortar and pestle using two mL of sterile 1X PBS.

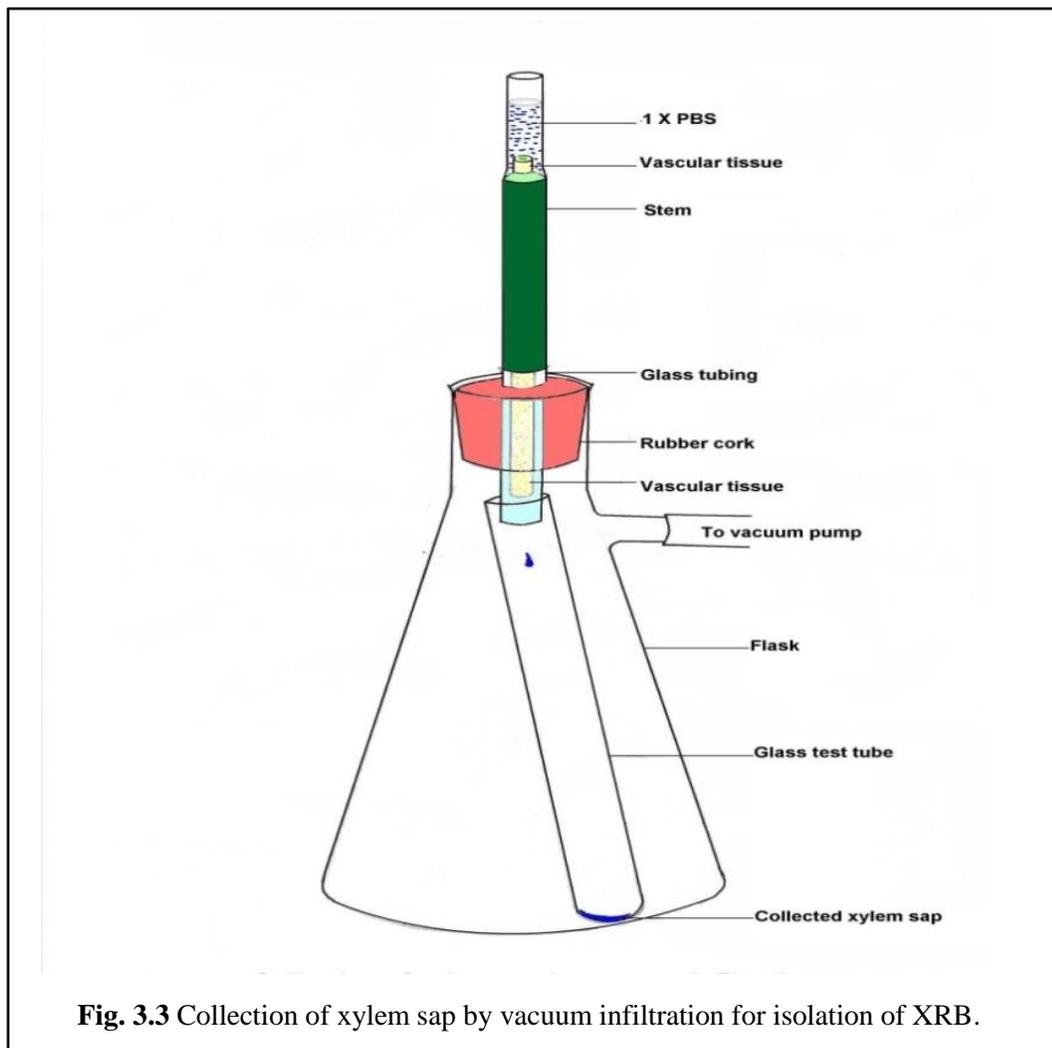


Fig. 3.3 Collection of xylem sap by vacuum infiltration for isolation of XRB.

3.2.1.2.3. Plating of xylem sap and macerate

One hundred microlitres of the vacuum in-filtered sap or macerate was plated onto Tryptic Soy agar (TSA) or medium 523 (Appendix A) (Viss *et al.*, 1991). The plates were incubated at $28 \pm 2^\circ\text{C}$ for 5 days. Sterility checks were performed by plating 100 μL of wash water from the last rinse used during surface sterilization on TSA plates. If contaminants were detected in the sterility check plates after incubation of one week, isolates from the respective extractions were not maintained. Different colonies from isolation plates were selected based on differences in their shape, color and texture and purified onto TSA or medium 523. Pure cultures of the XRB were maintained at -80°C in 25% glycerol for long term and 4°C for temporary storage. The xylem sap and macerate

were preserved at -80°C for subsequent analysis by culture independent method. Selected number of XRB used for different experiments presented herein and their selection criteria is presented in Table E1 in Appendix E.

3.2.2. Diversity analysis of XRB

3.2.2.1. Diversity analyses of XRB by Amplified rDNA Restriction Analysis

3.2.2.1.1. Isolation of genomic DNA from XRB

Isolation of genomic DNA from XRB was performed as described by Wilson, 1997. Buffers and reagents used for DNA isolation were prepared as given in Appendix C. Single isolated colony of XRB was inoculated in 5 mL King's B (KB) broth (Appendix A) and incubated at $28 \pm 2^{\circ}\text{C}$ for 24 to 48 h with constant shaking at 140 rpm. In each 1.5 mL centrifuge tube, one mL culture was centrifuged at 10000 rpm at 4°C for 2 min. The supernatant was discarded and the pellet was re-suspended in one mL sterile distilled water followed by centrifugation at 10000 rpm for 2 min at 4°C . The supernatant was discarded and the pellet was re-suspended in 564 μL of 1X TE buffer and 30 μL of 10% SDS and 6 μL of proteinase K ($10 \text{ mg}\cdot\text{mL}^{-1}$), contents were mixed and incubated at 37°C for 1h in the water bath. To the viscous solution, 100 μL of 5M NaCl was added and the contents were mixed thoroughly. Immediately 80 μL CTAB: NaCl solution (pre-warmed to 65°C) was added and contents were mixed thoroughly. The tubes were incubated for 10 min at 65°C in a water bath. Approximately an equal volume (0.7-0.8 mL) of chloroform: isoamyl-alcohol (24:1) was added and mixed thoroughly; the contents were centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred into a new micro centrifuge tube and equal volumes of phenol: chloroform: isoamyl-alcohol (25:24:1) was added to the aqueous layer and was centrifuged at 10000 rpm for 10 min. The aqueous layer was carefully pipetted and was transferred into a new micro centrifuge tube to

which 0.6 volumes of isopropanol (Merck, Germany) was added to precipitate the nucleic acid and incubated at room temperature (RT) after mixing thoroughly until a stringy white DNA precipitate was visible. Tubes were centrifuged to obtain the DNA pellet. The supernatant was discarded and the pellet was washed with 70% ethanol to remove any residual CTAB and the mixture was centrifuged at 10000 rpm at RT. The supernatant was drained off and the pellet was incubated at 60°C in a water bath until dry. Finally, the pellet was dissolved in 100 µL of 1X TE buffer containing 20 µg.mL⁻¹ RNase and incubated at 37°C for 30 min. DNA was stored at -20°C. Quality and quantity of the DNA was measured using Nanodrop 1000 (Thermo Scientific, USA) as per manufacturer's instructions in the user manual. The DNA samples were diluted to a concentration of 50 ng.µL⁻¹, stored at -20°C and used for subsequent PCR amplifications.

3.2.2.1.2. Agarose Gel Electrophoresis of DNA

Agarose gel electrophoretic analysis of DNA (Genomic DNA and PCR amplified DNA) was performed using horizontal midi submarine electrophoresis unit (Tarson Products Pvt. Ltd., India), with 10 x 16 cm gel tray. Trays were assembled in gel caster as per the manufacturer's instructions. Agarose (0.8% agarose with 0.5 µg.mL⁻¹ ethidium bromide) was prepared and run in 1X TAE buffer (Appendix C). Genomic DNA samples (5 µl) or PCR amplified product (10 µl) were mixed with 6X DNA loading buffer (Thermo Scientific, USA) and loaded into the wells of agarose gel submerged in 1X TAE running buffer. Appropriate DNA markers (viz. 100 bps and 5 kb, Thermo Scientific, USA) were also loaded on agarose gels in parallel wells with the DNA samples to determine the size of the DNA fragment (Sambrook and Russell, 2001). Electrophoresis was performed at 65 V until the dye front travelled 2/3 rd of the agarose gel. The gel was viewed and photographed using a gel documentation system (Alpha-Innotech Inc., USA).

3.2.2.1.3. Amplification of 16S rRNA gene fragment

16S rRNA gene from the XRB was amplified using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Twenty μL reaction mix contained 1X PCR buffer, 0.75 Units of Taq DNA polymerase (Sigma Aldrich, USA), 200 μM dNTPs (Sigma Aldrich, USA), 0.5 μM each primer (Chromous Biotech, Bangalore, India) and 50 $\text{ng}\cdot\mu\text{L}^{-1}$ of genomic DNA. Amplifications were carried out in Eppendorf Mastercycler Pro Thermal cycler (Eppendorf, Germany). Amplification cycle included a denaturation step of 94°C for 5 min followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 40 s and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The amplification of the 1500 bp PCR product was determined by agarose gel electrophoresis using 0.8 % agarose gel in 1 X TAE buffer as described above.

3.2.2.1.4. Restriction digestion of 16S rRNA gene fragments using *MspI* and *AluI*

Fifteen microlitres PCR product amplified as described above was digested separately with one unit of *MspI* and *AluI* (Thermo Scientific, USA) (Barzanti *et al.*, 2007) for 4 h at 37°C in 1 X tango buffer (Thermo Scientific, USA). Restriction fragments were separated by electrophoresis on a 2 % agarose gel in 1X TAE buffer containing 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide at 60 V for 2 h. Gel was documented using Alpha Imager (Alpha Innotech Inc., USA) as per manufacturer's instructions.

3.2.2.1.5. Analysis of ARDRA fingerprint

ARDRA restriction fingerprints were compared visually, scored manually as 1 for presence and 0 for absence of fragment and the binary data was entered in the NT Edit

software version 1.1 b (Applied Biostatistics Inc. USA). The similarity matrix derived using the binary data of ARDRA restriction fragment was subjected to cluster analysis using Un-weighted Pair Group Method for Arithmetic Average (UPGMA) using Dice coefficient in the NTSYSpc 2.02i software (Applied Biostatistics Inc. USA). Subsequent to analysis several clusters were obtained. Each cluster consisted of XRB having an identical restriction fragment profile. Strains having very unique restriction profile remained separable as independent clusters. Clusters obtained at 80 % similarity values of Dice coefficient were denoted as haplotypes. Haplotypes obtained using *AluI* were numbered as A80-1 to A80-46 whereas those obtained using *MspI* were numbered from M80-1 to M80-38.

3.2.2.2. Diversity analyses of XRB using culture independent method PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

3.2.2.2.1. Amplification of 16S rRNA gene from xylem sap samples

Xylem sap extracted from 13 eggplant samples and 2 chilli samples were analyzed by PCR-DGGE (Table 3.3). The 16S rRNA gene was amplified directly from the sap sample. About one mL xylem sap sample was centrifuged at 13000 rpm for 10 min at RT. The supernatant was discarded and the pellet was re-suspended in 10 µL of distilled water. Three µL suspension was used directly for amplification of 16S rRNA gene from the bacteria in the sap using GC clamped primers F968_LGC (5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-AACGCGAAGAACCTTAC-3') and R1378 (5'-CGGTGTGTACAAGGCCCGGGAACG-3') (Araújo *et al.*, 2002). PCR reaction mix for amplification of 16S rRNA gene from xylem sap contained 200 µM dNTPs, 1 %

formamide, 0.4 μ M each primer, 1 unit of Taq DNA polymerase, 1 X Taq buffer (Sigma Aldrich, USA) and three μ L xylem sap as template in a final volume of 50 μ L. PCR reactions were performed in Eppendorf master cycle pro (Eppendorf, Germany) using the programme described by Garbeva *et al.* (2001). The cycling program consisted of initial denaturation at 94°C for 4 min, two cycles of: 1 min at 94°C, 1 min at 60°C and 2 min at 72°C followed by 10 runs of the same cycle with every one degree decline in annealing temperature to 55°C, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension at 72°C for 10 min. After PCR, 450 bp fragment of the 16S rRNA gene was expected and its amplification was analyzed by running PCR products on 1% agarose gel in 1 X TAE at 70 volts for 1 hour. Fragment of 450 bp was excised from the gel and purified using GeneJet™ gel extraction kit as per the manufacturer's instructions (Thermo Scientific, USA) and used for DGGE.

Table 3.3 Xylem sap samples used for PCR-DGGE analysis of uncultured XRB.

Xylem sap sample	Host plant
XS8	Local eggplant
XS9	Local eggplant
XS10	Local chilli
XS11	Local eggplant
XS12	Local eggplant
XS13	Local eggplant
XS31	Local eggplant
XS34	Local eggplant
XS36	Local chilli
XS38	Eggplant cv. <i>Arka Keshav</i>
XS39	Eggplant cv. <i>Arka Anand</i>
XS40	Eggplant cv. <i>Arka Kusumakar</i>
XS42	Eggplant cv. <i>Agassaim</i> x <i>Surya</i> No. 13
XS43	Eggplant cv. <i>Agassaim</i> x <i>Surya</i> No. 14
XS44	Eggplant cv. <i>Agassaim</i> x <i>Surya</i> No. 20
XS47	Eggplant cv. <i>Agassaim</i> x <i>Surya</i> No. 12

3.2.2.2.2. Casting and running the gel

DGGE gels were run in Ingeny phorU unit (Ingeny, Leiden, Netherlands). Glass plates, spacer and combs were assembled as per the manufacturer's instruction. Reagents and gel recipes are given in Appendix C. Acrylamide solution containing high and low denaturant solutions were prepared and poured in separate compartments of the gradient maker provided with the unit and placed on a magnetic stirrer for casting the gel. The resolving gel consisted of 8% polyacrylamide and a gradient of 20%- 80% denaturant (100 % denaturant consisted of 7 M urea and 40% formamide). Five mL of water was added over the casted gel immediately and was allowed to polymerize for one hour. Ten mL of stacking gel solution containing 5% polyacrylamide (no denaturant) was added over the resolving gel and allowed to polymerize for one hour after placing the comb. After the stacking gel polymerized the gel assembly was then placed in the tank filled with 0.5 X TAE Buffer pre-warmed to 65°C. Twenty µL of 16S RNA gene amplified using GC clamed primers was loaded in each well. The gel was run at 70 V for one hour at 65°C. Later the current was increased to 170 volts and the gel was allowed to run for 22 h at 65°C. After the run was complete, the gel was removed from plates and placed in fixer solution for 5 min on plate rocker. The gel was immediately transferred in impregnation solution and incubated for 6-7 min. The gel was rinsed with deionized water for 1 min and incubated in developer solution for 3 -5 min. The gel was then incubated in stop solution for 2 min, rinsed and photographed under white light using Alpha Imager (Alpha Innotech Inc., USA) as per manufacturer's instructions. Random bands from six eggplant samples were then cut from the gel using sterile razor blade and preserved at -20°C for re-amplification of 16S rRNA gene and subsequent sequencing.

3.2.2.2.3. Elution and re-amplification of bands separated by DGGE

DGGE gel pieces containing DNA fragments (stored at -20°C) were thawed at room temperature and macerated in a sterile 1.5 mL centrifuge tube containing 50 µL of TE buffer pH 8, using a sterile Eppendorf tube pestle. The tubes were incubated at 37°C for 18 h. The tubes were centrifuged at 13000 rpm for 10 min at RT and supernatant was collected in a fresh tube. To the supernatant, five µL of 3 M Sodium acetate and 125 µL of 100% ice cold ethanol was added, mixed well and incubated at -20°C for 2 h to allow efficient re-precipitation of eluted DNA. The tubes were centrifuged at 13000 rpm for 10 min at 4°C, supernatant was discarded and the pellet was rinsed with 70% ethanol. The tubes were centrifuged at 13000 rpm for 10 min at 4°C. The pellet was dried and dissolved in ten µL sterile 1X TE buffer, pH 8. Three µL eluate was used in PCR for re-amplification. PCR was performed using primers F968_{LGC} and R1378 as described in section 3.2.2.2.1. A piece of excised gel containing no DNA fragment served as control to ensure no contaminating DNA was eluted from the gel.

3.2.2.2.4. Retrieval of sequence information of DNA fragments eluted from DGGE gel for identification of uncultured bacteria

Six re-amplified fragments were resolved on a 1% agarose gel run in 1 X TAE buffer at 70 V for 1 hour. The bands were excised and purified using GeneJET™ Gel extraction kit (Thermo Scientific, USA) following manufacturer's instructions. The purified band was sequenced using primer R1378 (Xcelris Labs Pvt. Ltd., India). Partial 16S rRNA gene sequences (about 400 nucleotides) obtained were matched against the sequences available in the nucleotide database from National Center for Biotechnology Information

(www.ncbi.nlm.nih.gov/BLAST) using the BLASTn (Basic Local Alignment Search Tool) program.

3.2.3. Characterization and identification of XRB

3.2.3.1. Antagonism of XRB towards *R. solanacearum* assessed by agar cup bioassay

Antagonism towards *R. solanacearum* was the most important criteria for choosing XRB for further characterization. In order to assess the inhibition of *R. solanacearum* by XRB, bioassay was performed by the agar cup method. XRB (n=167) were screened for inhibition of the BW pathogen *R. solanacearum*. Virulent strains of *R. solanacearum* isolated from BW infected eggplant cultivated in Goa, India varying in their pathogenicity on eggplant cv. *Agassaim* were chosen from culture collection of Plant Pathology Laboratory, ICAR Research Complex for Goa. *R. solanacearum* strains Rs-08-17 (highly virulent), Rs-09-100 (moderately virulent) and Rs-09-109 (mildly virulent) were selected for the bioassay. Agar cup bioassay was performed as described by Ramesh and Phadke (2012). Briefly, single colony of *R. solanacearum* strains and XRB was grown in 5 mL CPG broth and KB broth (Appendix A) respectively at $28 \pm 2^\circ\text{C}$ for 48 h with constant shaking at 140 rpm. 150 μL *R. solanacearum* culture was seeded every 100 mL molten cooled KB agar, mixed well and poured into plates. After the plates solidified, three wells were made in each plate by removing a circular agar piece with the help of cork borer (8 mm diameter). Twenty five μL of culture broth of XRB containing $10 \text{ Log CFU.mL}^{-1}$ was added into each of the three wells. All the plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Plates were observed for inhibition of *R. solanacearum*. Zones of inhibition were measured as radius in mm from the edge of the agar well. Strains that were found

antagonistic to *R. solanacearum* were characterized and identified by biochemical tests and 16S rRNA gene sequencing as described in following sections.

3.2.3.2. Morphological and biochemical characterization of XRB for identification

Morphological characterization of antagonistic XRB (n=28) was done following the method of Cruickshank *et al.* (1972). Isolates were Gram stained (Appendix B) and observed under the light microscope at 100 X magnifications to study the cell morphology using Olympus CX41 microscope (Olympus, Japan). The colony characteristics of bacterial isolates *viz.* size, shape, colour, margin, elevation, consistency and opacity were observed and recorded. Colonies grown on KB agar plates were also observed and photographed at 10X magnification using stereo-zoom microscope SZ2-ILST (Olympus, Japan). Common biochemical tests (Appendix B) were performed and results were interpreted according to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) for presumptive identification of XRB.

3.2.3.3 16S rRNA gene sequencing and sequence analysis for identification of XRB

A total of 55 XRB were chosen for identification which included 38 representatives from each *MspI* haplotype and additional 17 strains which were antagonistic to *R. solanacearum*. Fragments of the 16S rRNA gene of size of 1500 bp were amplified as described above in the ARDRA section. Amplicons were purified using GeneJetTM PCR purification kit (Thermo Scientific, USA) as per manufacturer's instructions and sequenced using 27F and/or 1492R primers (Xcelris Labs Pvt. Ltd., India). Partial 16S rRNA gene sequences (ranging from 400 to 1200 nucleotides) obtained were matched against the sequences available in the nucleotide database from National Center for

Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/BLAST) using the BLASTn (Basic Local Alignment Search Tool) program. The sequences were deposited in the GenBank database of NCBI, USA.

3.2.3.4. Detection of extracellular hydrolytic enzymes produced by XRB

3.2.3.4.1. Amylase

Amylase activities of antagonistic XRB were determined as described by Choi *et al.* (2005). XRB were spot inoculated on starch agar plates (Appendix A). Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h and flooded with lugol's iodine (Appendix A). Clear yellowish halo on a brown background around the point where the XRB were inoculated indicated production of amylase.

3.2.3.4.2. Caseinase

Antagonistic XRB were spot inoculated on skim milk agar plates (Appendix A). Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h. Zone of clearance around the point where the XRB were inoculated indicated production of caseinase (Bell *et al.*, 1995).

3.2.3.4.3. Endoglucanase

Antagonistic XRB were spot inoculated on agar medium containing carboxy methyl cellulose (Appendix A). Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h. Visualization of endoglucanase activity was done by staining the plates with 0.1% congo red solution for 15 min followed by destaining with 1 M NaCl solution (Appendix A). Yellowish halos on a red background around the point where the XRB were inoculated, indicated degradation of cellulose (Compant *et al.*, 2005b).

3.2.3.4.4. Chitinase

For testing chitinase activities of XRB colloidal chitin was prepared from commercial chitin as described by Hsu and Lockwood (1975). Antagonistic endophytes were spot inoculated on mineral salts medium (MSM) containing 0.2% colloidal chitin (Appendix A). Plates were incubated at 28 ± 2 °C for 7 days. Zone of clearance around the point where the XRB were inoculated indicated production of chitinase.

3.2.3.4.5. Pectinase

Antagonistic XRB were spot inoculated on pectin agar medium (Appendix A). Plates were incubated at 28 ± 2 °C for 48 h. To visualize pectinase activity the plates were flooded with 2% CTAB solution and incubated for 30 min and washed with 1M NaCl Solution (Appendix A). Pectin degradation was observed as a hazy halo around the point where the XRB were inoculated.

3.2.3.4.6. Polygalactouronase

Antagonistic XRB were spot inoculated on medium containing polygalatouronic acid (Appendix A). Plates were incubated at 28 ± 2 °C for 48 h. Visualization of polygalactouronase activity was done by staining the plates with 0.1% ruethenium red solution for 15 min followed by destaining with 1 M NaCl solution. Polygalacturonase activity was determined by the appearance of intense purple-red halos on a colorless background at the point where the XRB were inoculated (Compant *et al.*, 2005b)

3.2.4. Plant growth promotion by xylem residing bacteria

3.2.4.1. Production of plant growth promoting substances

3.2.4.1.1. 1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity

Antagonistic XRB were tested for their ability to use ACC as sole source of nitrogen by production of enzyme ACC deaminase was performed as per the method described by Godinho *et al.* (2010). Strains were streaked on Dworkin and Foster's DF salts agar containing 3.0 mM ACC (Appendix A) and incubated at $28 \pm 2^\circ\text{C}$ for 7 days. Ability of the strains to grow on the medium containing ACC as a sole nitrogen source was indicative of ACC deaminase production.

3.2.4.1.2. Indole Acetic Acid (IAA) production

Antagonistic XRB were tested for their ability to produce phytohormone IAA in presence of tryptophan as described by Gordon and Paleg (1957). Briefly, strains were grown in nutrient broth amended with 100 mg.L^{-1} of tryptophan (Appendix A) for 30 h at $28 \pm 2^\circ\text{C}$ at 140 rpm. The supernatants were obtained by centrifugation at 8000 rpm for 10 min. One mL of supernatant was mixed with one mL of Salkowsky's reagent (Appendix A). The mixture was allowed to stand at RT for five min and the absorbance was read at 530 nm using UV-Visible spectrophotometer (Varian Cary 50, Agilent Technologies, USA). A standard curve was prepared using analytical grade IAA and the concentrations of IAA in the culture supernatants of XRB were estimated based on the curve.

3.2.4.1.3. Nitrogen fixation

Antagonistic XRB were tested for their ability to fix atmospheric nitrogen. Ten microliter of 48 h old culture of antagonistic bacteria grown in KB broth was inoculated in 5mL Dobereiner's Nitrogen free semisolid medium (Nfb) (Appendix A) (Eckert *et al.*, 2001).

Tubes were incubated at $28 \pm 2^{\circ}\text{C}$ for five days under stationary conditions. Ten μL of pellicle growth was transferred to fresh Nfb medium. This procedure was repeated seven times consecutively after five days incubation to ensure the ability of each pellicle forming XRB to grow in absence of nitrogen (Goes *et al.*, 2012). XRB able to a change the colour of medium to bluish green and form pellicle growth in Nfb after the incubation after the seventh transfer were considered putative diazotrophs.

3.2.4.1.4. Phosphate Solubilization

Antagonistic XRB were tested for phosphate solubilization by a method described by Godinho *et al.* (2010). XRB were spot inoculated on Pikovskaya's agar plates (Hi Media Laboratories, Mumbai). Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h. Clearance zones around the point where the XRB were inoculated on the opaque white medium were indicative of solubilization phosphate.

3.2.4.2. Enhancement of seedling vigour in eggplant

Ability of antagonistic XRB to enhance seedling vigour in eggplant was tested *in vitro*. Seeds of eggplant cv. *Agassaim* were surface sterilized as described by Ramesh *et al.* (2011). Single colony of antagonistic XRB was grown in KB broth for 48 h at $28 \pm 2^{\circ}\text{C}$ with constant shaking at 140 rpm. After incubation 100 μL culture was taken to assess population of XRB in culture broth by serial dilution and plating. One mL culture broth was centrifuged in sterile Eppendorf tube for 2 min at 8000 rpm at 4°C . The supernatant was discarded and the pellet was re-suspended in one mL 1 X PBS, spinned for 2 min at 8000 rpm at 4°C . The pellet was re-suspended in 1 X PBS. After surface disinfection, seeds were incubated in the freshly prepared suspension of antagonistic XRB (containing

approximately $10 \text{ Log}_{10} \text{ CFU.mL}^{-1}$). In the untreated control, seeds were incubated in sterile 1 X PBS for 18 h. Germination was carried out by the paper towel method. Twenty treated and untreated seeds were placed on separate paper towels moistened with distilled water, rolled and wrapped with polypropylene sheet to prevent drying and incubated at $28 \pm 2^\circ\text{C}$ in dark. Each treatment consisted of two replications and ten seeds per replication were used. On the seventh day, the towels were unrolled and the number of germinated seeds was counted. The lengths of roots and hypocotyls of the germinated seedlings were measured using a ruler. The seedling vigor index (SVI) was calculated using the formula $\text{SVI} = (\text{mean root length} + \text{mean hypocotyl length}) \times \% \text{ seeds germinated}$ (Long *et al.*, 2008). Strains exhibiting SVI greater than control were screened for growth promotion in eggplant under greenhouse conditions.

3.2.4.3. Growth promotion in eggplant by XRB under greenhouse conditions

Twenty three strains of XRB exhibiting SVI greater than control (average SVI of 577.17) were studied for their effect on growth in eggplant. Ability to increase shoot length in eggplant cv. *Agassaim* was used as a measure to evaluate their growth promotion efficacy under greenhouse conditions. Thirty day old seedlings raised in non-sterile soil in greenhouse were transplanted in pots filled with standard non sterile pot mixture (soil: sand: farm yard manure at 2: 1: 1 ratio). Single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Culture broth (200 μL) was transferred to 20 mL KB broth in flasks and further incubated at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm for 48 h. After incubation 100 μL culture was taken to assess population of XRB in culture broth by serial dilution and plating. Remaining culture was centrifuged at 8000 rpm for 10 min at 4°C . The pellet was washed once with sterile 1X

PBS and centrifuged at 8000 rpm for 10 min at 4°C. The pellet was then dissolved in 20 mL sterile 1X PBS and diluted to 200 mL with sterile 1X PBS. Ten mL suspension of XRB (containing approximately 8 Log CFU.mL⁻¹) was applied per seedling by soil drenching in pots in greenhouse. Soil drenching was done by gently pouring the suspension of XRB near the base of the seedling stem in small volumes at a time to allow percolation near the root. Each treatment consisted of two replicates with two pots per replication and five seedlings per pot. Plants were maintained with suitable watering and plant height was measured from the soil level to the shoot tip 40 days post inoculation of XRB. Ability of antagonistic XRB to increase shoot length in eggplant was expressed as growth promotion efficacy (GPE) using the formula given by Aliye *et al.* (2008) ([shoot length increase in treatment] – [shoot length increase in control]/shoot length increase in control) × 100.

3.2.5. Antibiotic susceptibility tests of XRB

Susceptibility of XRB to 14 different antibiotics was tested using discs (Hi Media Laboratories, Mumbai). Single colony of XRB was grown in 5 mL KB broth for 24 h at 28 ± 2°C with constant shaking at 140 rpm. Culture was serially diluted in sterile 1X PBS to contain approximately 8 Log CFU.mL⁻¹ (approximately a 0.5 McFarlands standard). 100 µL suspension was spread plated on KB agar plates. Antibiotic discs (Ampicillin 25µg.mL⁻¹, Amoxycillin 30 µg.mL⁻¹, Carbenicillin 100 µg.mL⁻¹, Penicillin G 10 µg.mL⁻¹, Cloxacillin 30 µg.mL⁻¹, Chloramphenicol 30 µg.mL⁻¹, Kanamycin 30 µg.mL⁻¹, Ciprofloxacin 30 µg.mL⁻¹, Gentamicin 30 µg.mL⁻¹, Streptomycin 10 µg.mL⁻¹, Amikacin 30 µg.mL⁻¹, Tetracycline 30 µg.mL⁻¹, Cephatoxime 30 µg.mL⁻¹ and Rifampicin 30 µg.mL⁻¹) were placed (7-8 discs of different antibiotic per plate) on the plates and

incubated at $28 \pm 2^\circ\text{C}$ for 24-48 h. Diameter of inhibition zones were measured from the edge of the disc to the edge of the zone. Resistance or susceptibility of the XRB tested was determined based on the diameter of inhibition zones as per the manufacturer's instructions.

3.3. RESULTS AND DISCUSSION

3.3.1. Isolation of XRB from eggplant, chilli and *S. torvum*

Bacteria could be constantly isolated from the xylem of eggplant, chilli and *S. torvum* (collected from nine talukas notable for solanaceous vegetable cultivation) by vacuum infiltration and maceration techniques. Bacterial counts in each isolation ranged on an average from 10 to 10^2 CFU.mL⁻¹ of xylem sap or macerate. Colonies appeared between 24 and 120 h of aerobic incubation at $28 \pm 2^\circ\text{C}$. A total of 206 bacteria were isolated by vacuum infiltration or maceration from a total of 45 eggplant, chilli and *S. torvum* samples. However, 39 strains failed to grow upon revival from glycerol stocks preserved at -80°C . Amongst 167 viable isolates, 99 were Gram negative rods (59.28 %) and 68 were Gram positive bacteria (40.72%) comprising of 42 rods (61.76 %), 25 cocci (36.76%) and one filamentous actinomycete were isolated (Fig. 3.4 and Table 3.4). Colony characteristics of select isolates are presented in Table 3.5. Gardner *et al.* (1982) and Bell *et al.* (1995) have earlier reported higher count of Gram-negative rod shaped bacteria from xylem of citrus and grapevine using vacuum infiltration. Scholander pressure bomb was also useful in extraction of diverse Gram positive and Gram negative bacterial genera from xylem tissues in contrast to trituration methods that yielded higher number of Gram-positive rod shaped bacteria (Hallmann *et al.*, 1997). Though, Scholander pressure bomb was not used in this study, a combination of vacuum

infiltration (Gardner *et al.*, 1982; Bell *et al.*, 1995) and trituration of decorticated stems (Gagné *et al.*, 1987; Hallmann *et al.*, 1997) was employed with an aim to isolate diverse XRB from xylem tissues of eggplant, chilli, and *S. torvum*. Loss of viability of endophytic bacteria upon preservation at -80°C has been documented earlier (Bell *et al.*, 1995; Long *et al.*, 2010). Several researchers have reported that cultured bacteria may enter viable but non culturable state under stressful conditions such as freezing (Oliver, 2005; Vartoukian *et al.*, 2010) which may make resuscitation difficult.

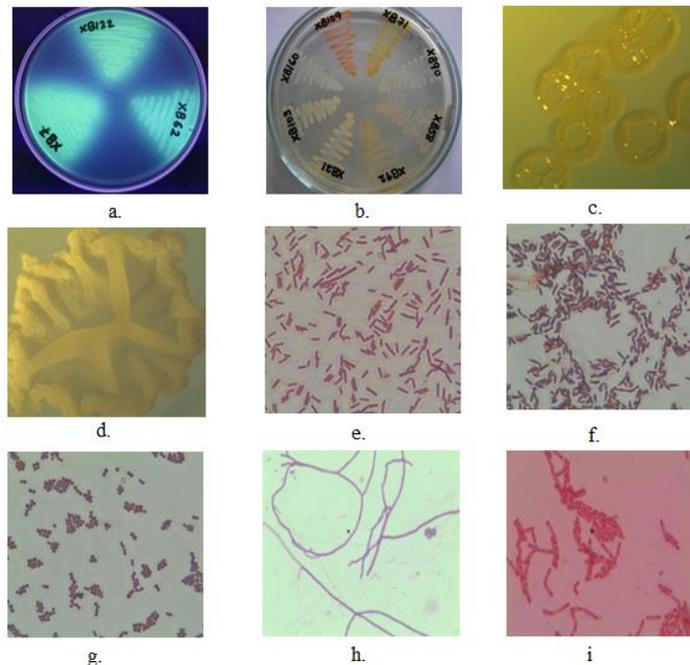


Fig. 3.4 Morphological characteristics and Gram staining of XRB. a. Fluorescence under UV, b. Pigmentation in XRB, c. and d. Colony of XB114 and XB153 grown on KB agar and observed under stereo-zoom microscope (Olympus, Japan) at 10X magnification, e. Gram stain of XB114, f. Gram stain of XB153. g. Gram stain of XB169, h. Gram stain of XB200, i. Spore stain of XB177.

Table 3.4 Isolation of XRB from eggplant, chilli and *S. torvum*.

Plant Host	No. of samples	Total isolates	Gram Negative	Gram Positive	Method of isolation
Eggplant (BW susceptible)	24	89	53	36	VI or M
Eggplant (BW resistant)	12	36	20	16	VI or M
Chilli	7	33	20	13	M only
<i>Solanum torvum</i>	2	9	6	3	VI only
Total	45	167	99	68	

VI: Vacuum infiltration, M: Maceration

Table 3.5 Colony characteristics of select XRB.

(Antagonistic XRB, representatives from each *MspI* haplotypes and strains with quorum quenching activities)

Strain	Col.Size	Shape	Color	Marg	Elev	Surf	Cons	OpCh	Gram Stain	Morp
XB1	1	Circular	Cream	Entire	Convex	Glistening	Smooth	Opaque	-	Long rods
XB7	3	Circular	White	Entire	Umbonate	Glistening	Smooth	Translucent	-	Long rods
XB8	1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci in tetrads
XB19	1	Circular	Yellow	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci in tetrads
XB20	1	Circular	White	Entire	Flat	Semi Shiny	Smooth	Opaque	+	Cocci in clusters
XB22	<1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Rods
XB25	<1	Circular	Yellow	Entire	Convex	Glistening	Smooth	Opaque	-	Rods
XB27	1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci in clusters
XB34	1	Circular	Off white	Entire	Convex	Glistening	Smooth	Translucent	+	Short rods
XB35	1	Circular	Off white	Entire	Convex	Glistening	Smooth	Translucent	+	Very short rods
XB36	<1	Circular	Light yellow	Entire	Convex	Glistening	Smooth	Opaque	-	Thin rods
XB37	2	Circular	Yellow	Entire	Convex	Semi Shiny	Smooth	Opaque	-	Short rods
XB40	2	Circular	Light yellow	Entire	Convex	Semi Shiny	Smooth	Opaque	+	Cocci in tetrads
XB41	2	Circular	Off white	Entire	Flat	Glistening	Smooth	Opaque	+	Rods
XB47	1	Circular	Brown	Entire	Flat	Glistening	Smooth	Translucent	-	Thin rods
XB53	1	Circular	Light yellow	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci in tetrads
XB62	1-2	Circular	White	Entire	Flat	Glistening	Slimy	Opaque	-	Short thick rods
XB64	4	Irregular	White	Wavy	Flate	Dull	Stringy	Opaque	+	Rods in chains
XB66	1	Scalloped	White	Wavy	Flat	Semi Shiny	Smooth	Translucent	-	Rods
XB70	<1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci
XB86	2	Circular	Cream	Entire	Convex	Glistening	Smooth	Opaque	-	Thin rods
XB87	2-3	Circular	White	Wavy	Raised	Dull	Smooth	Opaque	+	Rods in chains

Strain	Col.Size	Shape	Color	Marg	Elev	Surf	Cons	OpCh	Gram Stain	Morp
XB88	2	Oval	Light yellow	Wavy	Flat	Dull	Smooth	Opaque	+	Rods
XB92	<1	Circular	Red rusty	Entire	Convex	Semi shiny	Smooth	Translucent	-	Rods
XB93	2	Circular	White	Wavy	Raised	Dull	Smooth	Opaque	+	Thick rods
XB94	2	Oval	Dark yellow	Wavy	Flat	Semi shiny	Smooth	Opaque	+	Rods
XB98	1	Circular	White	Entire	Convex	Semi shiny	Smooth	Opaque	+	Curved rods
XB99	1-2	Circular	White	Entire	Flat	Glistening	Smooth	Opaque	-	Short rods
XB100	1-2	Circular	White	Entire	Flat	Glistening	Slimy	Opaque	-	Rods
XB102	2	Circular	Off white	Entire	Convex	Glistening	Butyrous	Opaque	-	2 mm
XB103	1	Circular	Yellow	Entire	Convex	Semi shiny	Smooth	Opaque	+	Thin rods
XB109	1	Circular	Organge	Entire	Convex	Glistening	Slimy	Opaque	+	Rods
XB114	1-2	Irregular	Light brown	Wavy	Raised border	Dull	Hard to pick	Opaque	-	Thick rods
XB115	1	Circular	Peach	Entire	Flat	Semi shiny	Smooth	Opaque	+	Rods
XB122	2- 3	Irregular	White	Rhizoidal	Flat	Dull	Butyrous	Opaque	-	Short rods
XB123	1-2	Circular	White	Entire	Flat	Dull	Butyrous	Opaque	-	Short thin rods
XB126	2	Circular	Yellowish	Entire	Convex	Semi shiny	Smooth	Translucent	-	Long rods
XB134	1	Circular	Off white	Entire	Flat	Dull	Butyrous	Opaque	-	Short rods
XB137	2-3	Circular	White	Entire	Flat	Semi shiny	Butyrous	Opaque	-	Rods
XB140	1	Circular	white	Entire	Convex	Glistening	Butyrous	Opaque	-	Short rods
XB153	2-3	Oval	white	Irregular	Scalloped	Dull	Sticky	Opaque	+	Thick rods
XB157	2	Circular	White	Entire	Drop like	Glistening	Slimy	Opaque	+	Rods
XB158	1	Circular	Off white	Entire	Convex	Glistening	Smooth	Translucent	-	Thin rods
XB159	<1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Thin rods
XB161	<1	Circular	Off white	Entire	Convex	Glistening	Smooth	Translucent	+	Short rods
XB165	1	Circular	White	Entire	Convex	Glistening	Smooth	Translucent	-	Short rods
XB167	<1	Circular	Yellowish	Entire	Convex	Glistening	Slimy	Translucent	+	Short rods

Strain	Col.Size	Shape	Color	Marg	Elev	Surf	Cons	OpCh	Gram Stain	Morp
XB168	2	Circular	Yellow	Entire	Convex	Glistening	Smooth	Opaque	+	Rods in chains
XB169	1	Irregular	White	Rhizoidal	Crumpled	Dull	Smooth	Opaque	+	Cocci in clusters
XB170	<1	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	+	Cocci in clusters
XB174	1-2	Circular	White	Entire	Flat	Semi shiny	Stringy	Opaque	-	Thick rods
XB177	4	Irregular	White	Rough	Flat	Semi shiny	Smooth	Opaque	+	Rods in chains
XB179	1	Circular	Yellow	Entire	Umbonate	Dull	Smooth	Opaque	+	Cocci in tetrads
XB188	<1	Circular	Yellow	Entire	Convex	Glistening	Smooth	translucent	-	Rods
XB190	1	Circular	Off white	Entire	Convex	Glistening	Smooth	Opaque	+	Rods
XB196	1-2	Circular	White	Entire	Convex	Glistening	Smooth	Opaque	-	Rods
XB197	1-2	Circular	Off white	Irregular	Wavy	Dull	Smooth	Opaque	-	Rods
XB200	2	Circular	Off white	Entire	Convex	Dull	Hard to pick	Opaque	+	Filamentous
XB202	2	Irregular	Yellow	Wavy	Crumpled	Glistening	Smooth	Opaque	-	Rods
XB203	1	Circular	Yellow	Entire	Convex	Glistening	Smooth	Translucent	-	Thin rods

Col. Size- Colony size, Marg-Margin, Elev-Elevation, Surf-Surface, Cons-Consistency, OpCh- Optical characteristics, Morp-Morphology.

3.3.2. Diversity analysis of XRB

3.3.2.1. Determination of diversity of XRB by ARDRA

Genomic DNA isolated from XRB yielded a distinct band when analyzed by agarose gel electrophoresis (Fig. 3.5). Genomic DNA was used as template for PCR amplification of 16S rRNA gene (Fig. 3.6) and restriction digestion for diversity analysis by ARDRA. ARDRA using *MspI* and *AluI* generated three to six restriction fragments of the 16S rRNA gene on resolving on 2% agarose gel (Fig. 3.7a and 3.7b). Analysis of ARDRA profiles by UPGMA using Dice's coefficient divided XRB with identical restriction profiles into several groups. At 80% similarity values of Dice coefficient in the dendrogram of ARDRA with *MspI* (Fig. 3.8), 167 strains of XRB were grouped into 38 haplotypes. A detailed representation of the ARDRA based analysis of 167 strains using *MspI* is presented in Table 3.6. Based on the ARDRA analysis of the collection of XRB, 153 strains were distributed over 24 different haplotypes and 14 XRB strains had unique profiles which formed 14 independent haplotypes with one strain in each. Antagonistic strains ($n = 28$) were distributed over 14 different ARDRA haplotypes wherein 6 antagonists formed independent haplotypes. Non antagonistic strains were distributed over 24 haplotypes. Haplotypes M80-9 and M80-15 were shared amongst BW susceptible and resistant eggplant, chilli, and *S. torvum*. Eleven haplotypes were unique to BW susceptible eggplant. Haplotypes M80-1, M8-12, and M80- 38 were unique to BW resistant eggplant whereas haplotypes M80-13, M80-19, and M80-26 comprised of strains isolated from chilli. Haplotype M80-36 had a strain isolated from *S. torvum*. Other haplotypes were a combination of strains isolated from different plant species.

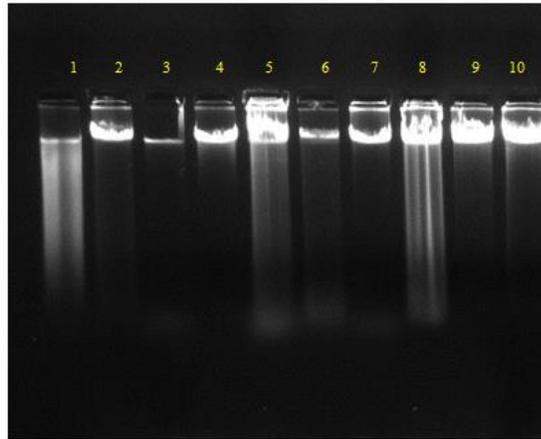


Fig. 3.5 Agarose gel electrophoresis of genomic DNA isolated from XRB. Lane 1. XB1, Lane 2. XB7, Lane 3. XB86, Lane 4. XB99, Lane 5. XB122, Lane 6. XB123, Lane 7. XB165, Lane 8. XB177, Lane 9. XB102, Lane 10. *E. coli* DH5 α .

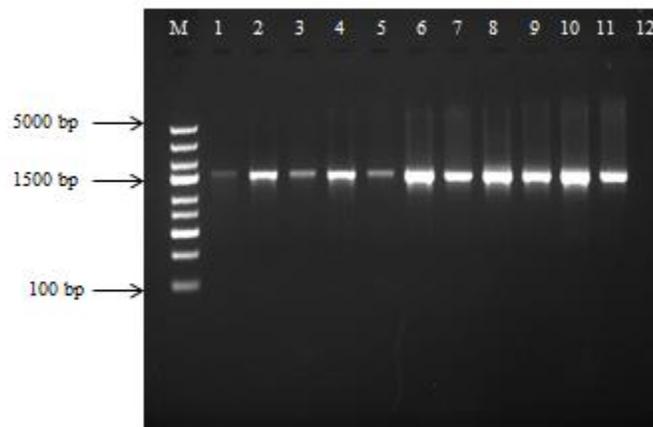
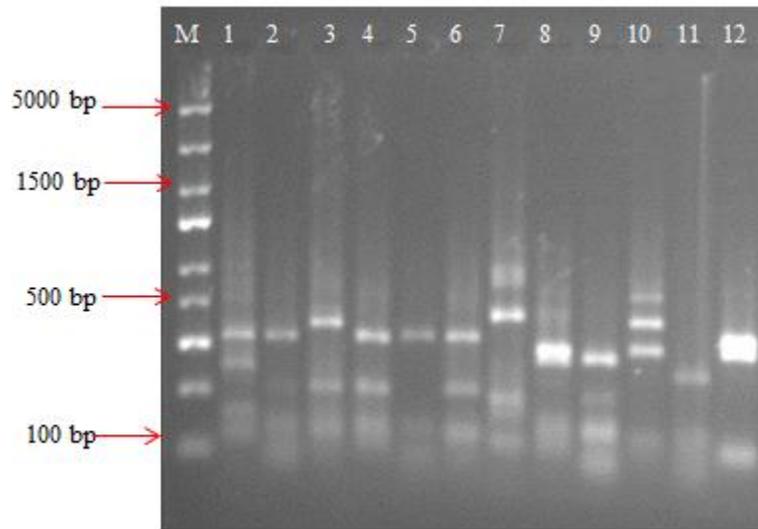
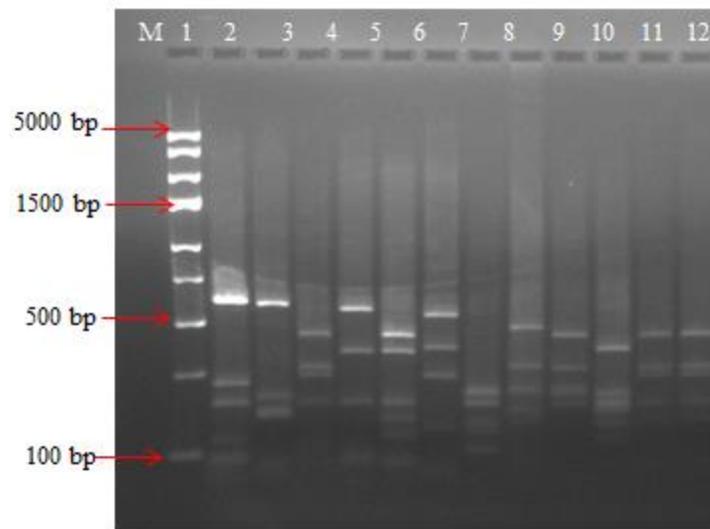


Fig. 3.6 Agarose gel electrophoresis of PCR amplified fragments of 16S rRNA gene from XRB. M: 100 bp to 5Kb ladder. 5 μ L PCR products loaded each lane. Lane 1. XB1, Lane 2. XB7, Lane 3. XB86, Lane 4. XB99, Lane 5. XB122, Lane 6. XB123, Lane 7. XB165, Lane 8. XB177, Lane 9. XB102, Lane 10. XB109, Lane 11. *E. coli* DH5 α , Lane 12: PCR negative control.



(a)



(b)

Fig. 3.7 ARDRA profiles of XRB. a. ARDRA using enzyme *MspI*. Lane 1- XB48, Lane 2- XB50, Lane 3- XB51, Lane 4- XB52, Lane 5- XB53, Lane 6- XB54, Lane 7- XB55, Lane 8- XB56, Lane 9- XB58, Lane 10- XB60, Lane 11- XB61 and Lane 12- XB62, b. ARDRA using enzyme *AluI*. Lane 1- XB94, Lane 2- 95, Lane 3- XB98, Lane 4- XB100, Lane 5- XB102, Lane 6- XB103, Lane 7- XB104, Lane 8- XB108, Lane 9- XB109, Lane 10- XB110, Lane 11- XB111 and Lane 12- XB112.

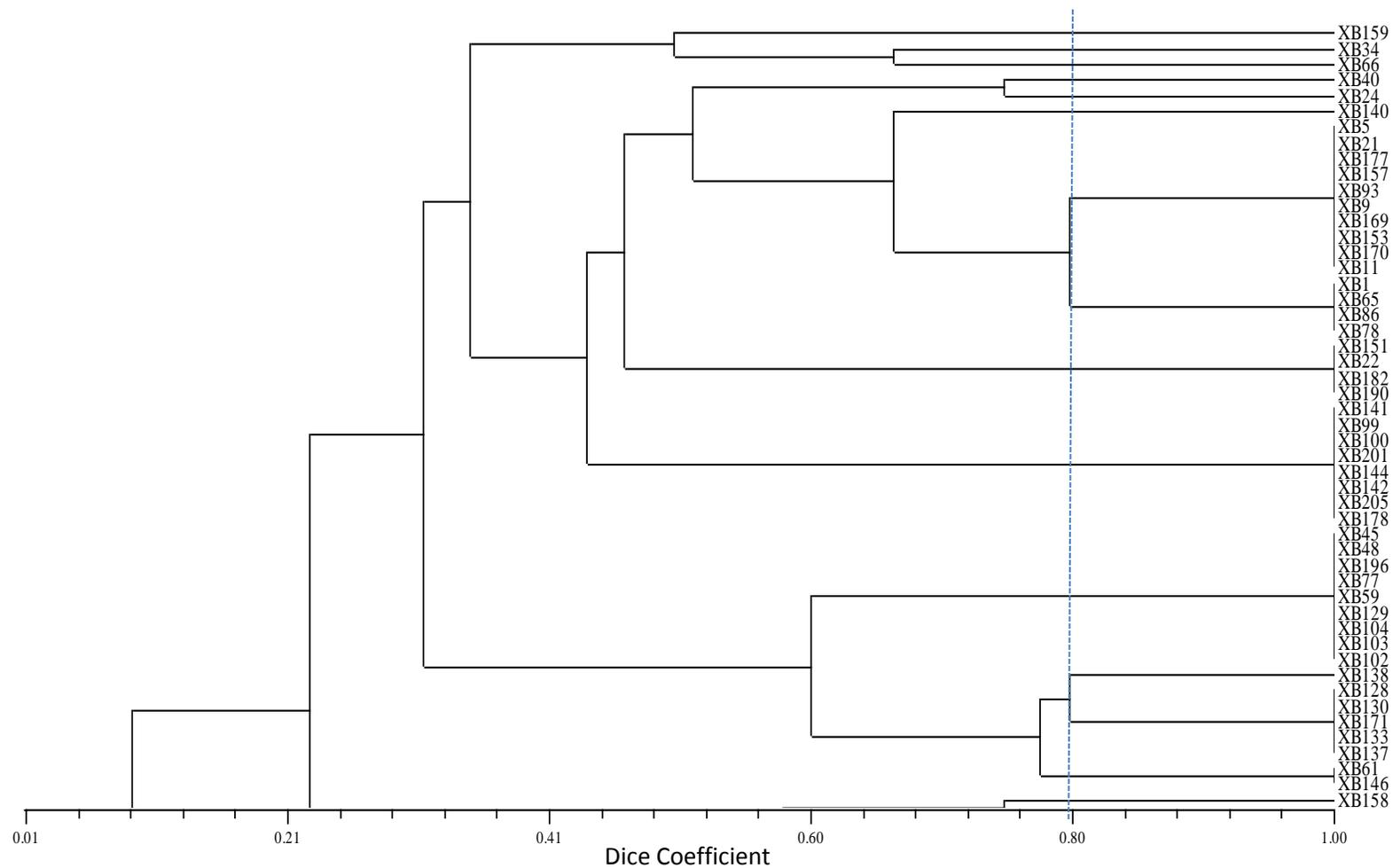


Fig. 3.8 Clustering of XRB by ARDRA using restriction enzyme *MspI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

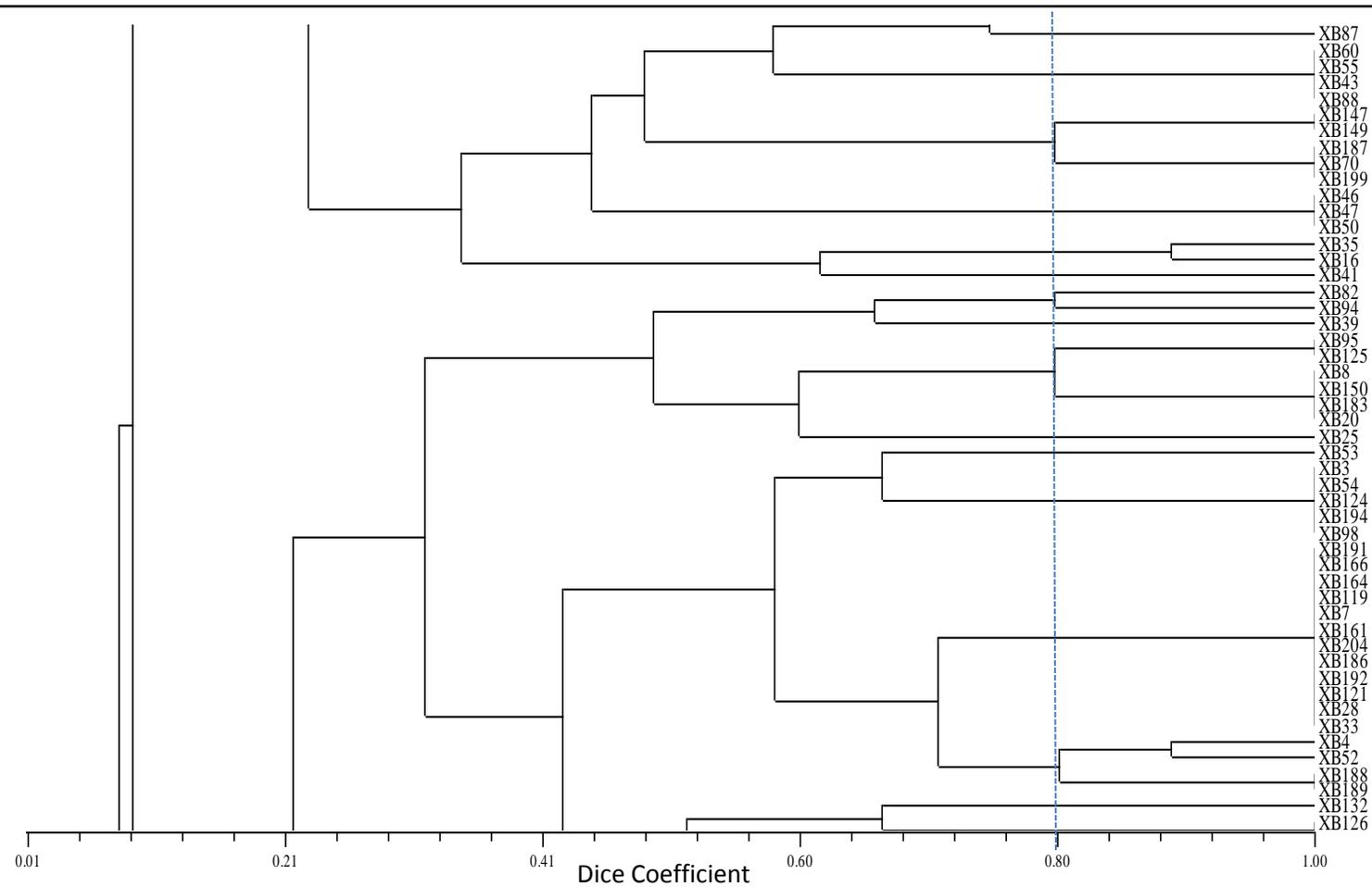


Fig. 3.8 contd. Clustering of XRB by ARDRA using restriction enzyme *MspI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

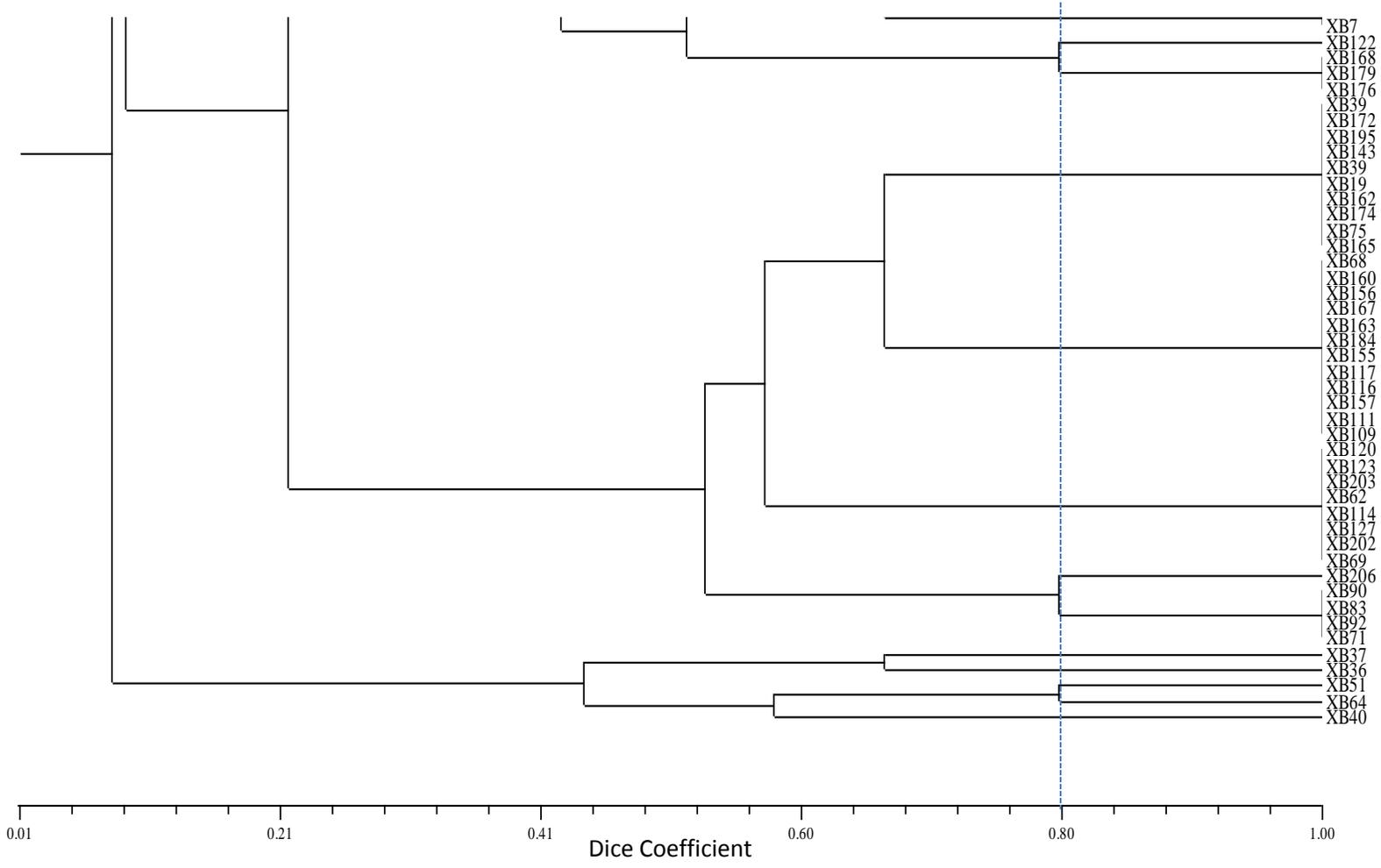


Fig. 3.8 contd. Clustering of XRB by ARDRA using restriction enzyme *MspI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

Table 3.6 Haplotypes of XRB at 80% similarity based on dendrogram of ARDRA using *MspI*.

Haplotype number	Host plant	No. of strains in each haplotype	Strains selected for identification
M80-1	RE	1	XB159
M80-2	SE	1	XB34
M80-3	SE	1	XB66
M80-4	SE, C	2	XB40
M80-5*	SE	1	XB140
M80-6*	SE, RE, C	10	XB177, XB157, XB93, XB169, XB153, XB170
M80-7*	SE, RE, C	6	XB1, XB86
M80-8	SE, RE	4	XB22, XB190
M80-9*	SE, RE, ST, C	8	XB99, XB100
M80-10*	SE, RE, C	10	XB196, XB103
M80-11	SE, RE, C	9	XB137
M80-12	RE	1	XB158
M80-13	C	1	XB87
M80-14	SE, C	5	XB88
M80-15*	SE, RE, ST, C	5	XB70
M80-16	SE	3	XB47
M80-17	SE	2	XB35
M80-18	SE	1	XB41
M80-19	C	2	XB94
M80-20*	SE, RE, C	13	XB8, XB20, XB27
M80-21	SE, ST, C	5	XB25
M80-22	SE	1	XB53
M80-23	SE, RE, C	5	XB98
M80-24	SE, RE, ST	11	XB161
M80-25	SE, RE	4	XB188
M80-26	C	1	XB168
M80-27*	SE, C	2	XB126, XB7
M80-28*	SE, RE	5	XB122
M80-29*	SE, RE, C	9	XB165
M80-30	SE, RE, C	15	XB167, XB109
M80-31*	SE, ST, C	11	XB123, XB203, XB62, XB114, XB202
M80-32	SE, ST, C	5	XB92
M80-33	SE	1	XB37
M80-34	SE	1	XB36
M80-35	SE	2	XB64
M80-36*	ST	1	XB200
M80-37*	SE	1	XB134
M80-38*	RE	1	XB197

* indicates haplotype comprises of XRB showing antagonistic property in the bioassays, SE: Bacterial Wilt susceptible eggplant, RE: Bacterial Wilt resistant eggplant, ST: *Solanum torvum*, C: Chilli plant.

At 80% similarity values of Dice coefficient in the dendrogram of ARDRA with *AluI*, 167 strains of XRB were grouped into 46 haplotypes (Fig. 3.9). A detailed representation of the ARDRA based analysis of 167 strains using *AluI* is presented in Table 3.7. Based on the ARDRA analysis of the collection of XRB, 147 strains were distributed over 26 different haplotypes and 20 XRB strains had unique profiles which formed 20 independent haplotypes with one strain in each. Antagonistic strains ($n = 28$) were distributed over 13 different ARDRA haplotypes wherein 5 antagonists formed independent haplotypes. Non antagonistic strains were distributed over 33 haplotypes. Haplotypes A80-2, A80-7, A80-15 and A80-24 were shared amongst BW susceptible and resistant eggplant, chilli, and *S. torvum*. Fifteen haplotypes were unique to BW susceptible eggplant. Haplotypes A80-5, A80-10, A80-14, A80-23, A80-29 and A80-31 were unique to BW resistant eggplant whereas haplotypes A80-18, A80-41 and A80-42 comprised of strains isolated from chilli. Haplotype M80-45 had a strain isolated from *S. torvum*. Other haplotypes were a combination of strains isolated from different plant species.

The results indicate that bacterial communities from the xylem of eggplant chilli and *S. torvum* cultivated in different locations in Goa comprise of diverse bacteria. The majority of haplotypes represent a blend of XRB isolated from different solanaceous plants from diverse locales. These results suggest that xylem of eggplant, chilli, and *S. torvum* largely possesses genetically similar population of XRB. Few strains having unique ARDRA fingerprint formed separable haplotypes. This observation hints an inference that only a minor population of xylem colonizing XRB is genetically distinct.

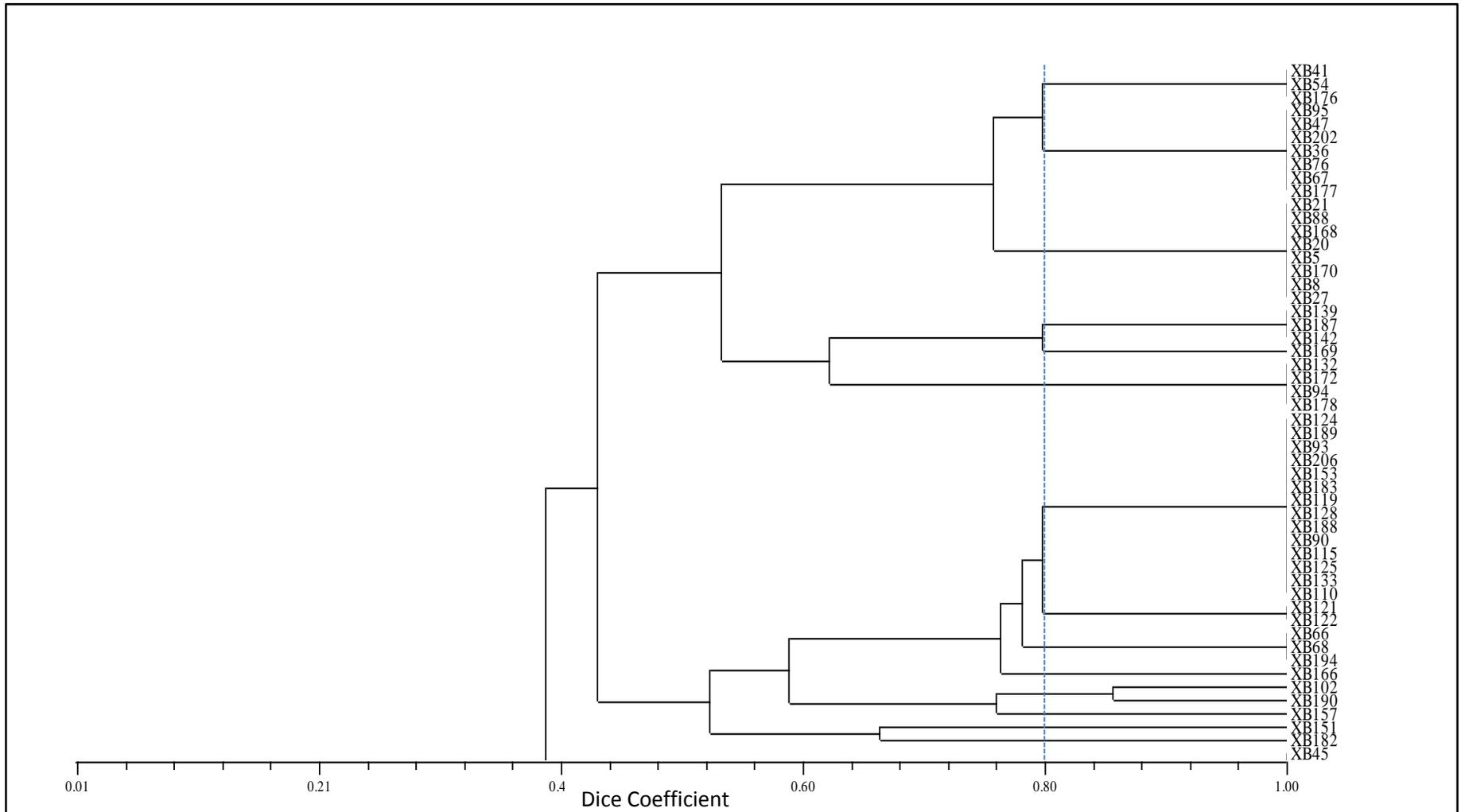


Fig. 3.9 Clustering of XRB by ARDRA using restriction enzyme *AluI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

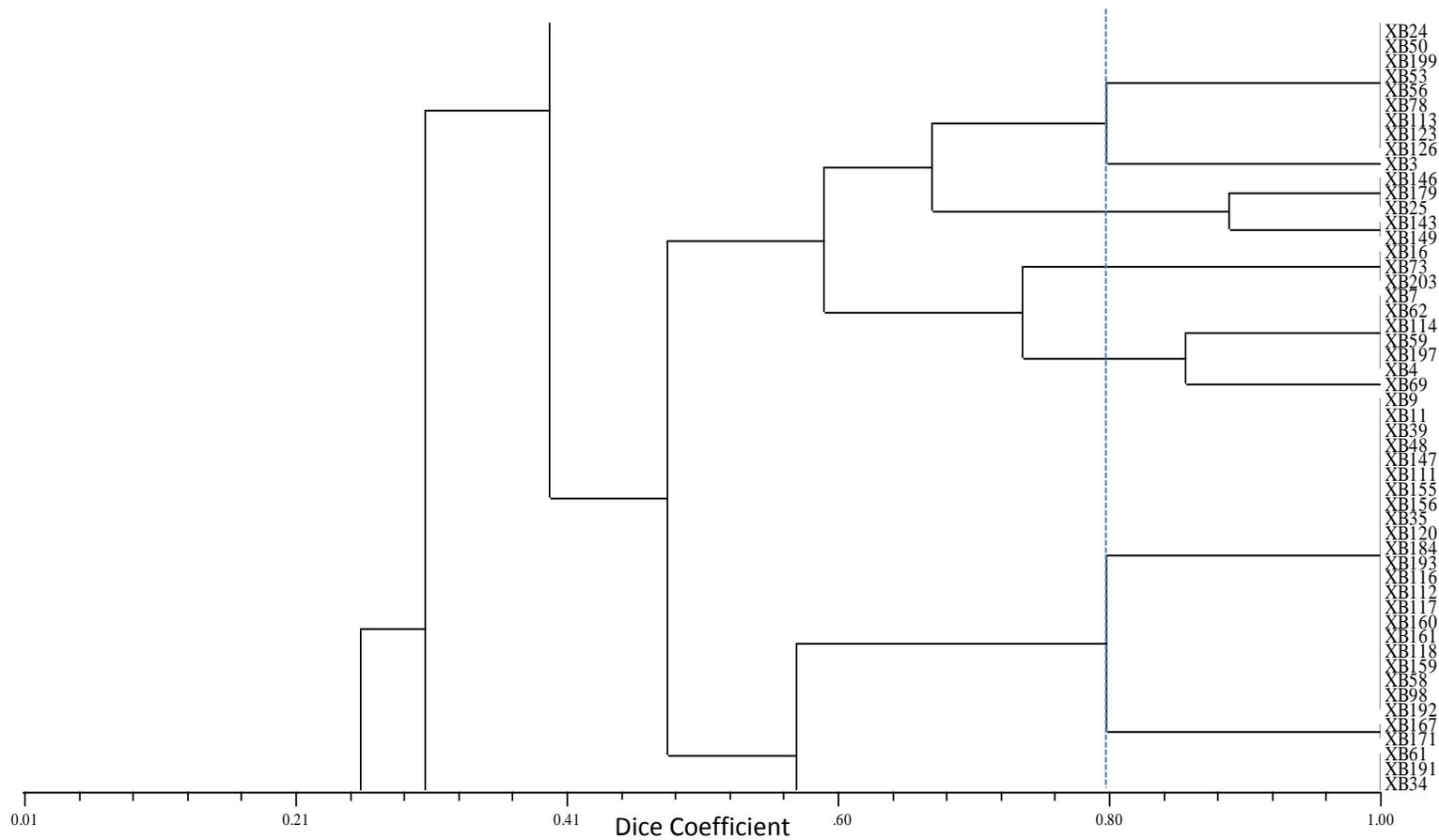


Fig. 3.9 contd. Clustering of XRB by ARDRA using restriction enzyme *AluI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

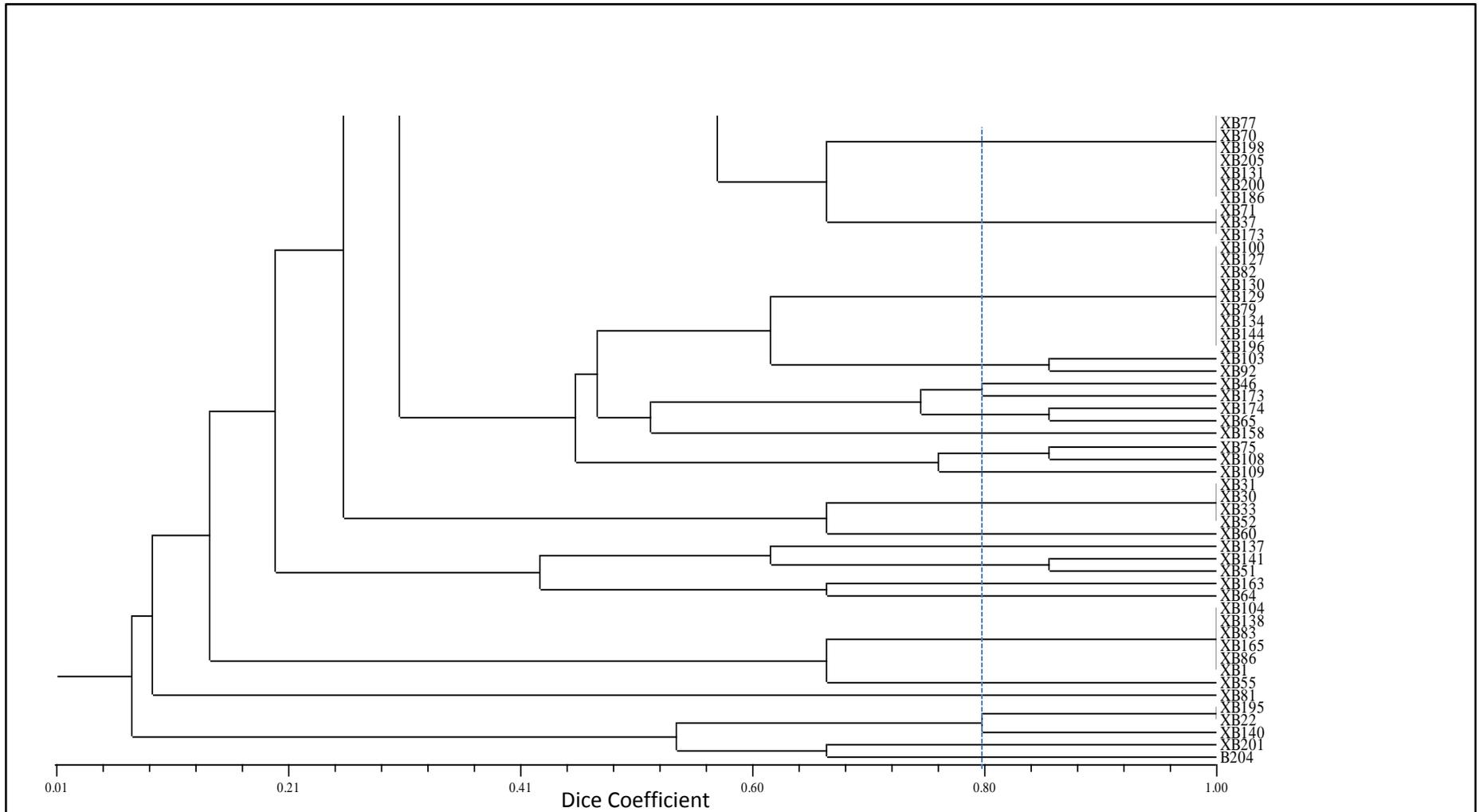


Fig. 3.9 contd. Clustering of XRB by ARDRA using restriction enzyme *AluI*. Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering was done using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Dendrogram illustrates the relation between XRB based on ARDRA patterns at 80% similarity (Dice's coefficient).

Table 3.7 Haplotypes of XRB at 80% similarity based on dendrogram of ARDRA using *AluI*.

Haplotype number	Host plant	No. of strains in each haplotype	Strain identified
A80-1	SE, RE	3	XB41
A80-2*	SE, RE, C, ST	7	XB177, XB47, XB202, XB36
A80-3*	SE, RE, C	7	XB170, XB88, XB8, XB27, XB20, XB168
A80-4	SE, RE	3	-
A80-5*	RE	1	XB169
A80-6	RE, C	4	XB94
A80-7*	SE, RE, C, ST	14	XB93, XB153, XB188
A80-8*	SE	2	XB122
A80-9	SE, RE	3	XB66
A80-10	RE	1	-
A80-11	SE, RE	2	XB190
A80-12*	SE	1	XB157
A80-13	SE	1	-
A80-14	RE	1	-
A80-15*	SE, RE, C, ST	10	XB53, XB126, XB123
A80-16	SE	1	-
A80-17	RE, C	3	XB25
A80-18	C	2	-
A80-19*	SE, ST	3	XB203
A80-20*	SE, RE	6	XB7, XB62, XB114, XB197
A80-21	SE	11	-
A80-22	SE, RE, C	22	XB159, XB35, XB98, XB161
A80-23	RE	2	XB167
A80-24*	SE,RE,C,ST	10	XB34, XB70, XB200
A80-25	SE,RE	3	XB37
A80-26*	SE, RE, C	9	XB99, XB100, XB196, XB134
A80-27	SE, C	2	XB103, XB92
A80-28	SE	1	-
A80-29	RE	1	-
A80-30	SE, RE	2	XB109
A80-31	RE	1	XB158
A80-32	SE	2	-
A80-33	SE	3	-
A80-34	SE	4	-
A80-35	SE	1	-
A80-36	SE	1	XB137
A80-37	SE	2	-
A80-38	SE	3	XB40
A80-39	SE	1	XB64

Haplotype number	Host plant	No. of strains in each haplotype	Strain identified
A80-40*	SE, RE, C	6	XB1, XB86, XB165
A80-41	C	1	-
A80-42	C	1	-
A80-43	SE, RE	2	XB22
A80-44*	SE	1	XB140
A80-45	ST	1	-
A80-46	C, ST	2	XB87

* indicates haplotype comprises of bacteria showing antagonistic property in the bioassays, SE: Bacterial Wilt susceptible eggplant, RE: Bacterial Wilt resistant eggplant, ST: *Solanum torvum*, C: Chilli plant,- No strain identified from particular haplotype.

Table 3.8 Number of ARDRA haplotypes from different hosts.

Host plant	No. of strains	Haplotypes	
		<i>MspI</i>	<i>AluI</i>
BW Susceptible eggplant	89	31	33
BW Resistant eggplant	36	17	24
Chilli	33	19	14
<i>S. torvum</i>	9	7	7

Plants are known to select endophytic colonization by specific bacteria (Rosenblueth and Martinez-Romero, 2006). Interestingly, the structure of endophytic community in *Nicotiana attenuata* a member of *Solanaceae* family is shown to be influenced by soil composition and ethylene homeostasis (Long *et al.*, 2010). Earlier evidence has shown that colonization by endophytic bacteria is also governed by plant genotype as well as root exudates (Fang *et al.*, 2005). Factors that regulate the selection of xylem colonists or the ability of XRB to colonize eggplant, chilli, and *S. torvum* has scope for further studies.

Based on the results presented in Table 3.8, ARDRA with *MspI* provided a better grouping of XRB in the collection as compared to *AluI*. Therefore, representative strains from haplotypes obtained using ARDRA with *MspI* were chosen for identification by 16S rRNA gene sequencing. Antagonism towards *R. solanacearum* was additional criteria used for choosing the isolates for identification. A total of 55 XRB were chosen for identification which included 38 representatives (both antagonistic and non-antagonists) from each *MspI* haplotype and 17 additional antagonistic XRB from random *MspI* haplotypes (Table 3.6). Selection of XRB for identification by 16S rRNA gene sequencing was not based on *AluI* haplotypes.

3.3.2.2. Diversity analyses of XRB using culture independent method PCR-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

Using DGGE, the 16S rRNA gene fragment (450 bp) amplified from total bacteria from xylem having different sequences was resolved on 8% polyacrylamide gel containing 20 to 80% denaturants, and later gels were silver stained (Fig. 3.10). Random bands from

high denaturant and low denaturant regions of the gel were eluted and 450 bp 16S rRNA gene fragment was re-amplified from eluate (Fig. 3.11).

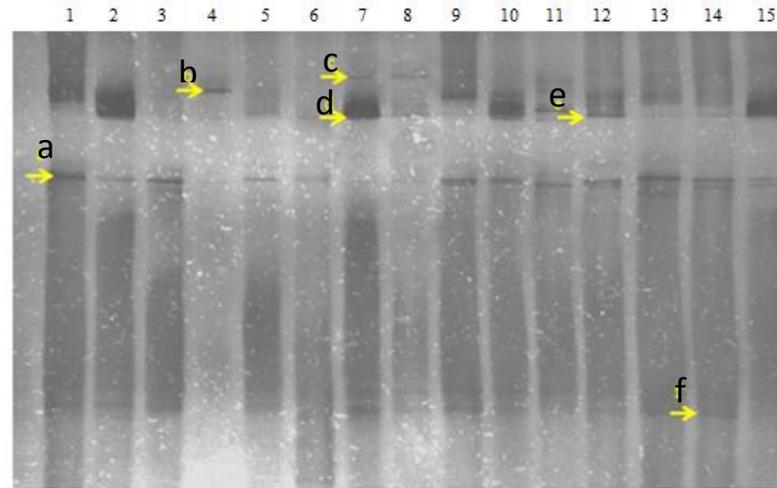


Fig. 3.10 DGGE of 16S rRNA gene amplified from xylem sap of eggplant and chilli. Lane 1- sample XS8, Lane 2-sample XS9, Lane 3-sample XS10, Lane 4-sample XS11, Lane 5-sample XS12, Lane 6 -sample XS13, Lane 7 -sample XS31, Lane 8 -sample XS36, Lane 9 -sample XS38, Lane 10 -sample XS39 , Lane 11 -sample XS43, Lane 12-sample XS40, Lane 13 -sample XS42, Lane 14 -sample XS44, Lane 15 -sample XS47. Arrows are pointing the fragment which was eluted and sequenced. a band DG4, b. band DG7, c. band DG10.1, d. band DG10.2, e. band DG15, f. band DG17.No fragments from lane 2, lane 3, lane 5, lane 6, lane 8, lane 9, lane 10, lane 11, lane 13 and lane 15 were sequenced.

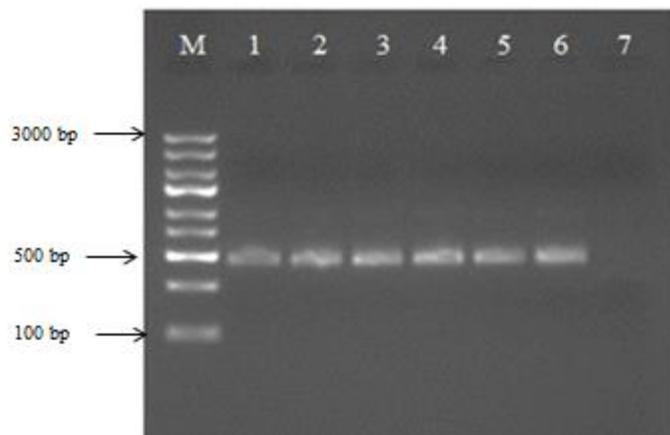


Fig. 3.11 Agarose gel electrophoresis of 16S rRNA gene fragments amplified from fragments eluted from DGGE gel. M- 100 bp to 1 Kb ladder. Lane 1- DG4, Lane 2- DG7, Lane 3- DG10.1, Lane 4- DG10.2, Lane 5- DG15, Lane 6- DG17 and Lane 7 –negative control.

Re-amplified fragments were sequenced using R1378 primer. Identification results of the sequence search in the NCBI database using BLAST is presented in Table 3.9. When compared to cultured bacteria from the same samples, it was found that *Enterobacter* sp. and other members of class *Gammaproteobacteria* could be detected by plating as well as PCR-DGGE (Table 3.10). In addition bacteria of genera *Pseudomonas*, *Bacillus*, *Pectobacterium* and *Janibacter* were not detected by PCR-DGGE. Since not all bands from DGGE gels were eluted and sequenced, there arises a possibility of missing these genera of bacteria in the DGGE analysis. Bacteria of genera *Arthrobacter*, *Curtobacterium* and *Acinetobacter* were not recovered from the same xylem sap plated for isolation. Bacteria of phyla *Proteobacteria* are long known to turn viable but non-culturable (VNBC), however recently, members of phyla *Actinobacteria* and *Firmicutes* have been reported to enter the VNBC state and can be resuscitated in presence of certain resuscitation promoting factors (Commichau and Halbedel, 2013).

Table 3.9 Sequence based identity of molecular isolates (fragments) eluted from DGGE gels.

Band Code	Accession No.	Identity*
DG4	KJ716880	Uncultured <i>Arthrobacter</i> sp.
DG7	KJ716881	Uncultured <i>Enterobacter</i> sp.
DG10.1	KJ716882	Uncultured bacterium
DG10.2	KJ716883	Uncultured <i>Curtobacterium</i> sp.
DG15	KJ716884	Uncultured <i>Curtobacterium</i> sp.
DG17	KJ716885	Uncultured <i>Acinetobacter</i> sp.

* Identity revealed by 16S rRNA gene sequencing.

Table 3.10 Comparison of culturable and non-culturable XRB from xylem sap of eggplant.

Species observed by 16S rRNA gene sequencing of isolates	Species observed by PCR-DGGE	Species observed by plating and PCR- DGGE
<i>Pseudomonas aeruginosa</i> <i>Enterobacter</i> sp.	<i>Arthrobacter</i> sp. <i>Curtobacterium</i> sp.	<i>Enterobacter</i> sp. and other Strains of <i>Gammaproteobacteria</i>
<i>Bacillus thuringiensis</i>	<i>Acinetobacter</i> sp.	
<i>Pectobacterium caratovorum</i>	<i>Enterobacter</i> sp.	
<i>Janibacter melonis</i>	Uncultured <i>Gammaproteobacteria</i>	

3.3.3. Characterization and identification of XRB

3.3.3.1. Antagonism of XRB towards *R. solanacearum* assessed by agar cup bioassay

Screening the collection of 167 XRB by using agar cup assay revealed antagonistic activities XRB against *R. solanacearum*. Screening was performed using three strains of *R. solanacearum* differing in their pathogenicity on eggplant cv. *Agassaim*. Results are summarized below and presented in Table 3.11 and Fig. 3.12.

3.3.3.1.1. Inhibition of *R. solanacearum* strain Rs-08-17, highly virulent strain on eggplant

Results of the *in vitro* screening against *R. solanacearum* strain Rs-08-17 revealed that 28 amongst 167 XRB exhibited antagonism towards the pathogen. Amongst the 28 antagonists, 11 strains, namely, XB62, XB93, XB100, XB114, XB122, XB123, XB126, XB197, XB200, XB202 and XB203 formed larger inhibition zones ranging from 4.0 mm to 6.83 mm. Antagonistic strains XB7, XB8, XB20, XB70, XB99, XB140, XB153, XB157, XB177, XB197 and XB196 produced inhibition zones ranging from 2.17 mm to 3.97 mm. XB1, XB27, XB86, XB134, XB165 and XB169 formed smaller inhibition zones less than 2.0 mm in radius. However, 139 strains of XRB did not inhibit *R. solanacearum* strain Rs-08-17 in the bioassay test.

3.3.3.1.2. Inhibition of *R. solanacearum* strain Rs-09-100, moderately virulent on eggplant

Results of the *in vitro* screening against *R. solanacearum* strain Rs-09-100 revealed that 28 amongst 167 XRB exhibited antagonism towards the pathogen. Amongst the 28 antagonists, 7 strains, namely, XB62, XB99, XB100, XB114, XB122, XB197 and XB202 formed larger inhibition zones against *R. solanacearum* ranging from 4.0 mm to 8.17

mm. The majority of the antagonistic strains ($n = 17$) produced inhibition zones ranging from 2.0 mm to 3.93 mm. XB27, XB134, XB165 and XB169 formed smaller inhibition zones ranging from 1.5 mm to 2.0 mm. However, 139 strains of XRB did not inhibit *R. solanacearum* strain Rs-09-100 in the bioassay test.

3.3.2.3.3. Inhibition of *R. solanacearum* strain Rs-09-109, mildly virulent strain on eggplant

Results of the *in vitro* screening against *R. solanacearum* strain Rs-09-109 revealed that 25 amongst 167 XRB exhibited antagonism towards the pathogen. Amongst the 25 antagonists, majority of the strains ($n=13$) formed larger inhibition zones against *R. solanacearum* ranging from 4.0 mm to 8.17 mm. Nine strains produced inhibition zones ranging from 2.0 mm to 3.83 mm. XB153, XB157, and XB165 formed inhibition less than 2.0 mm in radius. Strains XB134, XB202 and XB203 did not inhibit *R. solanacearum* strain Rs-09-109. A total of 142 strains of XRB did not inhibit *R. solanacearum* strain Rs-09-109 in the bioassay test.

Only 14.97% to 16.77% XRB out of 167 were antagonistic to three strains *R. solanacearum* based on *in vitro* assays. It was observed that the zones of inhibition by XRB against the highly virulent strain of the pathogen were smaller than those against less virulent strains. Strains XB134, XB202 and XB203 failed to antagonize mildly virulent strain Rs-09-109; however, these strains were included in the study based on their ability to inhibit the highly virulent strain of the pathogen. Twenty eight XRB antagonistic to *R. solanacearum* strains that are highly virulent (Rs-08-17) and moderately virulent (Rs-09-100) on eggplant were chosen for characterization and further studies.

Table 3.11 Inhibition of *R. solanacearum* by XRB in agar cup bioassay.

Strain	Radius of inhibition (mm) of <i>R. solanacearum</i>		
	Rs-08-17	Rs-09-100	Rs-09-109
	Highly virulent	Moderately virulent	Mildly virulent
XB1	1.03 ± 0.06	2.83 ± 0.29	2.83 ± 0.29
XB7	2.83 ± 0.29	3.33 ± 0.58	2.83 ± 1.44
XB8	2.47 ± 0.06	3.33 ± 0.29	5.47 ± 0.058
XB20	2.83 ± 0.29	3.27 ± 0.40	6.83 ± 0.58
XB27	1.67 ± 0.29	1.83 ± 0.29	4.83 ± 0.29
XB62	6.17 ± 0.29	8.17 ± 0.76	5.97 ± 0.058
XB70	3.47 ± 0.06	3.83 ± 0.58	4.0 ± 0.9
XB86	1.33 ± 0.29	2.33 ± 0.76	2.0 ± 0.5
XB93	4.0 ± 0.5	3.17 ± 0.29	3.17 ± 0.58
XB99	3.97 ± 0.06	6.83 ± 0.58	3.83 ± 0.29
XB100	4.33 ± 0.76	4.50 ± 0.50	7.5 ± 0.5
XB114	6.83 ± 0.58	6.50 ± 0.50	6.67 ± 1.26
XB122	6.67 ± 0.29	4.33 ± 0.58	5.17 ± 0.58
XB123	4.67 ± 0.29	3.00 ± 0.87	3.83 ± 0.58
XB126	4.83 ± 0.76	2.83 ± 0.29	3.83 ± 0.76
XB134	1.47 ± 0.06	1.57 ± 0.12	0
XB140	3.83 ± 0.29	3.67 ± 0.76	3.17 ± 0.76
XB153	3.17 ± 1.04	3.17 ± 0.29	1.83 ± 0.76
XB157	2.17 ± 0.29	2.50 ± 1.00	1.3 ± 0.7
XB165	1.83 ± 0.29	1.57 ± 0.12	1.83 ± 0.29
XB169	1.50 ± 0.5	1.53 ± 0.06	5.47 ± 0.06
XB170	2.83 ± 0.58	3.51 ± 0.02	4.0 ± 0.5
XB177	2.17 ± 0.29	1.99 ± 0.49	2.33 ± 0.29
XB196	3.47 ± 0.06	3.93 ± 0.12	4.47 ± 0.06
XB197	5.50 ± 0.5	4.33 ± 0.29	8.17 ± 0.29
XB200	5.17 ± 0.76	1.87 ± 0.23	4.83 ± 1.26
XB202	4.67 ± 0.29	4.67 ± 0.21	0
XB203	6.17 ± 0.29	3.67 ± 0.29	0

Inhibition zone was measured as radius from the outer edge of well. Inhibition zones are mean of three replications and showing standard deviation.

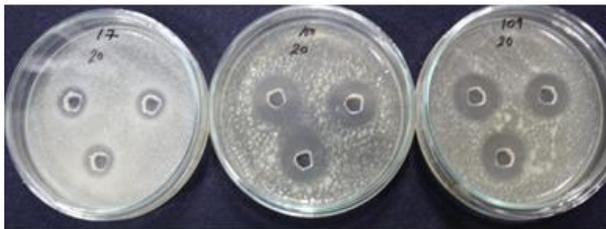


Fig. 3.12 Screening of XRB for antagonism towards *R. solanacearum*. Inhibition of *R. solanacearum* strains Rs-08-17, Rs-09-100 and Rs-09-109 by culture of XB20 added in wells.

In earlier reports, endophytic bacteria isolated from potato (Rasche *et al.*, 2006), tomato, chilli (Amaresan *et al.*, 2012) and eggplant (Ramesh and Phadke, 2012) have been shown to inhibit growth of BW pathogen *in vitro*. However, the antagonistic activities of xylem residing bacteria of solanaceous crops that share an ecological niche with the BW pathogen was unexplored and is demonstrated in this study.

3.3.3.2. Morphological characteristics and biochemical tests for identification of XRB

Based on the inhibition of *R. solanacearum*, 28 antagonistic XRB were chosen for identification by biochemical characteristics. Seventeen antagonists were Gram negative rods, four were Gram positive rods, six were Gram positive cocci, and one was filamentous actinomycete. Results of biochemical tests are presented in Table 3.12. Based on the colony morphology, Gram staining and biochemical tests, antagonistic XRB were tentatively identified using Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Majority of the antagonistic XRB were identified as *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp., *Agrobacterium* sp. and *Enterobacter* sp. Results of identification are presented in Table 3.13. XB200 was identified as an Actinomycete based on its filamentous morphology and Gram characters. XB70, XB197 and XB203 could not be identified with the given biochemical test results. Identity of the antagonistic XRB was confirmed by 16S rRNA gene sequencing.

Table 3.12 Biochemical characterization of XRB.

	Cat	Ox	I	M	Vi	C	Na	HLO	HLF	LD	NR	Gel	Ur
XB1	+	+	+	+	-	-	-	+	-	-	+	-	-
XB7	+	+	-	-	-	+	+	+	-	-	+	+	+
XB8	-	-	-	-	-	-	+	+	+	-	+	-	+
XB20	-	-	-	-	-	-	+	+	+	-	+	-	+
XB27	-	-	-	-	-	-	+	+	+	-	+	-	+
XB62	+	+	-	-	-	-	+	+	-	-	-	+	+
XB70	+	-	-	-	-	-	+	-	-	-	+	+	-
XB86	+	+	+	+	-	-	-	+	-	-	+	-	-
XB93	+	-	-	+	+	-	+	+	+	-	-	+	-
XB99	+	-	-	+	+	+	+	+	+	+	+	-	-
XB100	+	-	-	+	+	+	+	+	+	+	+	-	-
XB114	+	+	-	-	-	+	+	+	-	-	+	-	+
XB122	+	+	-	-	-	+	+	+	-	-	-	+	+
XB123	+	+	-	+	+	+	+	+	+	+	+	-	+
XB126	+	-	+	+	+	+	+	+	+	+	-	+	+
XB134	+	-	-	+	+	+	+	+	+	+	+	-	-
XB140	-	+	-	+	-	+	+	+	-	+	+	+	+
XB153	+	-	-	+	+	-	+	+	-	+	-	+	-
XB157	+	-	-	+	+	+	+	+	-	-	-	+	-
XB165	+	+	+	+	-	-	-	+	-	-	+	-	-
XB169	+	-	-	-	-	-	+	+	+	+	+	-	+
XB170	+	-	-	-	-	-	+	+	+	+	+	-	+
XB177	+	-	-	+	-	-	+	-	-	-	+	+	-
XB196	+	-	-	+	-	+	+	-	-	+	+	-	-
XB197	-	-	-	-	-	-	+	+	+	-	+	-	-
XB200	-	-	-	-	-	-	-	-	-	-	+	-	-
XB202	+	-	-	-	-	+	+	+	-	-	-	-	+
XB203	-	+	+	+	-	-	-	+	+	+	-	-	-

+ indicates presence of trait, - indicates absence of trait, cat: catalase, ox: oxidase, I: indole production, M: methyl red test, Vi: Voges Proskauer test, C: citrate utilization, Na: growth in 7% NaCl, HLO: Hugh Leifson oxidative test, HLF: Hugh Leifson fermentative test, LD: Lysine decarboxylase, NR: Nitrate reductase, Gel: gelatinase, Ur: urease.

Table 3.12 contd. Biochemical characterization of XRB.

	Fpg	Mot	Sp	Man	Lac	Fruc	Malt	Rib	Suc	Tre	Ara	Sor
XB1	-	+	-	+	-	+	+	+	+	+	+	-
XB7	+	+	-	-	-	-	-	-	-	-	-	-
XB8	-	-	-	-	+	-	+	-	+	+	-	-
XB20	-	-	-	-	+	-	+	-	+	+	-	-
XB27	-	-	-	-	+	-	+	-	+	+	-	-
XB62	+	+	-	-	-	-	-	-	-	-	-	-
XB70	-	+	-	+	-	-	+	-	+	+	-	-
XB86	-	+	-	+	-	+	+	+	+	+	+	-
XB93	-	+	+	-	-	+	-	+	+	+	-	-
XB99	-	+	-	+	+	(+)	(+)	+	(+)	+	+	(+)
XB100	-	+	-	+	+	(+)	(+)	+	(+)	+	+	(+)
XB114	-	+	-	-	-	-	+	-	-	-	-	-
XB122	+	+	-	-	-	-	-	-	-	-	-	-
XB123	-	+	-	+	+	(+)	(+)	+	(+)	+	-	(+)
XB126	-	+	-	-	+	+	-	+	+	+	-	+
XB134	-	+	-	+	+	(+)	+	+	(+)	+	-	(+)
XB140	-	+	-	-	-	+	+	-	-	-	+	-
XB153	-	+	+	-	-	+	-	-	+	-	-	-
XB157	-	+	+	+	-	+	-	-	+	-	-	-
XB165	-	+	-	+	-	+	+	+	+	+	+	-
XB169	-	-	-	-	+	+	+	-	+	+	-	-
XB170	-	-	-	-	+	+	+	-	+	+	-	+
XB177	-	+	+	-	-	+	-	+	-	+	-	-
XB196	-	+	-	+	+	+	+	+	+	+	+	-
XB197	-	-	-	-	-	+	+	-	+	-	-	-
XB200	-	-	-	-	-	-	-	-	-	-	-	-
XB202	-	+	-	-	-	-	-	-	-	+	-	-
XB203	-	-	-	+	-	+	+	+	+	+	-	-

+ indicates presence of trait, - indicates absence of trait, (+): acid and gas production. Fpg: Fluorescent pigment, Mot: motility, Sp: sporulation, Man: Mannitol fermentation, Lac: Lactose fermentation, Fruc: Fructose fermentation, Malt: maltose fermentation, Rib: ribose fermentation, Suc: sucrose fermentation, Tre: trehalose fermentation Ara: Arabinose fermentation, Sor: sorbitol fermentation.

Table 3.13 Antagonistic XRB and representative XRB from *MspI* (ARDRA) haplotypes identified by biochemical tests and/ or partial 16S rRNA gene sequencing, their closest NCBI match, % similarity and accession numbers.

Strain	Identity based on Biochemical tests	Closest NCBI match	% similarity	Accession no.
XB1*	<i>Agrobacterium</i> sp.	<i>Agrobacterium tumefaciens</i>	99	KF447383
XB7*	<i>Pseudomonas</i> sp.	<i>Pseudomonas aeruginosa</i>	99	KF447384
XB8*	<i>Staphylococcus</i> sp.	<i>Staphylococcus haemolyticus</i>	99	KF447385
XB20*	<i>Staphylococcus</i> sp.	<i>Staphylococcus haemolyticus</i>	100	KF447386
XB22	NI	<i>Brevibacterium casei</i>	99	KF447387
XB25	NI	<i>Enterobacter</i> sp.	95	KF447388
XB27*	<i>Staphylococcus</i> sp.	<i>Staphylococcus haemolyticus</i>	99	KF447389
XB34	NI	<i>Curtobacterium</i> sp.	99	KF447390
XB35	NI	<i>Microbacterium</i> sp.	99	KF447391
XB36	NI	<i>Xenophilus</i> sp.	99	KF447392
XB37	NI	<i>Chryseobacterium</i> sp.	99	KF447393
XB40	NI	<i>Micrococcus luteus</i>	89	KF447394
XB41	NI	<i>Bacillus</i> sp.	99	KF447395
XB47	NI	<i>Bacillus</i> sp.	99	KF447396
XB53	NI	<i>Micrococcus</i> sp.	99	KF447397
XB62*	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> sp.	99	KF447398
XB64	NI	<i>Bacillus thuringiensis</i>	100	KF447399
XB66	NI	<i>Pectobacterium carotovorum</i>	99	KF447400
XB70*	Unidentified	<i>Janibacter melonis</i>	99	KF447401
XB86*	<i>Agrobacterium</i> sp.	<i>Agrobacterium tumefaciens</i>	99	KF447402
XB87	NI	<i>Bacillus barbaricus</i>	99	KF447403
XB88	NI	<i>Bacillus</i> sp.	99	KF447404
XB92	NI	<i>Brevundimonas vesicularis</i>	99	KF447405
XB93*	<i>Bacillus</i> sp.	<i>Bacillus safensis</i>	100	KF447406
XB94	NI	<i>Bacillus</i> sp.	100	KF447407
XB98	NI	<i>Microbacterium</i> sp.	100	KF447408
XB99*	<i>Enterobacter</i> sp.	<i>Enterobacter</i> sp.	99	KF447409
XB100*	<i>Enterobacter</i> sp.	<i>Enterobacter cloacae</i>	98	KF447410
XB103	NI	<i>Brachybacterium phenoliresistens</i>	99	KF447411
XB109	NI	<i>Rhodococcus corynebacterioides</i>	99	KF447412
XB114*	<i>Pseudomonas</i> sp.	<i>Pseudomonas stutzeri</i>	99	KF447413
XB122*	<i>Pseudomonas</i> sp.	<i>Pseudomonas aeruginosa</i>	100	KF913446
XB123*	<i>Enterobacter</i> sp.	<i>Enterobacter</i> sp.	99	KF447414
XB126*	<i>Enterobacter</i> sp.	<i>Pantoea eucrina</i>	99	KF447415
XB134*	<i>Enterobacter</i> sp.	<i>Enterobacter</i> sp.	99	KF447416
XB137	NI	<i>Klebsiella</i> sp.	99	KF447417
XB140*	<i>Pseudomonas</i> sp.	<i>Burkholderia</i> sp.	99	KF447418

Strain	Identity based on Biochemical tests	Closest NCBI match	% similarity	Accession no.
XB153*	<i>Bacillus</i> sp.	<i>Bacillus amyloliquefaciens</i>	99	KF447419
XB157*	<i>Bacillus subtilis</i>	<i>Bacillus subtilis</i>	100	KF447420
XB158	NI	<i>Bosea</i> sp.	99	KF447421
XB159	NI	<i>Microbacterium xylanilyticum</i>	99	KF447422
XB161	NI	<i>Microbacterium aurum</i>	99	KF447423
XB165*	<i>Agrobacterium</i> sp.	<i>Agrobacterium</i> sp.	99	KF447424
XB167	NI	<i>Microbacterium aurum</i>	99	KF447425
XB168	NI	<i>Bacillus aryabhattai</i>	100	KF447426
XB169*	<i>Staphylococcus</i> sp.	<i>Staphylococcus gallinarum</i>	99	KF447427
XB170*	<i>Staphylococcus</i> sp.	<i>Staphylococcus</i> sp.	99	KF447428
XB177*	<i>Bacillus</i> sp.	<i>Bacillus cereus</i>	99	KF447429
XB188	NI	<i>Sphingomonas</i> sp.	99	KF447430
XB190	NI	<i>Brevibacterium casei</i>	99	KF447431
XB196*	<i>Enterobacter</i> sp.	<i>Enterobacter kobei</i>	99	KF447432
XB197*	Unidentified	<i>Sphingomonas</i> sp.	98	KF447433
XB200*	Actinomycete	<i>Streptomyces</i> sp.	100	KF913447
XB202*	<i>Pseudomonas</i> sp.	<i>Pseudomonas</i> sp.	99	KF447434
XB203*	Unidentified	<i>Flavobacterium</i> sp.	99	KF447435

* indicates strain is antagonistic to at least one strain *R. solanacearum* based on bioassay,
NI identification by biochemical test not performed.

3.3.3.3. Identification of XRB by 16S rRNA gene sequencing

16S rRNA gene sequencing based identity of 55 representative XRB from *MspI* haplotypes and their GenBank accessions are presented in Table 3.13. Overall, 23 different genera of bacteria were identified. Major genera identified are *Bacillus* sp. (11 strains), *Enterobacter* sp. (6 strains), *Microbacterium* sp. (5 strains), *Staphylococcus* sp. (5 strains), *Pseudomonas* sp. (5 strains) and *Agrobacterium* sp. (3 strains). Based on the identification, Phylum *Proteobacteria* consisting of Gram negative bacteria of subdivisions *Alphaproteobacteria* (12.73%), *Betaproteobacteria* (3.64%) and *Gammaproteobacteria* (25.45%) were predominant (41.81% strains identified), followed by phyla *Firmicutes* (29.09%), *Actinobacteria* (25.45%), and *Bacteroidetes* (3.64%) (Fig. 3.13).

Amongst the 28 antagonists, majority were identified as *Enterobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Bacillus* sp. (4 strains) and *Agrobacterium* sp. Consistent to our results, several studies have reported bacteria isolated from solanaceous crops and belonging to similar genera to be antagonistic to *R. solanacearum* (Sturz *et al.*, 2000; Ramesh *et al.*, 2009; Nawangsih *et al.*, 2011; Amaresan *et al.*, 2012). However, *Flavobacterium* sp. and *Janibacter melonis* have not been reported to be inhibitory to *R. solanacearum* earlier. Large numbers of the xylem inhabiting bacterial flora accounting to 83.23% to 85.02 % exhibited no antagonism towards *R. solanacearum*. Non antagonistic XRB were identified as *Microbacterium* sp. mainly. Endophytic persistence and nematocidal activities of *Microbacterium* sp. have been reported (Vargas-Ayala *et al.*, 2000; Zinniel *et al.*, 2002). Therefore the collection of XRB isolated in this study can be screened for inhibitory activities against other important agricultural pests.

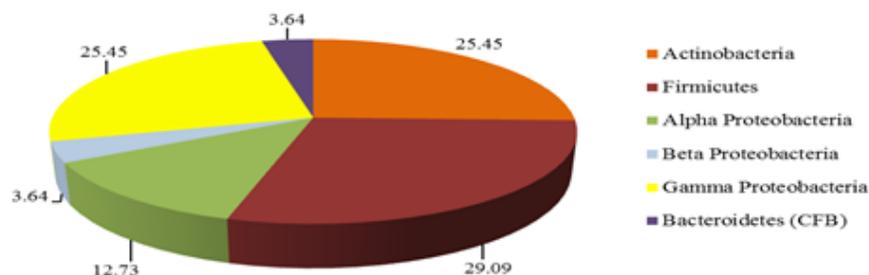


Fig. 3.13 Distribution of XRB (phylum/subdivision level) identified by 16S rRNA gene sequencing. Values indicate percentages of strains belonging to each phyla/ subdivision amongst the 55 identified strains.

Haplotypes M80-10, M80-28 and M80-31 contained XRB belonging to more than one phylum. Sklarz *et al.* (2009) have shown that ARDRA based grouping may not necessarily mirror 16S rRNA gene based phylogenetic analysis and ARDRA restriction profiles are often shared amongst members of family *Enterobacteriaceae* and phylum *Actinobacteria*. In this study ARDRA by digestion with a single restriction enzyme *MspI* was a useful tool to cluster isolates for determination of diversity of XRB from solanaceous vegetables.

3.3.3.4. Detection of extracellular hydrolytic enzymes produced by XRB

Results of the tests for extracellular hydrolytic enzymes produced by XRB is presented in Table 3.14 and Fig. 3.14. Results indicate that antagonistic strains isolated from xylem of eggplant, chilli and *S. torvum* produce multiple extracellular hydrolytic enzymes. XB93, XB153, XB157, XB177 were found to produce more than 3 enzymes tested. Considering their endophytic nature, production of endoglucanase, pectinase and polygalacturonase may be of importance for entering the plants, colonization and systemic spread through the xylem vessels (Rosenblueth and Martinez-Romero, 2006).

Table 3.14 Detection of extracellular hydrolytic enzymes of XRB.

	Amylase	Caseinase	Endoglucanase	Chitinase	Pectinase	Polygalacturonase
XB1	-	-	-	+	-	-
XB7	-	+	-	-	-	-
XB8	-	-	-	-	-	-
XB20	-	-	-	-	-	-
XB27	-	-	-	-	-	-
XB62	-	+	-	+	-	-
XB70	-	+	-	-	-	-
XB86	-	-	-	-	-	-
XB93	-	+	+	+	-	+
XB99	-	-	-	-	-	-
XB100	-	-	-	-	-	-
XB114	+	-	-	-	-	-
XB122	+	+	-	+	-	-
XB123	-	-	-	-	+	-
XB126	-	-	+	+	-	-
XB134	-	-	-	-	+	-
XB140	-	+	-	-	-	-
XB153	+	+	-	-	-	+
XB157	-	+	+	-	-	+
XB165	-	-	+	+	-	-
XB169	-	-	-	-	-	-
XB170	-	-	-	-	-	-
XB177	-	+	+	+	+	-
XB196	-	-	-	+	+	+
XB197	+	-	+	-	-	-
XB200	+	-	-	-	-	-
XB202	+	-	+	-	+	-
XB203	+	-	-	-	-	+

+ indicates presence of trait, - indicates absence of trait.

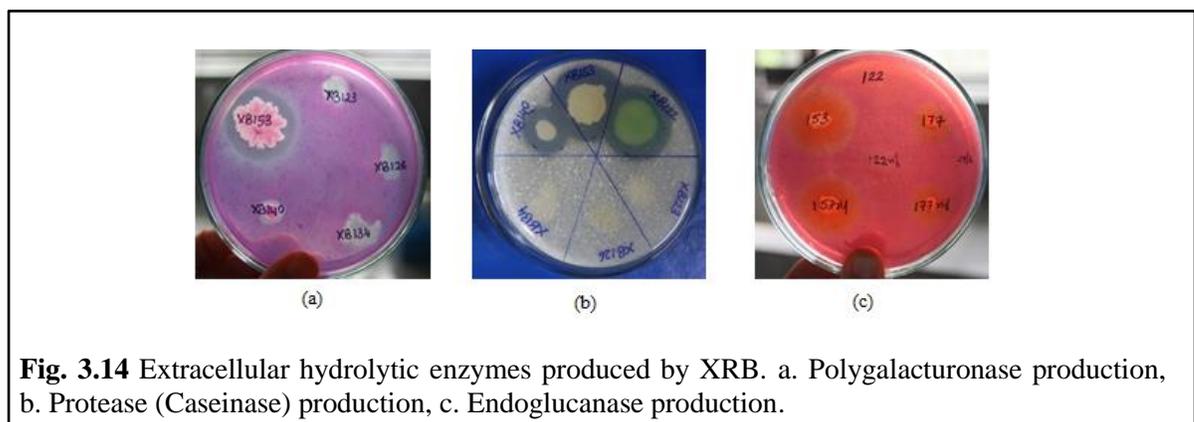


Fig. 3.14 Extracellular hydrolytic enzymes produced by XRB. a. Polygalacturonase production, b. Protease (Caseinase) production, c. Endoglucanase production.

Amylase, chitinase and endoglucanase may be beneficial to the endophytes during their saprophytic lifestyle in the soil abundant with dead insect and plant material. In addition chitinase producing bacteria can also be used as biocontrol agents against phytopathogenic fungi (Paul-Das *et al.*, 2012). In addition, the various hydrolytic enzymes produced by strains from xylem examined in this study may be novel sources of enzymes with biotechnological and industrial applications (Bhat *et al.*, 2000; Lang *et al.*, 2000; Panda and Gowrishankar, 2005; Sharma *et al.*, 2013b).

3.3.4. Plant growth promotion by xylem residing bacteria

3.3.4.1. Production of plant growth promoting substances

Results of screening of XRB for plant growth promoting substances *in vitro* is given in Table 3.15. Antagonistic strains XB1, XB7, XB62, XB86, XB122, XB140, XB165, XB202, XB203 were considered presumptive positive for nitrogen fixation ability, based on their ability to grow in nitrogen free medium even on seven successive transfers. Xylem is identified to be a non-nodular low oxygen niche ideal for nitrogen fixation by endophytic diazotrophs in non-leguminous plants (Cocking, 2003). Endophytic strains of *Enterobacter sp.*, *Burkholderia sp.* and *Klebsiella sp.* isolated from sweet potato and sugarcane are known to fix nitrogen (Tanaka *et al.*, 2006). Ability to provide fixed nitrogen to the plants may be a part of endophyte-plant symbiosis, where in return the, bacteria may be utilizing plant photosynthate for growth in xylem (James *et al.*, 2001). Atmospheric nitrogen is fixed to ammonium ions by symbiotic and non-symbiotic diazotrophs which can be easily assimilated by the plants. However acetylene reduction assay or PCR confirmation of *nifH*, *nifD* and *nifK* gene is of importance for confirming the nitrogen fixation by the putative diazotrophic bacteria (Ding *et al.*, 2005).

Table 3.15 Production of plant growth promoting substances, enhancement of seedling vigour and eggplant growth by antagonistic XRB.

Strain	ACC deaminase ^a	IAA ^b µg.mL ⁻¹	NF	PO ₄	SVI ^c	GPE
XB1	+++	105.00 ± 7.07	+	-	868.0 ± 43.8	19.4 ± 3.0
XB7	-	47.73 ± 3.21	+	+	893.5 ± 57.2	22.54 ± 3.90
XB8	-	19.09 ± 1.29	-	+	443.5 ± 64.3	ND
XB20	-	25.45 ± 1.71	-	+	514.5 ± 4.9	ND
XB27	-	15.91 ± 1.07	-	+	358.5 ± 20.5	ND
XB62	++++	73.18 ± 4.93	+	+	749.5 ± 31.8	8.9 ± 2.8
XB70	-	171.82 ± 11.57	-	-	652.0 ± 72.1	1.5 ± 0.9
XB86	+++	66.82 ± 4.50	+	-	782.0 ± 59.4	13.9 ± 1.0
XB93	-	57.27 ± 3.86	-	+	895.0 ± 46.6	7.0 ± 2.3
XB99	-	321.36 ± 21.64	-	+	859.5 ± 40.3	17.3 ± 0.7
XB100	-	238.64 ± 16.07	-	+	708.0 ± 42.4	20.3 ± 2.1
XB114	-	89.09 ± 6.00	-	+	808.5 ± 78.4	18.8 ± 2.1
XB122	-	66.82 ± 4.50	+	+	713.0 ± 111.7	28.2 ± 0.7
XB123	-	416.82 ± 28.07	-	+	828.0 ± 9.9	12.9 ± 3.8
XB126	-	60.45 ± 4.07	-	+	792.0 ± 50.9	12.4 ± 2.1
XB134	-	645.91 ± 43.50	-	+	754.0 ± 52.3	8.4 ± 2.1
XB140	++++	50.91 ± 3.43	+	+	803.5 ± 2.1	5.9 ± 4.2
XB153	-	155.91 ± 10.50	-	+	861.5 ± 13.4	26.9 ± 4.6
XB157	-	184.55 ± 12.43	-	+	909.0 ± 12.7	22.3 ± 1.9
XB165	+	190.91 ± 12.86	+	-	775.0 ± 50.9	12.4 ± 3.6
XB169	-	15.91 ± 1.07	-	-	848.5 ± 16.2	22.3 ± 8.4
XB170	-	28.64 ± 1.93	-	+	800.5 ± 27.5	14.9 ± 5.5
XB177	-	76.36 ± 5.14	-	-	1004.0 ± 56.5	12.2 ± 1.3
XB196	-	35.00 ± 2.36	-	-	510.5 ± 3.5	ND
XB197	-	41.36 ± 2.79	-	-	969.5 ± 4.9	14.9 ± 5.9
XB200	++	168.64 ± 11.36	-	-	449.0 ± 15.5	ND
XB202	+++	82.73 ± 5.57	+	+	785.0 ± 8.4	3.1 ± 2.9
XB203	-	70.00 ± 4.71	+	-	940.5 ± 50.2	-7.9 ± 2.6
					577.17 ± 109.7	
					(Control)	

+ indicates presence of trait, - indicates absence of trait in plate based assays, ^a Levels of ACC deaminase activities based on growth on plate based assay denoted as + for less growth, +++ for moderate growth, ++++ for luxuriant growth, +++++ for highly luxuriant growth, ^bIAA was estimated in culture filtrate and expressed as µg.mL⁻¹ using analytical grade IAA as standard, values indicate mean and standard deviation, NF: nitrogen fixation, PO₄ phosphate solubilization, ^cSeedling Vigour Index (SVI) was calculated using the formula (mean hypocotyl length + mean root length) x % seed germinated, values indicate mean and standard deviation. Growth promotion efficacy (GPE) calculated as [shoot length increase in treatment] - [shoot length increase in control]/shoot length increase in control) × 100 (Aliye *et al.*, 2008), values indicate mean and standard deviation. ND: not determined.

Majority of the antagonistic strains produced the phytohormone IAA with concentrations ranging from 15.91 $\mu\text{g.mL}^{-1}$ to 645.91 $\mu\text{g.mL}^{-1}$. IAA is a phytohormone produced by plants themselves and is involved in rapid root establishment by stimulating elongation of primary roots and by proliferation of lateral and adventitious roots (Patten and Glick, 2002). However, plants can perceive microbial IAA which is identical in structure and function to the indigenous plant IAA. Plant associated bacteria of genera *Agrobacterium* sp., *Enterobacter* sp., *Pseudomonas* sp., *Rhizobium* sp., *Bradyrhizobium* sp., *Acetobacter* sp. and *Alcaligenes* sp. produce plant growth stimulating IAA (Patten and Glick, 1996). XB202 was found to be the best ACC deaminase producing strain as seen from its luxuriant growth on DF salts medium supplemented with 3.0 mM ACC as sole nitrogen source. Other ACC deaminase producing strains include XB1, XB62, XB86, and XB140. Scarce growth of XB165 and XB200 was observed on DF salts medium. Rhizosphere and endophytic bacteria can lower levels of ethylene by producing enzyme ACC deaminase which degrades ACC and thus reduces ethylene formation. (Glick *et al.*, 2007a).

64.28% antagonistic XRB solubilized phosphates as indicated by clear haloes on Pikovskaya's agar plate. Phosphate solubilizing bacteria (PSB) can convert the insoluble forms of phosphorous to soluble orthophosphates, monobasic (H_2PO_4) and dibasic (HPO_2^{-4}) ions through production of organic acids and enhance the plant mineral nutrition (Bajpai and Rao 1971; Hariprasad and Niranjana, 2009).

3.3.4.2 Enhancement of seedling vigour by XRB and growth promotion in eggplant

Seedling vigor assay was used to screen the antagonistic XRB for their plant growth promotion ability, by determining their effects on seed germination, root and hypocotyl growth. XB177 exhibited highest seedling vigor index (SVI) of 1004 (Table 3.15, Fig.

3.15). Majority of the antagonistic XRB exhibited a high SVI in comparison to control. However, *Staphylococcus* sp. (XB8, XB20 and XB27), *Enterobacter* sp. (XB196) and XB200 exhibited SVI lower than control. None of the antagonistic XRB tested inhibited seedling germination. *In vitro* assessment of endophytes for growth promotion and seedling germination is useful as a preliminary screening test to identify plant growth promoting strains (Cattelan *et al.*, 1999; Hariprasad and Niranjana, 2009). Strains of XRB namely XB8, XB20, XB27, XB196 and XB200 exhibited SVI less than control.

3.3.4.3. Growth promotion in eggplant by XRB under greenhouse conditions

XRB (n=23) exhibiting high SVI were chosen for growth promotion experiment using eggplant. Highest growth promotion efficacy (GPE) was exhibited by XB122 (28.2%) (Table 3.15, Fig. 3.16 and Fig. 3.17). GPE above 20% was recorded in XB153, XB7, XB157 and XB169. Eleven antagonistic strains had a GPE ranging between 10-20%. However, strains XB62, XB70, XB93, XB134, XB140 and XB202 had GPE less than 10%. Endophytes are known to enhance plant growth since they are in a close association with plants (de Weert and Bloemberg, 2006). However, production of plant growth promoting substances *in planta* is also affected by several biotic and abiotic factors (Taghavi *et al.*, 2010).

XB203 identified as *Flavobacterium* sp. stunted shoot growth in eggplant cv. *Agassaim*, with shoot length less than control being recorded, leading to a negative GPE value. However, no visible symptoms of disease were observed in plants inoculated with XB203.

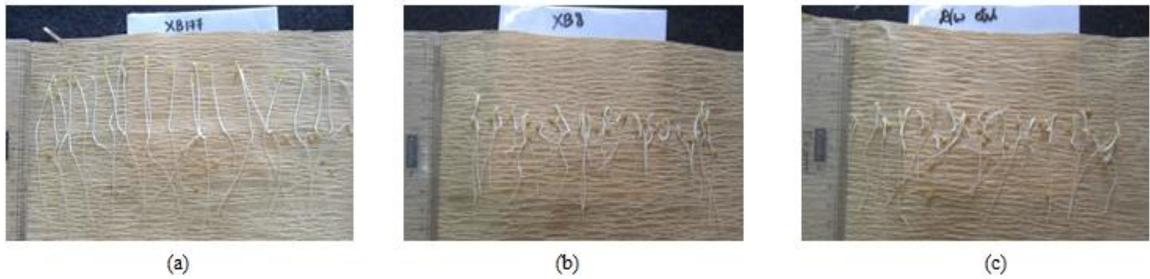


Fig. 3.15 Enhancement of seedling vigour by XRB in eggplant cv. *Agassaim* assessed by roll towel method. a. Enhancement of seedling vigour by XB177, b. Stunted radicle and shoot growth in eggplant seedlings on treatment with XB8, c. Control (treatment with 1XPBS).



Fig. 3.16 Assessment of plant growth promotion in eggplant by antagonistic XRB in greenhouse. a: growth promotion in eggplant treated with XB99, b. growth promotion in eggplant treated with XB7 with their respective controls treated with 1XPBS.

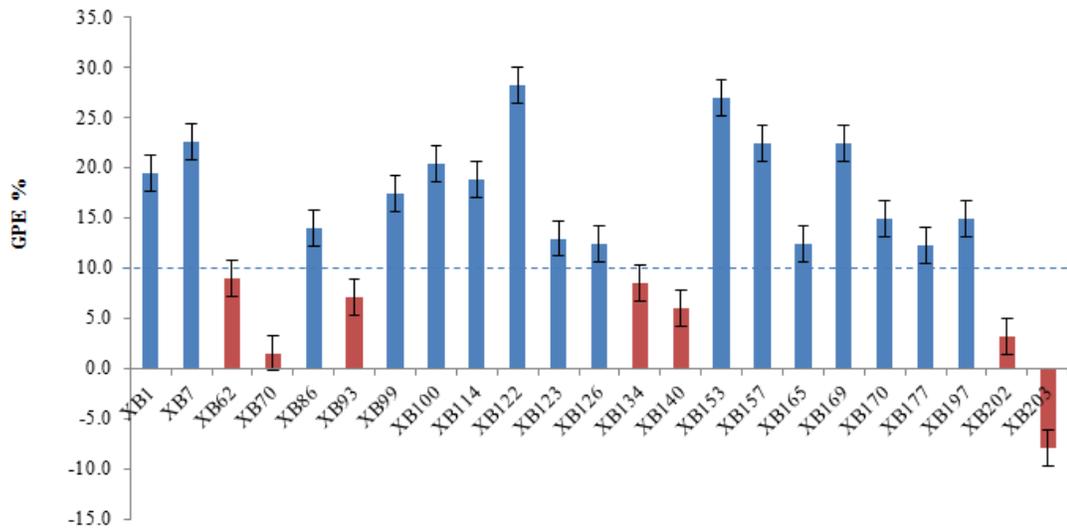


Fig. 3.17 Growth promotion by XRB in eggplant under greenhouse conditions. Each treatment consisted of two replicates with two pots per replication and five seedlings per pot. Bars indicate mean values of Growth promotion efficacy (GPE) % and error bars represent standard deviation.

Gardner *et al.*, (1985) have reported that xylem residing bacteria from lemon caused vascular plugging and produced certain metabolites that were toxic to plant cells but not cell viability. Bell *et al.* (1995) reported presence of endophytic plant pathogens in symptomless plants. Plant growth promoting *Flavobacterium* sp. are known to colonize solanaceous plants (Kolton *et al.*, 2012), however our study suggests that certain strains of *Flavobacterium* may stunt shoot growth in eggplant. Endophytes have possibly evolved from plant pathogens and may have retained parasitic behavior, leading to incompatible interactions with certain plants under certain circumstances (Kogel *et al.*, 2006).

3.3.5. Antibiotic susceptibility tests of XRB

Antibiotic resistance profiles of the antagonistic bacteria are given in Table 3.16. XB197 and XB140 were resistant to at least seven antibiotics. XB114, XB153 and XB157 were sensitive to all the antibiotics tested. Determining antibiotic sensitivity profiles of endophytic bacteria is a useful for tagging with reporter plasmids containing antibiotic resistance marker for *in planta* colonization studies. Alternatively based on the antibiotic resistance profile, introduced endophytic bacteria can be easily tracked in plants by re-isolating on selective medium designed by incorporating antibiotics (Shishido *et al.*, 1999; Tanuja *et al.*, 2013).

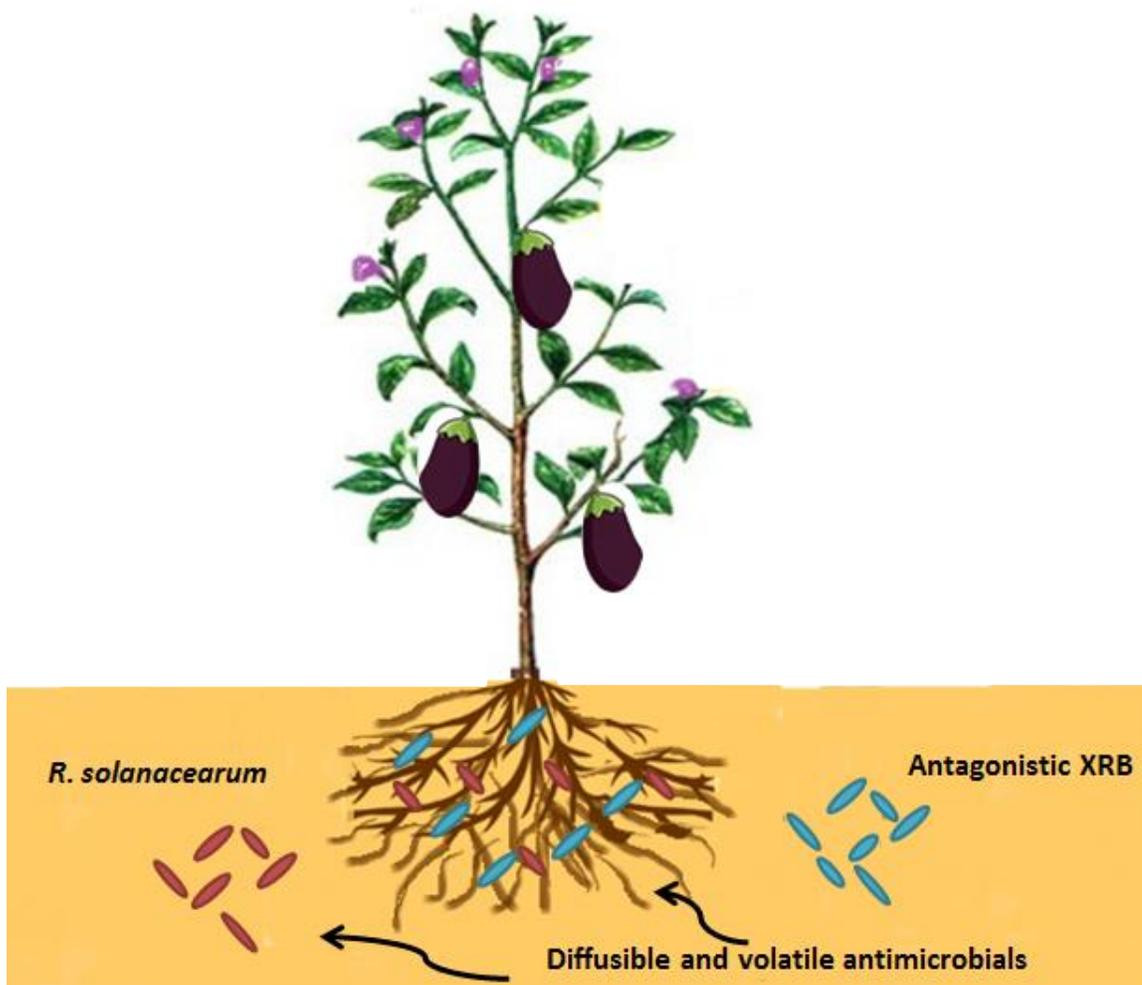
Table 3.16 Antibiotic susceptibility profiles of XRB.

Strains	A25	Am30	Cb100	Pg10	Clx30	C30	K30	Cf30	G30	S10	AK30	T30	Ce30	R30
XB1	S	S	S	R	R	I	R	S	S	R	R	S	S	S
XB7	R	R	S	R	R	I	R	S	S	I	S	R	S	S
XB8	I	R	S	R	S	S	S	S	S	S	S	R	I	S
XB20	R	R	R	R	S	S	R	S	S	S	S	S	I	S
XB27	R	R	R	R	S	S	S	S	S	S	S	R	R	S
XB62	R	R	S	R	R	R	S	S	S	R	S	R	R	S
XB70	S	R	S	S	S	S	S	S	S	I	R	S	R	S
XB86	I	I	S	R	R	S	I	S	S	I	I	S	S	S
XB93	S	S	R	S	S	S	S	S	S	S	S	S	S	S
XB99	R	R	S	R	R	S	S	S	S	S	S	S	S	S
XB100	I	R	S	R	R	S	S	S	S	S	S	S	S	I
XB114	S	S	S	S	S	S	S	S	S	S	S	S	S	S
XB122	R	R	R	R	R	S	R	S	S	I	S	R	R	S
XB123	R	R	I	R	R	S	S	S	S	S	I	I	S	S
XB126	I	S	R	R	I	R	S	S	S	S	S	S	S	S
XB134	R	R	S	R	R	S	S	S	S	S	S	S	S	S
XB140	R	R	R	R	R	S	R	S	S	R	S	S	S	S
XB153	S	S	S	S	S	S	S	S	S	S	S	S	S	S
XB157	S	S	S	S	S	S	S	S	S	S	S	S	S	S
XB165	S	S	S	R	R	I	R	S	S	S	S	S	S	S
XB169	I	I	S	I	S	S	S	I	S	I	I	S	S	S
XB170	S	S	S	S	S	S	S	S	S	S	I	S	S	S
XB177	I	R	R	R	I	S	S	S	S	S	S	S	S	S
XB196	R	R	I	R	S	S	S	S	S	S	S	S	S	S
XB197	R	R	R	R	R	S	R	S	S	S	S	S	R	S
XB200	S	S	S	I	S	S	S	S	S	S	S	S	I	S
XB202	S	S	S	I	R	S	S	S	S	S	S	S	S	S
XB203	S	S	S	S	R	S	R	S	I	I	I	S	S	S

S: susceptible, I: intermediate susceptibility, R: resistant (results were interpreted as per chart provided with Hi-Media antimicrobial susceptibility test discs). A25: Ampicillin 25 $\mu\text{g.mL}^{-1}$, Am30: Amoxycillin 30 $\mu\text{g.mL}^{-1}$, Cb100: Carbenicillin 100 $\mu\text{g.mL}^{-1}$, Pg10: Penicillin G 10 $\mu\text{g.mL}^{-1}$, Clx30: Cloxacillin 30 $\mu\text{g.mL}^{-1}$, C30: Chloramphenicol 30 $\mu\text{g.mL}^{-1}$, K30: Kanamycin 30 $\mu\text{g.mL}^{-1}$, Cf30: Ciprofloxacin 30 $\mu\text{g.mL}^{-1}$, G30: Gentamicin 30 $\mu\text{g.mL}^{-1}$, S10: Streptomycin 10 $\mu\text{g.mL}^{-1}$, AK30: Amikacin 30 $\mu\text{g.mL}^{-1}$, T30: Tetracycline 30 $\mu\text{g.mL}^{-1}$, Ce30: Cephatoxime 30 $\mu\text{g.mL}^{-1}$ and R30: Rifampicin 30 $\mu\text{g.mL}^{-1}$.

CHAPTER IV

In vitro and *in planta* interactions between xylem residing bacteria and *R. solanacearum*



4.1. INTRODUCTION

Several interactions are known to exist amongst the communities in the plant microbiome. Antagonistic interactions amongst beneficial microbes and plant pathogens have been harnessed for management of several plant diseases (Whipps, 2001). Antibiosis, competition for space and nutrients, and induction of systemic resistance are well known antagonistic interactions exhibited by plant associated bacteria against phytopathogens (Handelsman and Stabb, 1996).

Earlier studies have shown that endophytic bacteria isolated from solanaceous vegetables exhibit antagonism towards *R. solanacearum* (Ramesh *et al.*, 2009). Interestingly, antagonists isolated from eggplant from Goa were very effective as biocontrol agents against BW when tested under greenhouse and field conditions (Ramesh and Phadke, 2012). Biocontrol of is an emerging alternative to the existing management practices and suitable for sustainable agriculture in Goa. Antagonistic XRB could be suitable as biocontrol agents for management of BW.

Colonization of common niches by endophytes and pathogen is the basis for competition and interactions in the plant microenvironment (de Weert and Bloemberg, 2006). Ability of putative XRB to re-colonize plants is important to confirm their endophytic lifestyle and would also ensure efficient delivery of the antagonistic compounds for limiting *in planta* pathogen growth and colonization. Understanding the underlying mechanism(s) of antagonistic interactions between the XRB and *R. solanacearum* may aid in refining biocontrol strategies for BW management. This chapter presents the results pertaining to the objective ‘**to study the interactions between *R. solanacearum* and xylem residing bacteria.**’

4.2. MATERIALS AND METHODS

4.2.1. Mechanisms of direct interactions through production of diffusible and volatile inhibitory compounds

XRB (n=28) found to inhibit *R. solanacearum* growth by agar cup bioassay as reported in chapter III were screened for additional volatile and diffusible antimicrobial compounds namely bacteriocins, hydrogen cyanide (HCN), ammonia, acetoin and siderophores.

4.2.1.1. Bacteriocin production

Bacteriocins produced by XRB were extracted as described by Burianek and Yousef (2000). Briefly, single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Twenty μL culture was transferred to 5 mL KB broth and grown for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Culture (500 μL) was centrifuged at 11000 rpm for 2 min. Pellet was discarded and the supernatant was collected in a fresh tube. To the supernatant equal volumes of chloroform was added. Extraction was done by vigorous vortexing for 1 min. Tubes were centrifuged at 11000 rpm for 2 min. Three layers namely upper aqueous, middle interface and lower chloroform were obtained after centrifugation. The aqueous layer was pipetted and discarded carefully without disturbing the interface layer. The interface left above the chloroform layer was collected in another tube by careful pipetting to avoid pipetting of chloroform layer. The interface was evaporated in vacufuge vacuum concentrator (Eppendorf, Germany) at 60°C until dry. The residue was dissolved in 100 μL distilled water and assayed for inhibition of Rs-09-100 by agar cup method as described in section

3.2.3.1 in chapter III. All tests had three replications and plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Zone of inhibition of Rs-09-100 due to inhibitory effect of extract was measured as radius from outer edge of the well.

4.2.1.2. HCN production

Antagonistic XRB were tested for production of volatile HCN in presence of glycine as described by Saraf *et al.* (2013), a slight modification being the use of broth for the HCN test. Single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Twenty microliter culture was later transferred to five mL KB broth containing 4.4 g.L^{-1} of glycine (Appendix A). Immediately after inoculation sterile filter paper strips dipped in picric acid solution (Appendix A) were introduced taking care that the strips did not touch the medium and walls of the tube. The tubes were sealed with parafilm and incubated at $28 \pm 2^\circ\text{C}$ for four days with constant shaking at 140 rpm. The color change of the filter paper strips from yellow to brick red during incubation indicated the production of HCN by the XRB tested.

4.2.1.3. Ammonia production

Ammonia production by antagonistic XRB was determined using Nessler's reagent (Appendix A) as described by Marques *et al.* (2010). Single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Twenty μL culture was later transferred to 5 mL peptone water (Appendix A) and incubated at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm for 48 h. One mL of Nessler's reagent was mixed with one mL of the culture, mixed and incubated at RT for 10 min. Appearance of brown color indicated production of ammonia.

4.2.2. Mechanisms of indirect interactions through production of diffusible and volatile inhibitory compounds

4.2.2.1. Siderophore production

Antagonistic XRB were tested for siderophore production on KB agar containing Chrome Azurol Sulfonate (CAS) (Schwyn and Neilands, 1987) (Appendix A). Single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Ten μL culture was spot inoculated on CAS agar and incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Appearance of orange haloes around the point of inoculation of XRB after incubation at $28 \pm 2^\circ\text{C}$ for 48 h on the bluish green colored agar medium indicated a positive result for siderophore production.

4.2.2.2. Acetoin production

Acetoin production by antagonistic XRB was tested in MR-VP broth (Appendix B) as described by Hao *et al.* (2012). Single colony of XRB was grown in five mL KB broth for 48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Twenty μL culture was later transferred to MR-VP broth and incubated for 30 h at $28 \pm 2^\circ\text{C}$ at 140 rpm. One mL each of 5% α naphthol and 40% KOH (Appendix B) were added to the culture broth and mixed well. Appearance of wine red color indicated production of acetoin by the XRB tested.

4.2.3. Biocontrol efficacy of XRB against BW in eggplant under greenhouse conditions

Sixteen strains of antagonistic XRB which exhibited SVI greater than 577.17 and GPE greater than 10% in eggplant under greenhouse were selected for determining their wilt prevention ability/ biocontrol efficacy (BCE) in eggplant. Experimental conditions for

culturing and inoculation of XRB in plants were performed as given in 3.2.4.3 of chapter III, except growth measurements were not performed.

Twenty days after treatment with the antagonistic XRB the above seedlings were challenge inoculated with *R. solanacearum* strain Rs-09-100. Single colony of Rs-09-100 was grown in five mL CPG broth (Appendix A) for 48 h at $28 \pm 2^{\circ}\text{C}$ with continuous shaking at 140 rpm. Culture broth (200 μL) was transferred to 20 mL CPG broth in flasks and further incubated at $28 \pm 2^{\circ}\text{C}$ with constant shaking at 140 rpm for 36 h. After incubation 100 μL culture was taken to assess population of Rs-09-100 by serial dilution and plating. One mL culture was diluted to 100 mL with 1X PBS. Then, ten mL diluted culture suspension was diluted to 100 mL with 1X PBS. Ten mL (1000 fold diluted) suspension of Rs-09-100 (approximately $7.0 \text{ Log CFU.mL}^{-1}$) was used for challenging each XRB treated seedling by soil drenching. Plants not treated with XRB, but challenged with Rs-09-100 served as control. Plants were maintained in greenhouse at 30°C with regular watering. Percentage of plants infected with wilt was noted until 25 days after challenging with Rs-09-100. Ability of the XRB to prevent wilt in eggplant was expressed as biocontrol efficacy (BCE) and was determined using the formula $\text{BCE} = ([\text{percent disease in control}] - [\text{percent disease in treatment}] / \text{percent disease in control}) \times 100$ (Guo *et al.*, 2004). Strains with BCE greater than 20% were evaluated for their effect on growth in eggplant under greenhouse conditions.

4.2.4. Competition by XRB for *in planta* space (niche exclusion)

Eleven strains of XRB which exhibited a BCE greater than 20% were evaluated for their ability to colonize eggplant stem and rhizosphere under greenhouse conditions, using their rifampicin resistant (RR) mutants.

4.2.4.1. Generation of rifampicin resistant mutants

RR mutants of 11 XRB were generated as described by Sevastopoulos and Glaser (1977) on KB agar containing rifampicin ($100 \mu\text{g.mL}^{-1}$) (KBRif) (Appendix A). Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 -72 h. Colonies that appeared on the plates after incubation in presence of rifampicin were spontaneous RR mutants of the XRB. The colonies were purified onto KBRif and maintained in 25% glycerol at -80°C . RR mutants of each XRB were designated with the parent name followed a suffix 'R'.

4.2.4.2. Stability of rifampin resistance, growth curve and BOX-PCR fingerprinting of RR mutants

Stability of RR mutants was determined a by 15 serial passages on KB agar and KBRif as described by Ghirardi *et al.* (2012). Briefly, single colony of RR mutants grown on KBRif agar were suspended in one mL sterile distilled water and serially diluted 10^6 fold. One hundred μL diluted culture was spread plated on KB and KBRif agar plates. Two plates were maintained for each dilution plated on each medium. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 h and the number of colonies was counted. This procedure was repeated for 15 successive transfers to estimate the number of rifampicin resistant colonies.

Growth rates of rifampicin derivatives of each of the XRB was checked in KB broth and KBRif broth and compared to the wild type parent strain. Briefly, 20 μL (48 h old culture grown in KBRif broth) culture of RR mutants of XRB was inoculated in two separate tubes containing five mL KB and KBRif broth. Simultaneously, wild type parent strains were inoculated in KB broth. Tubes were incubated at $28 \pm 2^\circ\text{C}$ with shaking at 140 rpm. Optical density of the cultures at 600 nm (OD_{600}) was determined at intervals of

0 h, 6 h, 12 h, 24 h, 36 h, 48 h and 72 h. Growth curve for each strain was generated by plotting OD₆₀₀ vs time.

In addition, genetic similarity of rifampicin mutants and wild type XRB was confirmed by BOX PCR fingerprinting (Versalovic *et al.*, 1994). Genomic DNA from the rifampicin mutants of XRB were isolated as described in section 3.2.2.1.1 in chapter III. Twenty five µL reaction mix contained 1 X Gitschier's buffer (Appendix C) (Kogan *et al.*, 1987), 0.16 mg.mL⁻¹ BSA, 10% DMSO, 250 µM dNTPs, 30 pmol primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'), 2.5 units of Taq DNA polymerase and 100 ng template DNA. For XB7 and XB122 (*Pseudomonas* sp.), XB1, XB86 and XB165 (*Agrobacterium* sp.) and XB99 and XB123 (*Enterobacter* sp.) having a G+C content greater than 50% the PCR cycle consisted of initial denaturation of 95°C for 7 min, followed by 33 cycles of denaturation of 94°C for 1 min, annealing at 52°C for 1 min and extension at 65°C for 8 min, followed by a final extension of 65°C for 16 min. In case of XB177 (*Bacillus cereus*) having G+C less than 50% the PCR cycle consisted of initial denaturation of 95°C for 7 min, followed by 33 cycles of denaturation of 90°C for 30 s, annealing at 52°C for 1 min and extension at 65°C for 8 min, followed by a final extension of 65°C for 16 min (Versalovic *et al.*, 1994). The PCR fragments were separated on 2% agarose gel in 1 X TAE buffer as described in section 3.2.4.1.2 and gel was documented using Alpha Imager (Alpha- Innotech Inc. USA). The fingerprints of the wild type and rifampicin derivatives of XRB obtained after electrophoresis were compared.

4.2.4.3. Inoculation of RR mutants in plants

Single colony of RR mutants was grown in 5 mL KBRif broth at $28 \pm 2^{\circ}\text{C}$ at 140 rpm for 48 h. Fifty microliters of overnight culture was transferred to 40 mL KBRif broth and grown at $28 \pm 2^{\circ}\text{C}$ at 140 rpm for 48 h. Culture was centrifuged at 8000 rpm for 8 min at 4°C . Pellet was re-suspended in 200 mL 1X PBS. Ten mL suspension of RR mutants containing approx. $8\text{-}9 \text{ Log CFU.mL}^{-1}$ was applied to 30 day old eggplant seedlings planted in pots in greenhouse by soil drenching as described in section 3.2.4.3 in chapter III, except no growth parameters were measured. Five pots with four plants per pot were maintained. For determining colonization, plant samples were collected immediately after inoculation and at intervals of 15 and 30 days after inoculation (except for XB177R samples were collected upto 60 days). At each sampling point two plant samples were collected to assess population as described below in section 4.2.4.4.

In a separate experiment, parent strains XB1, XB86 and XB165 were tested to cause crown gall in eggplant, chilli, tomato and *Kalanchoe* sp. as described by Chen *et al.* (1999), since they are identified as *Agrobacterium* sp. Briefly, a longitudinal slash was made on the upper and lower part of the *Kalanchoe* leaf and bacterial suspension (overnight growth in KB broth at 140 rpm $28 \pm 2^{\circ}\text{C}$) was inoculated over the cut using a pipette. Stems of eggplant, chilli and tomato were pricked at the crown region using a sterile needle and 10 μL suspension of *Agrobacterium* sp. was inoculated. Greenhouse conditions were maintained as described in section 3.2.4.3 in chapter III.

4.2.4.4. Re-isolation of RR mutants from eggplant

Surface sterilization of eggplant stem pieces (6-8 cm length) and maceration was performed as described in section 3.2.1.2.2 in chapter III, except the stems were not decorticated. Appropriate dilutions (undiluted and 10^{-2} for leaf samples, 10^{-3} and 10^{-4} for

stem samples) of the macerate were plated on KBRif agar. Sterility check was performed by plating wash water from last rinse of the stem on KB and KBRif agar plates. Isolation of inoculated strains from the rhizosphere was performed as described by Buyer (1995). Briefly, loosely adhering soil was removed and root sample was shaken in 10 mL sterile distilled water for 30 min at 140 rpm at $28 \pm 2^\circ\text{C}$, serially diluted and plated on KBRif agar (dilutions 10^{-4} and 10^{-5}). Each serially diluted sample was plated in duplicates. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 48 to 72 h until visible colonies appeared. Rifampicin resistant colonies with morphological characteristics identical to parent strains were counted and purified by streaking on KBRif plates. For each plant sample, CFU per gram fresh weight of sample were calculated. Re-isolated bacteria were preserved in 30% glycerol at -80°C . Genomic DNA from re-isolated bacteria was isolated by method given by Wilson (1997) as described in section 3.2.2.1.1 in chapter III. To confirm that the re-isolated bacteria were identical to introduced RR mutant strains, genomic fingerprinting was performed by BOX PCR as described in section 4.2.4.2.

4.2.5. Population dynamics of XB177 during wilt infection

XB177 prevented wilt in eggplant and was a systemic colonizer of eggplant as seen from the results of colonization experiments. Therefore its population dynamics during wilt infection was studied. This experiment consisted of three types of treatments to eggplant, namely pre-treatment with XB177 before challenging with Rs-09-100, co-inoculation of XB177 and Rs-09-100 together and inoculation of XB177 after challenging with *R. solanacearum* Rs-09-100.

In the pre-treatment experiment, inoculation of XB177 and Rs-09-100 in eggplant was performed as described above in 4.2.3 in this chapter. For co-inoculation studies equal volumes of suspension of XB177 ($8.0 \text{ Log CFU.mL}^{-1}$) and Rs-09-100 (7.0 Log

CFU.mL⁻¹) in 1X PBS were mixed. Ten mL mixed suspension was inoculated by soil drenching in eggplant as described in section 4.2.3. In the third treatment, inoculations were performed as described in section 4.2.3 only difference being that Rs-09-100 was inoculated first by soil drenching. Seven days later XB177 was inoculated in eggplant by soil drenching. Control pots consisted of eggplant inoculated with either XB177 or Rs-09-100 alone and untreated plants in separate pots.

Each treatment consisted of three replications with five seedlings per pot. Plants samples collected for assessing the population of introduced bacteria included healthy plants as well as wilted plants from each treatment. Healthy plants were available only in the pre-treatment experiment and control, and they were assessed for population of XB177 and Rs-09-100, after 23 days of pathogen inoculation (that is 43 days post inoculation of XB177). Wilted plants from all the treatments and control were sampled for isolation of XB177 and Rs-09-100 as soon as the symptoms of wilt were observed (5-7 DAI of Rs-09-100, that is 25- 27 DAI of XB177). Two plants were collected at each sampling point. Populations of XB177 and Rs-09-100 in stem and rhizosphere soil were assessed as described in section 4.2.4.4. KB agar was chosen for isolation of XB177 from stem and rhizosphere of eggplant. Colonies of XB177 were easily recognizable based on its colony characteristics on KB agar (Fig. 4.1).

Genetic similarities of introduced and re-isolated colonies of XB177 were confirmed by BOX-PCR as described in section 4.2.4.2. SMSA agar (Semi-selective Medium from South Africa, Kelman, 1954; Pradhanang *et al.*, 2000) (Appendix A) was chosen for selective isolation of Rs-09-100 from stem and rhizosphere of eggplant.

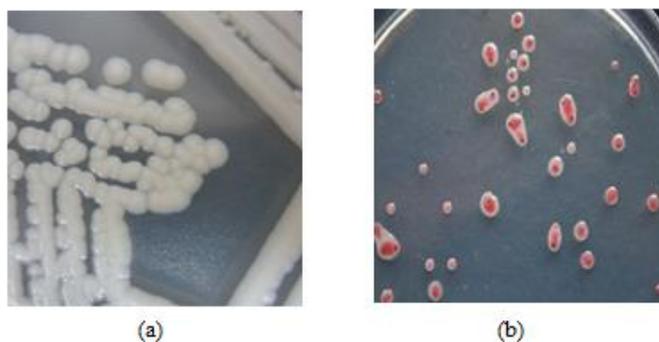


Fig. 4.1 Colony morphology XB177 and Rs-09-100. a. colonies of XB177 on KB agar, b. colonies of Rs-09-100 on SMSA agar. Plates were incubated at $28 \pm 2^\circ\text{C}$ for 24 and 48 h respectively for XB177 and Rs-09-100.

Colonies of Rs-09-100 were easily recognizable based on its colony characteristics on SMSA agar (Fig. 4.1) Re-isolated colonies were confirmed by *R. solanacearum* species specific 759f-760r primers (Opina *et al.*, 1997). This primer pair amplifies 281 bp upstream region of *lpxC* gene (encodes Uridine di-Phosphate-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase; involved in the biosynthesis of lipid A in Gram negative bacteria) from all *R. solanacearum* strains. This portion of the genome of *R. solanacearum* is conserved and essential for the function of all *R. solanacearum* strains (Opina *et al.*, 1997) and is a standard PCR for confirmation of identity of *R. solanacearum* strains worldwide (Nouri *et al.*, 2009). Single colony was dissolved in 100 μL sterile distilled water and one μL was used as a template for colony PCR. Ten μL reaction mix contained 1x Taq buffer (Sigma Aldrich, USA), 200 μM each dNTPs, 10% DMSO, 20 μg BSA, 1.0 μM each primer 759f (5'-GTCGCCGTCAACTCACTTTCC-3') and primer 760r (5'-GTCGCCGTCAGCAATGCGGAATCG-3'). PCR cycle consisted of initial step with denaturation of 94 for 3 min, annealing at 53 for 1 min and extension at 72 for 1 min followed by 30 cycles with denaturation at 94 for 15s, annealing at 60 for 15 s and extension at 72 for 15 s in each cycle and final extension at 72 for 5 min.

4.2.6. Visualization of pattern of colonization of XB177 by Green Fluorescent Protein (GFP) tagging

4.2.6.1. Isolation of GFP plasmid pAD43-25 from *E. coli* DH5 α

XB177 was tagged with GFP to study the pattern of its colonization in stem and rhizosphere of eggplant, chilli and *S. torvum*. GFP vector pAD43-25 (Dunn and Handelsman, 1999) (Fig. 4.2) was obtained from Bacillus Genetic Stock Centre, The Ohio State University, USA (BGSC accession number ECE166). Vector pAD43-25 was isolated from *E. coli* DH5 α by alkaline lysis method (Birnboim and Doly, 1979). Recipes for alkaline lysis solutions and buffers are given in Appendix C. Briefly, *E. coli* strain DH5 α harboring pAD43-25 was grown overnight at $37 \pm 2^{\circ}\text{C}$ in LB broth containing ampicillin ($100 \mu\text{g.mL}^{-1}$) and chloramphenicol ($10 \mu\text{g.mL}^{-1}$) with constant shaking at 140 rpm. Culture (1.5 mL) was centrifuged at 8000 rpm for 5 min at room temperature. Supernatant was discarded. The pellet was re-suspended in 100 μL of cold (4°C) alkaline lysis solution I by pipetting.

Freshly prepared alkaline lysis solution II (150 μL) was added and the contents were mixed by inverting several times and tubes were incubated on ice for 5 min. After incubation, 200 μl of cold (4°C) alkaline lysis solution III was added and the contents were mixed by inverting several times and incubated on ice for 5 min. The tubes were centrifuged at 10000 rpm for 10 min at RT. Supernatant was removed in separate tube. Thrice the volume of 100% ice cold ethanol was added and tubes were inverted several times to precipitate plasmid DNA.

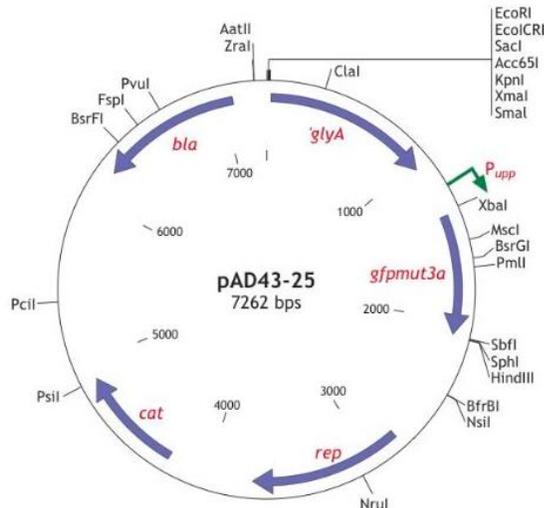


Fig. 4.2 Map of *Bacillus-E. coli* shuttle vector pAD43-25 used for GFP Tagging of XB177. pAD43-25 (BGSC accession no. ECE166) is a 7262 bp low copy number plasmid and maintains approx. 25 -30 copies per cell in *E. coli*. It replicates in *E. coli* from the pBR322 origin and in *Bacillus* sp. from the pTA1060 origin. It has *rep* (replication initiation protein) from cryptic rolling circle plasmid pTA1060. It constitutively expresses GFP *mut3a* gene under the control of *upp* (uracil phosphoribosyl transferase) gene promoter of *Bacillus cereus* UW85. It has *cat* (Chloramphenicol acetyl transferase) gene selectable in *E. coli* and *Bacillus* sp. and *bla* (β -lactamase) marker gene selectable in *E. coli* only and *glyA* which has last 1010 bp of the *Bacillus cereus* structural gene for glycine/serine hydroxymethyl transferase.

Tubes were centrifuged at 10000 rpm for 10 min at RT. Pellet was re-suspended in one mL 70% ethanol and centrifuged at 10000 rpm for 10 min at RT. Pellet was incubated in a 55°C water bath until dry and dissolved in 100 μ L sterile distilled water. Quantity and quality of plasmid DNA was checked using NanoDrop1000 (Thermo Scientific, USA).

4.2.6.2 Transformation of the pAD43-25 in *E. coli* GM2929 (*dam⁻dcm⁻*)

Certain strains of *Bacillus* sp. are known possess modification dependent restriction endonucleases which restrict methylated plasmid DNA (Macaluso and Mettus, 1991; Dunn *et al.*, 2003; Xu *et al.*, 2012; Loenen and Raleigh, 2013). *E. coli* DH5 α has methylases which methylate the adenine and cytosine bases of DNA, thus a plasmid

DNA isolated from *E. coli* DH5 α is methylated. Attempts to electroporate XB177 (*Bacillus cereus*) using plasmid pAD43-25 isolated from *E. coli* DH5 α resulted in no transformants. Therefore un-methylated plasmid DNA obtained from *E. coli* GM2929 (*dam*⁻*dcm*⁻) was used. *E. coli* GM2929 is a mutant strain of *E. coli* deficient in *dam* and *dcm* methylases, deficient for plasmid recombination, lacks the Mcr and Hsd restriction systems (*dam-13::Tn9*, *dcm-6*, *hsdR2*, *recF143*, McrA⁻, MctB⁻) and transforms well with foreign plasmid DNA (Palmer and Marinus, 1994).

Plasmid pAD43-25 isolated from *E. coli* DH5 α was transformed to *E. coli* GM2929 (*dam*⁻*dcm*⁻) (Dunn *et al.*, 2003) by heat shock at 42°C. Buffers (TFBI and TFBII) for making chemically competent *E. coli* GM2929 cells were prepared as described by Hanahan (1983) with slight modification being that no cobalt chloride was used (Appendix C). *E. coli* GM2929 was streaked out on LB agar (Appendix A) and grown overnight 37 \pm 2°C. Single colony was inoculated in three mL of LB broth and grown overnight 37 \pm 2°C with constant shaking at 120 rpm. Overnight culture (200 μ L) was inoculated to sterile 10 mL of LB broth supplemented with 10 mM MgSO₄ (Appendix A) and at 37 \pm 2°C with constant shaking at 120 rpm for 3-4 h until the OD₆₀₀ is between 0.5 to 0.75. Culture was centrifuged in 50 mL polypropylene tubes 8000 rpm for 8 minutes at 4°C. Four mL of cold (4°C) TFB-I solution was gently added to the pellet and left on ice until the cell pellet dissolved. To aid dissolving pellet, tubes were gently swirled intermittently. The tubes were centrifuged at 8000 rpm for 8 minutes at 4°C. Cells were re-suspended in 400 μ l of cold (4°C) TFB-II solution. Aliquots of 100 μ L cells were transferred to cold 1.5 mL tubes and stored on ice for instant transformation using plasmid DNA. One μ g (10 μ L) plasmid DNA was mixed with 100 μ L of CaCl₂ competent cells (stored on ice) and incubated further on ice for 15-30 minutes in 1.5 mL

Eppendorf tubes. Cells were heat shocked for 2 minutes at 42°C by placing tubes in a water bath. Immediately one mL of LB broth was added to the heat shocked cells and shaken at 37 ± 2°C for one hour. Cells were centrifuged at 12000 rpm for one minute and re-suspended in 200 µL of LB broth, spread plated on to LB agar containing ampicillin (100 µg.mL⁻¹) and choramphenicol (10 µg.mL⁻¹) and allowed to grow overnight at 37 ± 2°C. *E. coli* GM2929 transformants growing in presence of antibiotics were re-streaked on LB agar containing ampicillin (100 µg.mL⁻¹) and choramphenicol (10 µg.mL⁻¹) and allowed to grow overnight at 37°C. Success of transformation was assessed by isolating plasmid DNA from the *E. coli* GM2929 cells and checking by restriction digestion of the plasmid using restriction enzymes.

4.2.6.3. Restriction enzyme digestion to confirm transformation of *E. coli* GM2929 with pAD43-25

Based on the plasmid map of pAD43-25, three restriction enzymes were chosen for confirmation of re-isolated plasmid. Enzymes *Hind*III and *Xba*I were chosen for double digest to release 760 bp fragment, *Eco*RI and *Xba*I were chosen for double digest to release 1337 bp fragment, *Eco*RI and *Hind*III for double digest to release 2097 bp fragment and *Kpn*I to linearize the plasmid. Reaction mix contained 1X tango buffer (Thermo Scientific, USA, compatible for all the restriction enzymes for double digest), 200 ng plasmid DNA and five units of each restriction enzyme in a total volume of 20 µL. The reactions were incubated in a water bath at 37 ± 2°C for 4 h. The restriction digests were run on 1.5% agarose gel containing 0.5 µg.mL⁻¹ ethidium bromide as described in section 3.2.2.1.2 in chapter III. Plasmid DNA from one positive clone was

isolated as described in section 4.2.6.2 in this chapter and transformed into XB177 by electroporation as described below.

4.2.6.4. Preparation of electrocompetent cells and electroporation

Electrocompetent XB177 (*Bacillus cereus*) was prepared as described by Dunn *et al.* (2003). XB177 was streaked on LB agar and incubated overnight at $28 \pm 2^\circ\text{C}$. Single colony was inoculated in 5 mL LB broth and incubated overnight at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. After incubation, 200 μL of culture of XB177 was transferred to 50 mL LB broth and incubated at 180 rpm at $37 \pm 2^\circ\text{C}$ until the OD_{600} reached 0.3. Culture was allowed to cool on ice in a pre-chilled 50 mL centrifuge tube for 15 min. Culture was centrifuged at 8000 g for 8 min at 4°C and re-suspended gently by swirling in 40 mL chilled EP buffer (Appendix C). Cell pellet was re-suspended in 500 μL EP buffer by pipetting and incubated on ice. One μg of un-methylated pAD43-25 (isolated from *E. coli* GM2929) was added in 100 μL cells and incubated on ice for 5 min. Contents were transferred to pre-chilled one mm electroporation cuvette (Eppendorf, Germany) and cells were electroporated at 610 V, 10 μF , 600 Ω using e-porator (Eppendorf, Germany). Immediately one mL LB broth was added to electroporated cells, contents were quickly transferred in tubes and incubated at $28 \pm 2^\circ\text{C}$ at 120 rpm for 1 h. Cells were centrifuged at 8000 rpm for 2 min at RT and re-suspended in 100 μL LB broth and spread onto LB agar containing chloramphenicol ($10 \mu\text{g}\cdot\text{mL}^{-1}$) (LBC agar). Plates were incubated at $28 \pm 2^\circ\text{C}$ overnight. Chloramphenicol resistant colonies of XB177 obtained on the plates were checked for GFP fluorescence directly under 4 X magnification using Olympus CX41 microscope (Olympus, Tokyo, Japan), equipped with a B filter (dichromatic mirror DM500, excitation filter BP475, barrier filter

O515IF). Cells from colonies emitting green fluorescence were smeared on a glass slide and expression of GFP was confirmed by observing under oil immersion lens at 100 X magnification using fluorescence microscopy (Olympus CX41 equipped with a B filter). GFP positive transformants of XB177 were preserved as 25% glycerol stocks at -80°C.

4.2.6.5. Plasmid stability experiment and generation time

Single colony of GFP tagged XB177 (designated as XB177G) was inoculated in 5 mL LB broth amended with chloramphenicol (10 µg.mL⁻¹) (LBC broth) and grown at 28 ± 2°C with shaking at 140 rpm overnight. Fifty µL of overnight culture was transferred to 5 mL LB broth and incubated for 10 h. Next, 50 µL of overnight culture was transferred to fresh 5 mL LB broth and incubated for 10-11 h. In the third transfer 50 µL of overnight culture was transferred to 5 mL LB broth and incubated for 10 to 12 h. Population (CFU.mL⁻¹) of XB177G in the broth was assessed after each transfer and before each transfer by plating appropriate dilutions (10⁴ fold dilution just after transfer and 10⁷ fold dilution before transfer) on LB and LBC agar. Number of generations of XB177G were determined as described by Ramos *et al.* (2002) using formula $n = [\text{Ln}(N_t) - \text{Ln}(N_0)] / \text{Ln}2$, where n is the number of generations, N_t is the number of colonies before each successive dilution and N₀ is the number of colonies after each successive dilution. Percentage of chloramphenicol resistant (Cm^R) GFP positive colonies present at the end of each transfer were counted for an estimate of stability of plasmid without antibiotics over several generations over 34 hours.

4.2.6.6. Inoculation of XB177G in eggplant, chilli and tomato

Single colony of XB177G was grown in 5 mL KB broth with chloramphenicol (10µg.mL⁻¹) (KBC) at 28 ± 2°C at 140 rpm overnight. Fifty µL of overnight culture was

transferred to 40 mL KBC at $28 \pm 2^\circ\text{C}$ at 140 rpm overnight. Culture was centrifuged at 8000 rpm for 8 min at 4°C . Pellet was re-suspended in 200 mL 1X PBS. Ten mL suspension of XB177G containing approx. $8\text{-}9 \text{ Log CFU.mL}^{-1}$ was applied to 30 day old eggplant, chilli and tomato plants planted in pots in greenhouse by soil drenching as described in section 3.2.4.3 in chapter III, except no growth parameters were measured. Five pots with four plants per pot were maintained. For observation of colonization by XB177G, eggplant samples were collected immediately after inoculation and at intervals of 7, 20 and 30 and 45 days after inoculation. Tomato and chilli plants were sampled only at 30 days post inoculation for microscopy. At each sampling point two plant samples were collected for observations by fluorescence microscopy. At 30 DAI additional two plants were collected for assessing population of XB177G by plating. For assessing population of XB177G, KB agar and KBC agar was used. Population was assessed as described section 4.2.4.4 in this chapter.

For microscopy, loosely adhering soil from the roots was shaken off and roots were washed under running water and dabbed dry using tissue paper. Root hair samples were directly mounted in 10% glycerol (v/v in distilled water) on a slide and observed under oil immersion lens of Olympus CX41 microscope (Olympus, Tokyo, Japan), equipped with a B filter (dichromatic mirror DM500, excitation filter BP475, barrier filter O515IF). Thin stem sections were cut manually using a sharp blade. Stem sections were stained with 0.05% methyl violet (w/v) for 30 s as described by Germaine *et al.* (2004). Sections were transferred in sterile distilled water to wash excess stain, mounted in 10% glycerol and observed under oil immersion lens of Olympus CX41 microscope (Olympus, Tokyo, Japan), equipped with a B filter. Fields showing GFP positive

XB177G cells in the plant tissue were captured using ProgRes Capture Pro 2.8 software using CT3 camera (Jenoptik AG, Germany).

4.3. RESULTS AND DISCUSSION

4.3.1. Mechanisms of direct interactions through production of diffusible and volatile inhibitory compounds.

4.3.1.1. Bacteriocin production

Nine strains of antagonistic XRB produced bacteriocin when screened by agar cup assay using chloroform extracts (Table 4.1). Radius of inhibition caused by bacteriocin extracts ranged from 0.83 to 3.67 mm. Largest zone of inhibition was formed by bacteriocin extract from XB153, whereas the smallest was from XB134 and XB202. Bacteriocins are microbial peptides which inhibit bacteria by targeting cell membrane (Abee *et al.*, 1995). In this study bacteriocins extracted from both Gram positive and Gram negative XRB inhibited *R. solanacearum*. Bacteriocins were earlier known to act only on closely related bacteria of same genera. But recent studies show that they may have a broad spectrum antibacterial effect (Oscariz *et al.*, 1999; Bizani *et al.*, 2005). In addition to the inhibition of *R. solanacearum*, bacteriocinogenic XRB may have a competitive advantage for endophytic colonization of plants (Muñoz-Rojas *et al.*, 2005) which may aid in wilt prevention.

4.3.1.2. Ammonia and HCN production

Five strains of XRB namely XB99, XB100, XB123, XB134 and XB140 produced volatile inhibitory compound ammonia and XB7, XB62, XB93, XB122 and XB170 produced HCN (Table 4.1). Ammonia concentrations of upto 500 ppm may reduce the growth of *R. solanacearum* when used as organic soil amendment (Michel and Mew,

1998). In addition ammonia is a known plant growth promoting agent (Ahmad *et al.*, 2008), and may simultaneously contribute to growth promotion in eggplant.

HCN is a broad spectrum antimicrobial which affects bacterial cytochrome c oxidase and is involved in biocontrol of soil borne pathogens by fluorescent *Pseudomonas* sp. (Ramette *et al.*, 2003). *R. solanacearum* has cytochrome cbb(3) type oxidase of haem-copper oxidase family which are sensitive to micromolar concentrations of HCN (Ray and Williams, 1997). Cytochrome cbb (3) type oxidase of *R. solanacearum* is also involved in respiration in micro-aerophilic environments such as the xylem vessels during plant colonization. A mutant lacking this oxidase exhibited delayed wilting symptoms in tomato (Clifford-Colburn and Allen, 2010). HCN produced by endophytic XRB may aid in controlling *R. solanacearum* population *in planta*.

4.3.2. Mechanisms of indirect interactions through production of diffusible and volatile inhibitory compounds

4.3.2.1. Siderophore and acetoin production

Four strains of XRB namely XB7, XB114, XB122 and XB140 produced siderophore molecules as seen from the results of CAS agar assay (Fig. 4.3, Table 4.1). There is scarcity of bioavailable iron in rhizosphere soil which leads to competition between the rhizosphere inhabitants. Siderophores scavenge iron making it unavailable to pathogen resulting in its growth inhibition (Compant *et al.*, 2005a). The ability to produce siderophores is also of competitive advantage to biocontrol agents for plant colonization (Raaijmakers *et al.*, 1995; de Weert and Bloemberg, 2006). Siderophore producing *Bacillus* sp. and *Serratia* sp. isolated from tomato and chili have been reported to control

BW in tomato (Amaresan *et al.*, 2012). In addition bacterial siderophores are known elicitors of induced systemic resistance (ISR) in plants (Choudhary *et al.*, 2007).

ISR occurs when the plant immune system is stimulated by benign bacteria and is primed to resist infection by plant pathogens. ISR occurs mainly by bacterial contact by colonization which is dependent on jasmonic acid (JA), salicylic acid (SA) and NPR1. Whereas ISR by exposure (without colonization or contact) to bacterial volatiles leads to activation of ISR via ethylene signaling and SA pathways and is independent of JA and NPR1 (Ryu *et al.*, 2004; Kim *et al.*, 2011).

Interestingly, it was shown that plant can sense and respond to bacterial volatiles namely acetoin (3-hydroxy butanone) and 2, 3-butanediol resulting in induction of ISR (Ryu *et al.*, 2004). In the present study, nine strains of XRB namely XB93, XB99, XB100, XB123, XB126, XB134, XB153, XB157 and XB197 produced acetoin. Bacterial acetoin production occurs via the alternate pyruvate metabolism under low oxygen concentration (Xiao and Xu, 2007). Xylem of plants is also known to be a low oxygen niche (Cocking, 2003). Production of acetoin by XRB during xylem colonization may lead to induction of ISR against *R. solanacearum*.

4.3.3. Biocontrol efficacy of XRB against BW in eggplant under greenhouse conditions

Biocontrol efficacy of 16 XRB (GPE greater than 20%) against BW was tested in eggplant cv. *Agassaim* under greenhouse conditions. XB86 and XB169 exhibited 100% BCE against BW (Table 4.1, Fig. 4.4), followed by XB177 (93.75%), XB122 (87.5 %) and XB7 (77.5%). Seven strains of XRB exhibited a BCE ranging from 12.5% to 71.5%.

Table 4.1 Diffusible and volatile antimicrobial compounds produced by antagonistic XRB and their biocontrol efficacy (BCE) against BW.

Strain	Bacteriocin	Ammonia	HCN	Acetoin	Siderophores	BCE%
XB1	0.00	-	-	-	-	42.8 ± 9.8
XB7	0.00	-	+	-	+	77.5 ± 3.5
XB8	0.00	-	-	-	-	ND
XB20	1.33 ± 0.58	-	-	-	-	ND
XB27	0.00	-	-	-	-	ND
XB62	0.00	-	+	-	-	ND
XB70	0.00	-	-	-	-	ND
XB86	0.00	-	-	-	-	100.0 ± 0.0
XB93	0.00	-	+	+	-	ND
XB99	0.00	+	-	+	-	25.0 ± 14.1
XB100	2.67 ± 0.58	+	-	+	-	0.0
XB114	0.00	-	-	-	+	-12.5 ± 7.1
XB122	1.33 ± 0.58	-	+	-	+	87.5 ± 17.7
XB123	0.00	+	-	+	-	57.0 ± 2.8
XB126	0.00	-	-	+	-	12.5 ± 7.8
XB134	0.83 ± 0.29	+	-	+	-	ND
XB140	0.00	+	-	-	+	ND
XB153	3.67 ± 0.58	-	-	+	-	0.0
XB157	0.00	-	-	+	-	0.0
XB165	0.00	-	-	-	-	28.6 ± 6.9
XB169	1.67 ± 0.58	-	-	-	-	100.0 ± 0.0
XB170	2.67 ± 0.58	-	+	-	-	71.4 ± 9.1
XB177	2.67 ± 0.58	-	-	-	-	93.75 ± 8.8
XB196	0.00	-	-	-	-	ND
XB197	0.00	-	-	+	-	71.5 ± 9.0
XB200	0.00	-	-	-	-	ND
XB202	0.83 ± 0.29	-	-	-	-	ND
XB203	0.00	-	-	-	-	ND

Bacteriocin inhibition zones are mean of three replications and showing standard deviation. Inhibition zone was measured as radius from the outer edge of well. + indicates presence of trait, - indicates absence of trait. All *in vitro* experiments were conducted at 28 ± 2°C. Experiments to estimate BCE were performed greenhouse conditions, values of BCE are mean of two replications and showing standard deviation. ND: Not determined

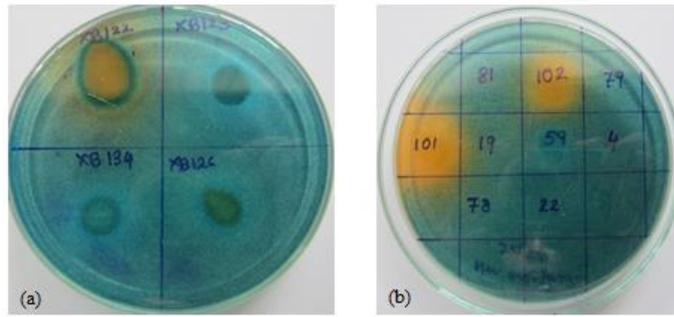


Fig. 4.3 Siderophore production by XRB inoculated on CAS agar. Appearance of orange haloes around the point of inoculation indicates siderophore production.

Apparent wilt prevention abilities of the XRB tested in this study may be attributed to their ability to produce multiple pathogen inhibitory compounds which are tested *in vitro* and reported above. Endophytic niche is known to be a protected environment aiding in direct delivery of antagonistic compounds to the pathogen (de Weert and Bloemberg, 2006). However, antagonistic strains namely XB1, XB86 and XB165 which prevented wilt were found negative for production of additional volatile and diffusible inhibitory compounds tested.

XB100, XB153 and XB157 did not prevent wilt in eggplant whereas XB126 exhibited a BCE less than 20%. Biocontrol effect of inoculated bacteria may fail because of their inability to re-colonize plants which is in turn affected by several biotic and abiotic factors (Benizri *et al.*, 2001). In addition, failure in biocontrol may occur if the bacteria are unable to produce the antibacterial compound in the rhizosphere of eggplant or its antibacterial effect is reduced due to environmental conditions (Lugtenberg *et al.*, 2001). In this study for determination of wilt prevention ability non sterile soil was used which may have affected colonization of eggplant due to competition and/or inhibition by normal flora of resulting in less or no biocontrol effect by introduced bacteria (Compant

et al., 2005a). Similar results in failure of highly antagonistic bacteria to prevent BW under greenhouse and field conditions in tomato and eggplant have been reported earlier (Ran *et al.*, 2005a; Lemessa and Zeller, 2007; Ramesh and Phadke, 2012).

XB114 had less BCE than control, though it was antagonistic to *R. solanacearum* *in vitro*. Similar results on reduced biocontrol effect against BW on inoculation of antagonistic *Pseudomonas* sp. strain Rbh40 in eggplant is reported earlier (Ramesh and Phadke, 2012). In the present study, concentrations of 8 Log CFU/ mL of XB114 were used for plant inoculation for both growth promotion and wilt prevention experiments. Though this population of XB114 caused increase in seedling vigour and growth, it possibly increased susceptibility of eggplant to wilt infection. Endophytic *Methylobacterium* sp. when inoculated at high concentration resulted in susceptibility of potato plants to *Pectobacterium atrosepticum* whereas at low concentrations it induced resistance (Ardanov *et al.*, 2012). Determination of effect of lower population of XB114 in wilt prevention would give insights into its mechanism of interaction with *R. solanacearum* and plant host.

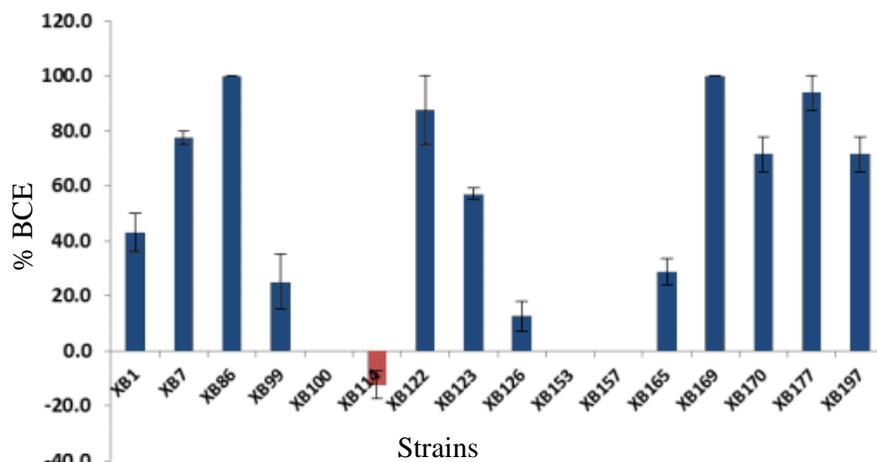


Fig. 4.4 Biocontrol efficacy of plant growth promoting antagonistic XRB. Bars indicate % BCE, a mean of two replications and error bars indicate standard deviation.

4.3.4. Competition by XRB for *in planta* space (niche exclusion)

Rifampicin resistance of spontaneous RR mutants was found to be stable after 15 transfers on medium without rifampicin (Table 4.2). As seen from the results the population of RR colonies obtained on KB and KBRif agar after 15 transfers on medium without rifampicin were the same indicating the resistance is stable. In addition the growth rates of the RR mutants in KB and KBRif broth was found similar to that of the wild type parent when tested over a period of 72 h (Fig. 4.5 to 4.8). BOX PCR results indicated that genetic fingerprints of RR mutants and the parent XRB were the same (Fig. 4.9a and 4.9b). Therefore the RR mutants were regarded as representatives of their respective parent wild type XRB for studying their colonization in eggplant cv. *Agassaim* under greenhouse conditions. RR mutants re-isolated from eggplant samples after 15 and/or 30 days after inoculation (DAI) were confirmed similar to introduced RR mutants in eggplant based on their similar colony characteristics and BOX PCR fingerprints (Fig. 4.9a and 4.9b). Results indicate that eight out of 11 XRB evaluated were able to re-colonize eggplant stem and/ or rhizosphere under given conditions.

Table 4.2 Population of rifampin resistant cells of XRB in a single colony before and after 15 serial transfers on KB and KBRif agar.

Strain	KB (Log CFU.mL⁻¹)	KBRif (Log CFU.mL⁻¹)
XB1R (T1)	9.5 ± 0.04	9.5 ± 0.03
XB1R (T15)	9.5 ± 0.05	9.6 ± 0.02
XB86R (T1)	9.5 ± 0.04	9.4 ± 0.05
XB86R (T15)	9.6 ± 0.09	9.6 ± 0.04
XB165R (T1)	9.6 ± 0.02	9.5 ± 0.04
XB165R (T15)	9.7 ± 0.01	9.6 ± 0.01
XB7R (T1)	10.1 ± 0.00	10.05 ± 0.00
XB7R (T15)	10.1 ± 0.00	10.0 ± 0.00
XB122R (T1)	9.9 ± 0.01	9.9 ± 0.01
XB122R (T15)	10.0 ± 0.01	10.0 ± 0.01
XB99R (T1)	9.6 ± 0.02	9.6 ± 0.01
XB99R (T15)	9.8 ± 0.02	9.9 ± 0.01
XB123R (T1)	9.7 ± 0.03	9.7 ± 0.01
XB123R (T15)	9.9 ± 0.02	9.9 ± 0.00
XB177R (T1)	8.5 ± 0.02	8.5 ± 0.03
XB177R (T15)	8.7 ± 0.05	8.9 ± 0.07

T1: colonies obtained after the first transfer, T15: colonies obtained after the 15th transfer, KB: KB agar, KBRif: KB agar with rifampin (100 µg.mL⁻¹). Assessment of population was done on KB and KBRif agar. All experiments were conducted at 28 ± 2°C, values are mean colony counts in Log CFU.mL⁻¹ of two replications per isolate per dilution and showing standard deviations.

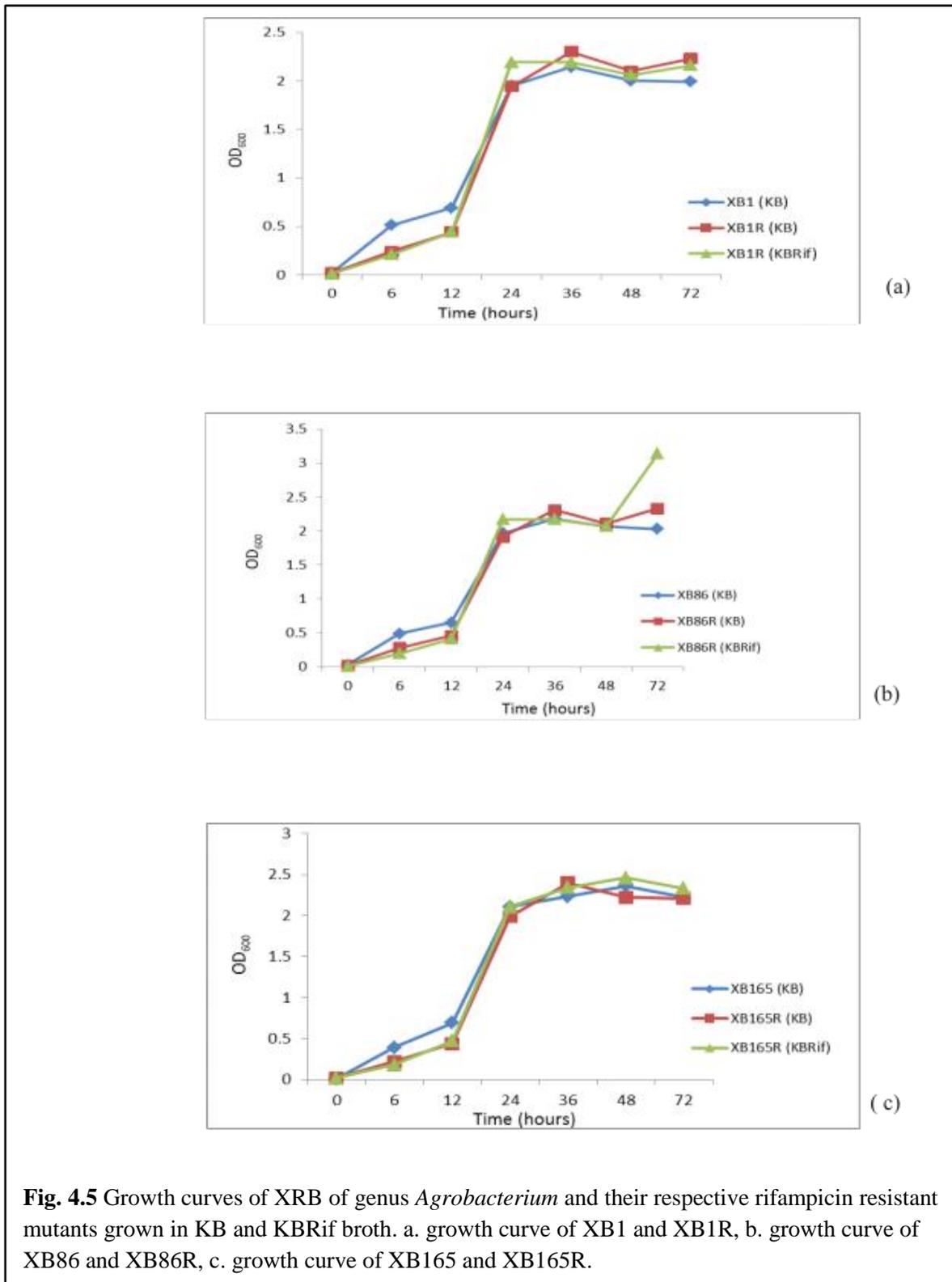
4.3.4.1. Colonization of *Agrobacterium* group of XRB

Population of XB1R (RR mutant of XB1), XB86R (RR mutant of XB86) and XB165R (RR mutant of XB165) immediately after inoculation, 15 DAI and 30 DAI in eggplant stem and rhizosphere is presented in Table 4.3 and Fig. 4.10. At 15 DAI rhizosphere population of XB1 was steady at $4.96 \text{ Log CFU.g}^{-1}$ fresh weight and also colonized the stem endophytically. At 30 DAI, endophytic population of XB1R decreased to $3.53 \pm 0.04 \text{ Log CFU.g}^{-1}$ fresh weight of stem whereas its population in the rhizosphere increased to $5.78 \pm 0.08 \text{ Log CFU.g}^{-1}$. XB86 colonized endophytic tissues and its rhizosphere population remained steady in the range of $5.0 \text{ Log CFU.g}^{-1}$ fresh weight over 30 day period in eggplant. XB165 colonized only the rhizosphere but its population reduced to $5.23 \pm 0.01 \text{ Log CFU.g}^{-1}$ rhizosphere soil at 30DAI.

When tested as per the method described by Chen *et al.* (1999) and Tkalec *et al.* (2012) strains XB1, XB86 and XB165 did not cause tumors in eggplant, chilli, tomato and *Kalanchoe* sp. until 60 days post inoculation. No galls appeared when these strains were inoculated by soil drenching in root zone. The results indicate that XB1, XB86 and XB165 are non-pathogenic endophytes of eggplant cv. *Agassaim* possibly because they may be lacking the T-DNA or have mutated *virG* genes on the Ti plasmid (Belanger *et al.*, 1995; Tzfira and Citovsky, 2000). However, studying their host range for ability to cause crown gall in other plants species is of importance (Anderson and Moore, 1979).

Congruent to the present results, endophytic colonization of several plants by strains of *Agrobacterium* sp. is reported. Non-pathogenic forms of *Agrobacterium* sp. are known to move systemically through the xylem vessels of plants (Bazzi *et al.*, 1987; Cubero *et al.*, 2006; Kawaguchi *et al.*, 2007; Llop *et al.*, 2009; Souza *et al.*, 2013). Production of antagonistic compounds and efficient niche exclusion by XB1, XB86 and

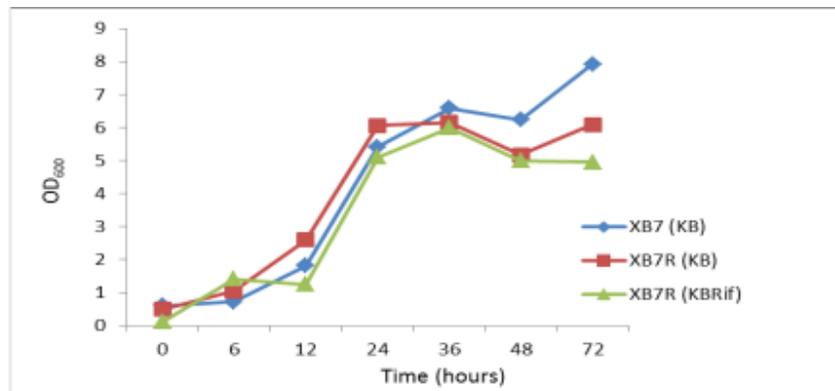
XB165 may have contributed the observed BCE (28.6 to 100%) of these strains against wilt in eggplant.



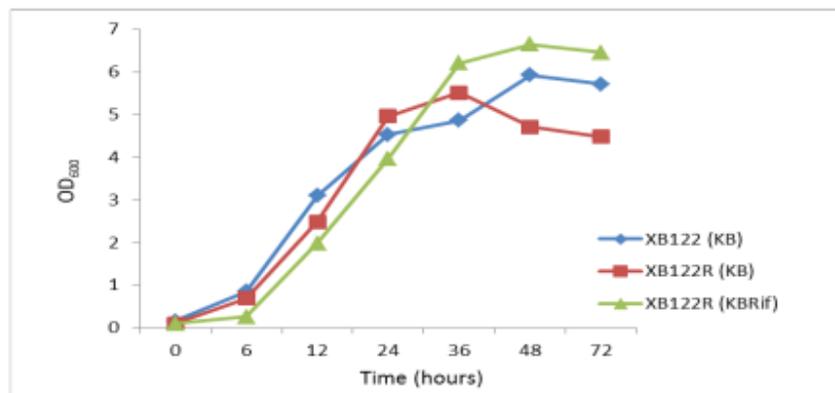
4.3.4.2. Colonization of *Pseudomonas aeruginosa* group of XRB:

Population of XB7R (RR mutant of XB7) and XB122R (RR mutant of XB122) after inoculation, 15 DAI and 30 DAI in eggplant stem and rhizosphere is presented in Table 4.3 and Fig. 4.11. XB7R and XB122R colonized rhizosphere. Population of XB7R reduced to 4.67 Log CFU.g⁻¹ fresh weight after 15 DAI whereas that of XB122R remained in the range of 4.6 Log CFU.g⁻¹ at 15 DAI and 30 DAI. No endophytic colonization by XB7R and XB122R was detected.

XB7 and XB122 are identified as *Pseudomonas aeruginosa*. Strains of *P. aeruginosa* have been reported as endophytic and rhizosphere colonists of eggplant, pepper, mustard, millet, castor and other plants (Pandey *et al.*, 2005; Aravind *et al.*, 2009; Ramesh *et al.*, 2009; Kumar *et al.*, 2012; Gupta *et al.*, 2013; Lakshmi *et al.*, 2014). Ability of *P. aeruginosa* and other fluorescent pseudomonads to inhibit soil borne plant pathogens is attributed to production of inhibitory compounds namely HCN, siderophores, pyoverdine, pyrrolnitrin and induce ISR in plants (Lugtenberg *et al.*, 2001; Cazorla *et al.*, 2006). In this study XB7 and XB122 were found to be efficient rhizosphere colonists of eggplant when tested until 30 days after inoculation and produced HCN and siderophores. Efficient colonization of rhizosphere is an essential factor for delivery of antimicrobial compounds and induction of ISR in plants for BW management (Ran *et al.*, 2005b). Niche exclusion and production of multiple inhibitory compounds in rhizosphere of eggplant may have led to the observed biocontrol effect against Rs-09-100 in plants treated XB7 and XB122. Thus rhizosphere colonizing strains XB7 and XB122 are potential antagonists for BW prevention in eggplant.



(a)



(b)

Fig. 4.6 Growth curves of XRB identified as *Pseudomonas aeruginosa* and their respective rifampicin resistant mutants grown in KB and KBRif broth. a. growth curve of XB7 and XB7R, b. growth curve of XB122 and XB122R.

4.3.4.3. Colonization of *Enterobacter* group of XRB

Population of XB99R (RR mutant of XB99) and XB123R (RR mutant of XB123) after inoculation, 15 DAI and 30 DAI in eggplant stem and rhizosphere is presented in Table 4.3 and Fig. 4.12. Endophytic population XB99R in stem tissue of eggplant reduced from 15 DAI to 30 DAI. Rhizosphere population of XB99R decreased to 4.82 ± 0.23 Log CFU.g⁻¹ rhizosphere soil at 15 DAI and at 30 DAI was almost steady at 4.83 ± 0.11 Log CFU.g⁻¹ rhizosphere soil. XB123R colonized only the rhizosphere of eggplant its population was 5.5 Log CFU.g⁻¹ and 4.68 CFU.g⁻¹ at 15 and 30 DAI respectively.

XB99 and XB123 are identified as *Enterobacter* sp. Presence of *Enterobacter* sp. has been reported in cotton, poplar, tomato, corn and several other plants (Hinton and Bacon, 1995; Quadt-Hallmann *et al.*, 1997; Taghavi *et al.*, 2009; Seleim *et al.*, 2011). Antagonistic *Enterobacter* sp. isolated from rhizosphere of eggplant and tomato inhibited *R. solanacearum* in plate based assays and greenhouse studies (Long *et al.*, 2004; Ramesh *et al.*, 2009; Seleim *et al.*, 2011; Nawangsih *et al.*, 2011). In other studies isolates of *Enterobacter* sp. from soil are reported to prevent BW (Xue *et al.*, 2009; Nguyen *et al.*, 2011). Plant associated *Enterobacter* sp. have a positive effect on plant growth by the virtue of production of plant growth promoting substances such as IAA, ammonia and solubilization of phosphate. Their antagonistic activities are attributed to ability to produce antimicrobial compounds and acetoin which elicits ISR in plants. In addition *Enterobacter* sp. respond and move towards root exudates chemotactically and have adapted to colonize endophytic tissues and plant rhizosphere by utilizing plant sugars (Taghavi *et al.*, 2010). High BCE exhibited by XB99 and XB123 may be attributed to their ability to produce ammonia and acetoin which are inhibitory to *R. solanacearum* and known to induce ISR in plants respectively.

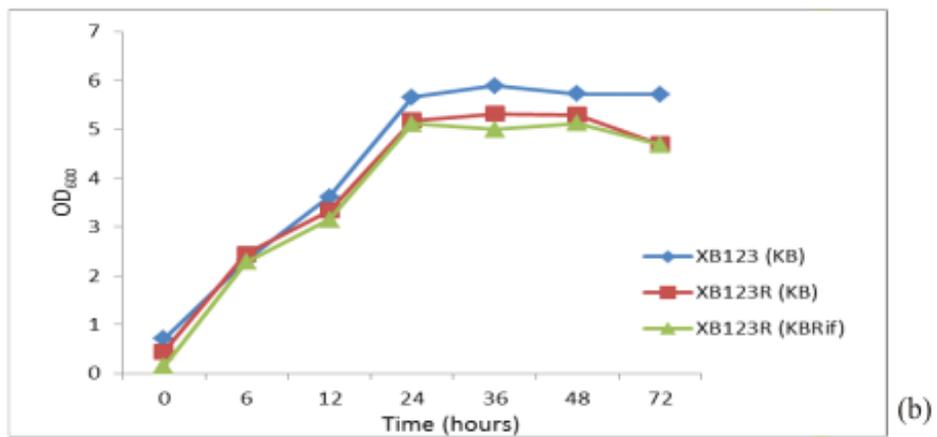
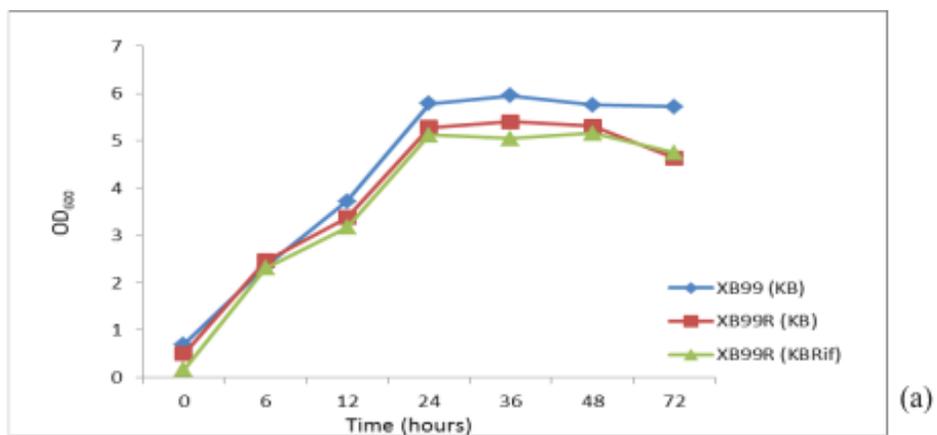


Fig. 4.7 Growth curves of XRB identified as *Enterobacter* sp. and their respective rifampicin resistant mutants grown in KB and KBRif broth. a. growth curve of XB99 and XB99R, b. growth curve of XB123 and XB123R.

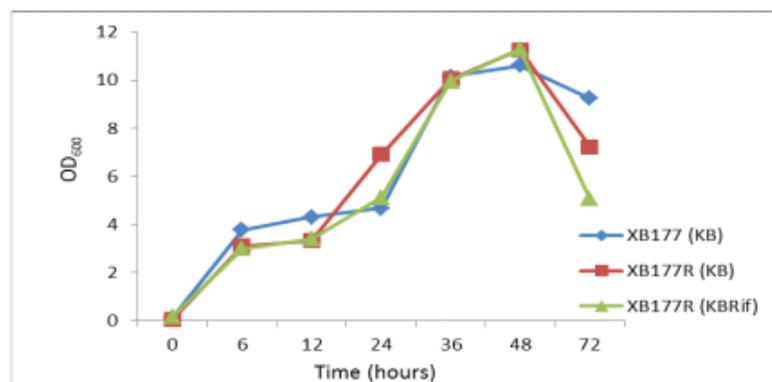


Fig. 4.8 Growth curves of 177 (*Bacillus cereus*) and its rifampicin resistant mutant XB177R grown in KB and KBRif broth.

XB99 and XB123 also solubilized phosphate and produced IAA which may be involved in observed increase in eggplant growth in this study.

4.3.4.4. Colonization by *Bacillus cereus* (XB177)

XB177R (RR mutant of XB177) was found to colonize eggplant systemically from rhizosphere to leaves (Table 4.4 and Fig. 4.13). Colonization of eggplant by XB177R was studied until 60 DAI under greenhouse conditions. Population of XB177R in rhizosphere of eggplant was found to remain steady in the range of 6.0 Log CFU.g⁻¹ rhizosphere soil upto 60 DAI. Endophytic colonization of XB177R in the stem was observed starting from 7 DAI, remained steady until 15 DAI at 5.0 Log CFU.g⁻¹ fresh weight and at 30 DAI it increased to 5.65 ± 0.07 Log CFU.g⁻¹ fresh weight. However its endophytic population reduced at 45 DAI to 4.99 Log CFU.g⁻¹ at 45 DAI and then increased to 5.28 Log CFU.g⁻¹ at the 60th day. XB177R colonized the leaf at 15 DAI with a population of 4.04 ± 0.65 Log CFU.g⁻¹ fresh weight which remained steady in the range of 4.0 Log CFU.g⁻¹ until the 45th day and then reduced to 3.73 ± 0.07 Log CFU.g⁻¹ fresh weight at 60 DAI.

XB177 is identified as *Bacillus cereus* and formed sub-terminal spores when observed by malachite green staining. Bacilli including *Bacillus cereus* are known antagonist of *R. solanacearum* (Anith *et al.*, 2004; Aliye *et al.*, 2008; Ji *et al.*, 2008; Chen *et al.*, 2012; Feng *et al.*, 2013). *B. cereus* is naturally present in soil (McSpadden Gardener, 2004) and has been isolated from several plant species namely tomato, soybean, maize, tobacco, groundnut, grapevine and others (Bai *et al.*, 2002; Figueiredo *et al.*, 2009; West *et al.*, 2010; Xiao *et al.*, 2012; Dutta *et al.*, 2013). Strains of *B. cereus* produce bacteriocins, tunicamycin, zwitterimycin and other antibacterial and antifungal

metabolites and improve plant health (Handelsman *et al.*, 1990; Silo-Suh *et al.*, 1994; Oscariz *et al.*, 1999; Bizani *et al.*, 2005).

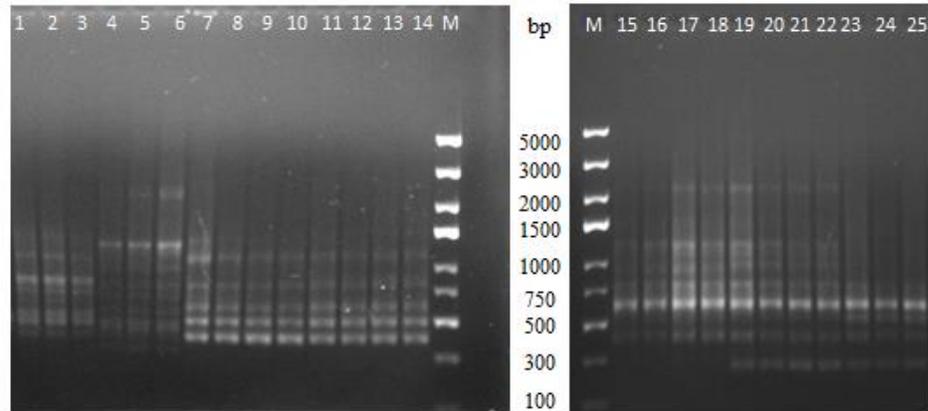


Fig. 4.9a BOX PCR fingerprints of XRB, rifampin mutants and re-isolated strains from eggplant. M- 100 bp to 5000 bp ladder, Lane 1-XB99, Lane 2-XB99R, Lane 3-XB99R-30D-Rh, Lane 4- XB123, Lane 5-XB123R, Lane 6-XB123R-30D-Rh, Lane 7-XB122, Lane 8-XB122R, Lane 9- XB122R-15D-Rh, Lane 10-XB122R-30D-Rh, Lane 11- XB7, Lane 12-XB7R, Lane 13-XB7R- 15D-Rh, Lane 14-XB7R-30D-Rh, Lane 15- XB1, Lane 16-XB1R, Lane 17-XB1R-30D-E, Lane 18-XB1R-30D-Rh, Lane 19-XB86, Lane 20-XB86R, Lane 21-XB86R-30D-E, Lane 22-XB86R- 30D-Rh, Lane 23-XB165, Lane 24-XB165R, Lane 25-XB165R-30D-Rh. D-days after inoculation, R- Rifampicin resistant mutant, Rh-Rhizosphere, E- endophytic.

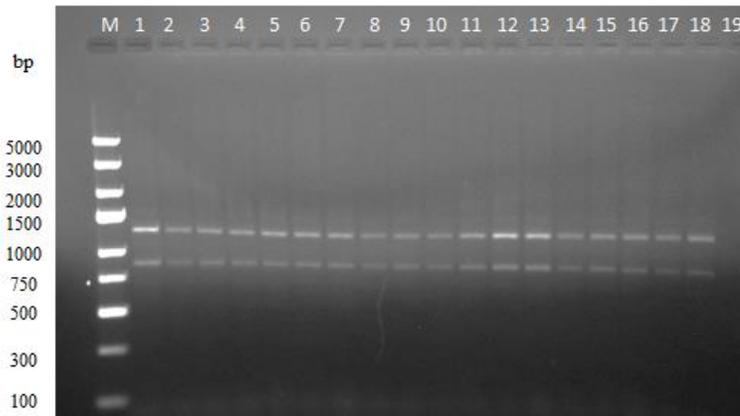
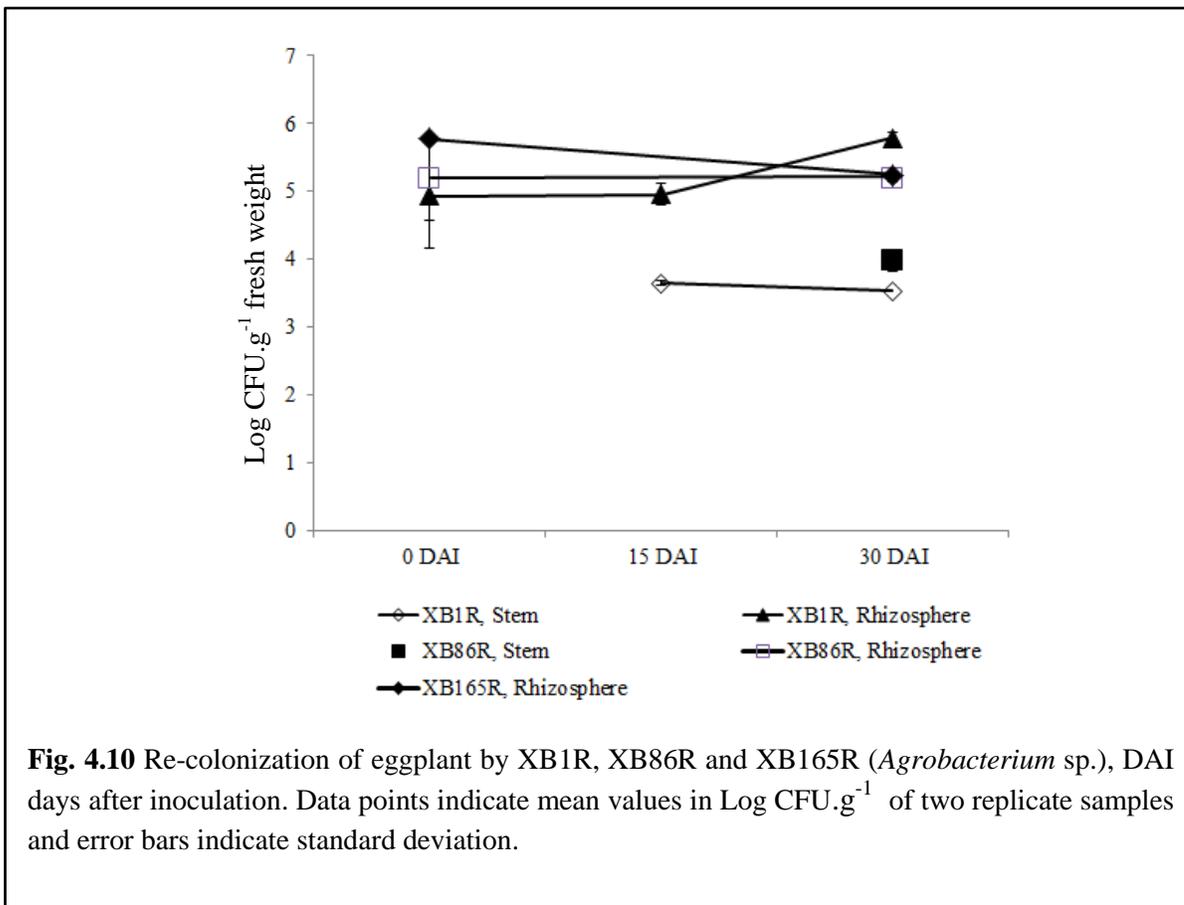


Fig. 4.9b BOX PCR fingerprints of XB177, rifampin mutant and re-isolated strains from eggplant at various time periods. M- 100 bp to 5000 bp ladder, Lane 1- XB177, Lane 2- XB177R, Lane 3- XB177R-15D-L, Lane 4- XB177R-15D-E, Lane 5- XB177R-15D-E, Lane 6- XB177R-15D-Rh, Lane 7- XB177R-30D-L, Lane 8- XB177R-30D-E, Lane 9- XB177R-30D-E, Lane 10- XB177R- 30D-Rh, Lane 11- XB177R-45D-L, Lane 12- XB177R-45D-E, Lane 13- XB177R-45D-E, Lane 14- XB177R-45D-Rh, Lane 15- XB177R-60D-L, Lane 16- XB177R-60D-E, Lane 17- XB177R- 60D-E, Lane 18- XB177R-60D-Rh, Lane 19- Negative control, D-days after inoculation, R- Rifampicin mutant, Rh-Rhizosphere, E- endophytic, L-leaf.

Table 4.3 Population of XRB re-isolated from eggplant at different point of times.

	0 DAI		15 DAI		30 DAI	
	Stem	Rhizosphere	Stem	Rhizosphere	Stem	Rhizosphere
XB1R	ND	4.93± 0.78	3.65 ± 0.01	4.96 ± 0.16	3.53 ± 0.04	5.78 ± 0.08
XB86R	ND	5.20 ± 0.63	ND	ND	3.98 ± 0.15	5.19 ± 0.05
XB165R	ND	5.77 ± 0.75	ND	ND	0	5.23 ± 0.01
XB7R	ND	5.24 ± 0.07	0	4.67 ± 0.04	0	4.67 ± 0.07
XB122R	ND	5.10 ± 0.07	0	4.64 ± 0.14	0	4.60 ± 0.14
XB99R	ND	5.42 ± 0.66	3.94 ± 0.13	4.82 ± 0.23	3.70 ± 0.03	4.83 ± 0.11
XB123R	ND	5.25 ± 0.69	0	5.50 ± 0.10	0	4.68 ± 0.14

Values are mean of population in Log CFU.g⁻¹ fresh weight from two plants with two replicates per dilution and showing standard deviation, DAI: days after inoculation, ND: not determined. All experiments were performed using eggplant cv. *Agassaim* under greenhouse conditions. *In vitro* assessment of population was done on KBRif agar at 28 ± 2°C.



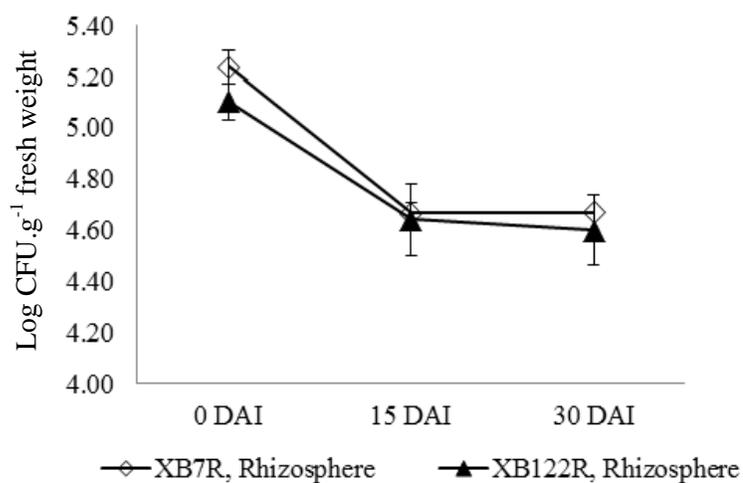


Fig. 4.11 Re-colonization of eggplant by XB7R and XB122R (*Pseudomonas aeruginosa*), DAI days after inoculation. Data points indicate mean values in Log CFU.g⁻¹ of two replicate samples and error bars indicate standard deviation.

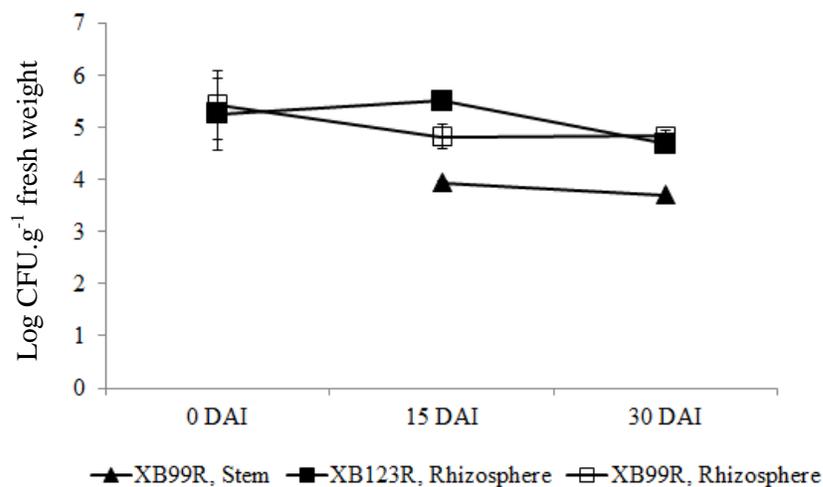


Fig. 4.12 Re-colonization of eggplant by XB99R and XB123R (*Enterobacter* sp.), DAI days after inoculation. Data points indicate mean values in Log CFU.g⁻¹ of two replicate samples and error bars indicate standard deviation.

Table 4.4 Population XB177R in re-isolated from leaves, stem and rhizosphere of eggplant.

	0DAI	7DAI	15DAI	30DAI	45DAI	60DAI
Rhizosphere	6.57 ± 0.54	6.19 ± 0.02	6.71 ± 0.04	6.50 ± 0.33	6.70 ± 0.26	6.33 ± 0.26
Stem	ND	5.17 ± 0.32	5.05 ± 0.11	5.65 ± 0.07	4.99 ± 0.00	5.28 ± 0.36
Leaf	ND	ND	4.04 ± 0.65	4.34 ± 0.23	4.99 ± 0.14	3.73 ± 0.07

Values are mean of population in Log CFU.g⁻¹ fresh weight from two plants with two replicates per dilution and showing standard deviation, DAI: days after inoculation, ND: not determined. All experiments were performed using eggplant cv. *Agassaim* under greenhouse conditions. *In vitro* assessment of population was done on KBRif agar at 28 ± 2°C.

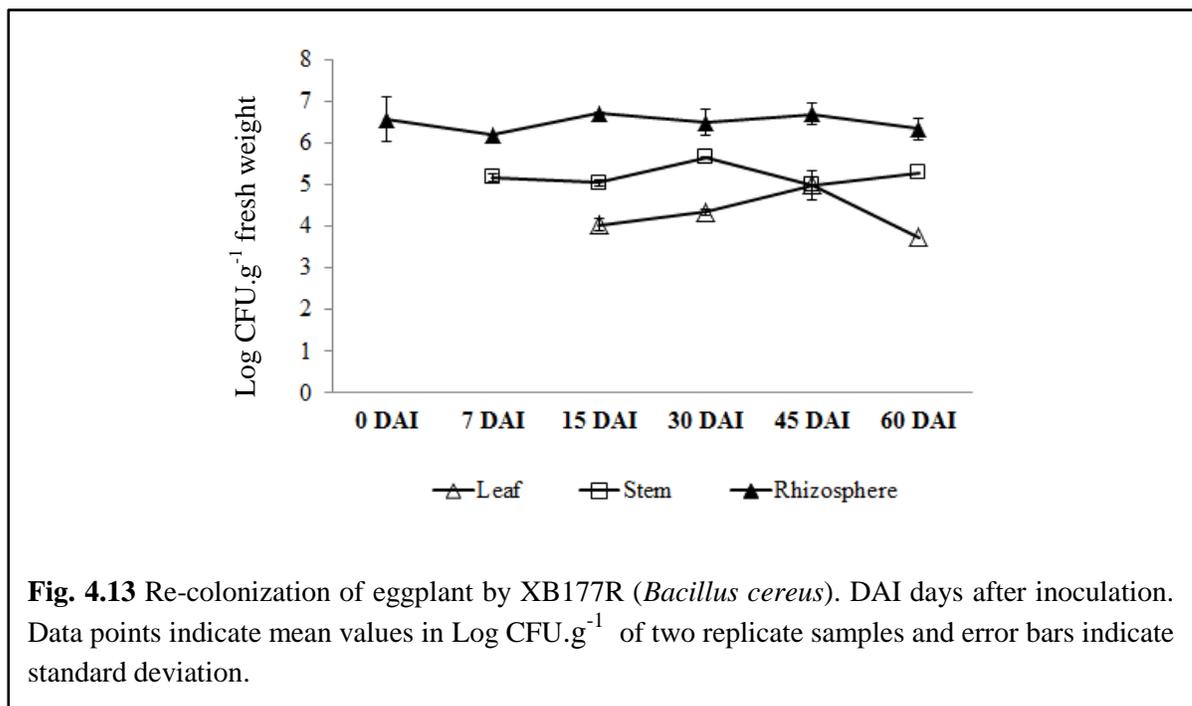


Fig. 4.13 Re-colonization of eggplant by XB177R (*Bacillus cereus*). DAI days after inoculation. Data points indicate mean values in Log CFU.g⁻¹ of two replicate samples and error bars indicate standard deviation.

As seen in the present study, systemic colonization by other Bacilli from rhizosphere to leaves and BW prevention through ISR has been reported (Ji *et al.*, 2008). *Bacillus* sp. are well known to stimulate plant growth by production of phytohormones mainly IAA (Jetiyanon and Kloepper, 2002; Choudhary and Johri, 2009; Kumar *et al.*, 2011).

In this study antagonistic XB177 produced bacteriocin inhibitory to *R. solanacearum* which may have contributed to its high BCE of 93.75%. Efficient colonization of eggplant by XB177 may have contributed to the biocontrol of BW in greenhouse tests. In addition XB177 produced IAA which may have led to increase in growth in eggplant as seen in this study. Persistence in leaves and in rhizosphere over extended periods is attributed to spore formation by *B. cereus* (Melnick *et al.*, 2008) and can be correlated to the current results.

Other strains of XRB namely, XB169 (*Staphylococcus gallinarum*), XB170 (*Staphylococcus* sp.), XB197 (*Sphingomonas* sp.) could not be recovered from rhizosphere and endophytic tissues of eggplant.

4.3.5. Population dynamics of XB177 during wilt infection

In planta interactions of XB177 and Rs-09-100 in eggplant during wilt infection were studied. Rs-09-100 colonized stem and rhizosphere of eggplant irrespective whether the plants were pre-treated, co-inoculated or post-treated with XB177 (Table 4.5) that is Rs-09-100 caused latent infection in healthy looking plants in the treatments. Wilt incidence in co-inoculated and post-treated plants was higher and ranged between $86.66 \pm 23.09\%$ to $93.33 \pm 11.54\%$, whereas in pre-treated plants wilt incidence was only $20.0 \pm 16.32\%$. Appearance of wilt symptoms was delayed by 4-6 days in the pre-treated plants and 80.0% plants remained healthy at 23 DAI (Table 4.6).

Population of XB177 in stem and rhizosphere of eggplant in unchallenged control (not inoculated with Rs-09-100) was found to be highest (5.92 Log \pm 0.07 and 7.03 Log \pm 0.68 CFU.g⁻¹ stem and rhizosphere soil) (Table 4.5). However population of XB177 in co-inoculated plants which remained healthy (3.72 Log \pm 0.46 and 5.70 Log \pm 0.03 CFU.g⁻¹ stem and rhizosphere soil) and healthy plants in post-treated pots (4.84 Log and 5.74 Log \pm 0.03 CFU.g⁻¹ stem and rhizosphere soil) was found to be low. Population of XB177 in wilted plants in the three treatments ranged from 4 Log CFU.g⁻¹ of stem and 5-6 Log CFU.g⁻¹ of rhizosphere soil. However, when XB177 is inoculated before the pathogen it achieves a somewhat higher population of 5.48 Log \pm 0.04 and 6.18 Log \pm 0.11 CFU.g⁻¹ stem and rhizosphere soil. This suggests that Rs-09-100 affects endophytic and rhizosphere colonization by XB177 in eggplant.

Population of Rs-09-100 in stem and rhizosphere of wilted eggplant in untreated control was highest (11.28 Log \pm 0.02 and 10.98 Log \pm 0.06 CFU.g⁻¹) and it caused a wilt incidence of 90.0 \pm 20.0% (Table 4.5 and Table 4.6). Population of Rs-09-100 in a few plants which remained healthy/ latently infected (at 23DAI of Rs-09-100) in co-inoculated and post-treated pots was found to be less than 10.0 Log CFU.g⁻¹ stem and rhizosphere soil. However, population of Rs-09-100 in all three treatments after appearance of wilt symptoms was found to be ten-fold higher (10.0 Log CFU.g⁻¹ of stem and 9-10 Log CFU.g⁻¹ rhizosphere soil) (Table 4.5). This suggests when endophytic population of Rs-09-100 exceeds above 10.0 Log CFU.g⁻¹ in stem, wilting occurs.

In agreement with this observation it was found that the population of Rs-09-100 in pre-treated healthy plants was lower than Log 10.0 CFU.g⁻¹ (7.55 Log \pm 0.08 and 7.34 Log \pm 0.08 CFU.g⁻¹ of stem and rhizosphere soil respectively), no wilt symptoms appeared and plants were visually healthy.

Table 4.5 Population dynamics of XB177 and Rs-09-100 in eggplant during wilt infection.

XB177	Healthy		Wilted	
	Stem	Rhizosphere	Stem	Rhizosphere
Pre-treated	5.48 ± 0.04	6.18 ± 0.11	4.98 ± 0.59	6.64 ± 0.04
Co-inoculated	3.72 ± 0.46	5.70 ± 0.03	4.89 ± 0.65	5.58 ± 0.70
Post-treated	4.84 ± 0.00	5.74 ± 0.03	4.83 ± 0.15	6.36 ± 0.03
Control XB177	5.92 ± 0.07	7.03 ± 0.68	NA	NA
Rs-09-100				
Pre-treated	7.55 ± 0.08	7.34 ± 0.08	10.64 ± 0.31	9.98 ± 0.54
Co-inoculated	9.41 ± 0.01	9.20 ± 0.18	10.63 ± 0.38	10.34 ± 0.18
Post-treated	9.39 ± 0.10	9.25 ± 0.05	10.62 ± 0.36	10.03 ± 0.52
Control Rs-09-100	ND	ND	11.28 ± 0.02	10.98 ± 0.06

Values are mean of population in Log CFU.g⁻¹ fresh weight from two plants with two replicates per dilution and showing standard deviation, DAI: days after inoculation, NA: not applicable, ND: not determined. All experiments were performed using eggplant cv. *Agassaim* under greenhouse conditions. Healthy plants to assess population of XB177 and Rs-09-100 were taken after 23 DAI (after inoculation of Rs-09-100 i.e 43 DAI after XB177) and wilted plants to assess population of Rs-09-100 and XB177 were taken after the plants showed wilt symptoms (approx. 5-7 DAI of inoculation of Rs-09-100 i.e. 25-27 DAI of XB177). *In vitro* assessment of population was done on KB and SMSA agar for XB177 and Rs-09-100 respectively at 28 ± 2°C.

Table 4.6 Wilt incidence in eggplant recorded during population dynamics studies.

Treatment	Appearance of wilt symptoms (DAI)	Wilt incidence (%) at 23DAI
Pre-treated	10	20.00 ± 16.32
Co-inoculated	4	86.66 ± 23.09
Post-treated	6	93.33 ± 11.54
Untreated Control	4	90.0 ± 20.0

Values are mean % wilt incidence observed in two replications. DAI: days after inoculation of Rs-09-100. All experiments were performed using eggplant cv. *Agassaim* under greenhouse conditions.

This leads to conclusion that XB177 does not prevent entry and colonization of Rs-09-100 in eggplant stem and rhizosphere. However, treatment with XB177 may delay appearance of wilt and control wilt to the extent of 80% only when it is able to achieve a population of greater than 5.0 Log CFU.g⁻¹ in stem and 6.0 Log CFU.g⁻¹ in the rhizosphere of eggplant. Population of *R. solanacearum* in tomato during wilt infection has been reported to reach levels greater than 10.0 Log CFU.cm⁻¹ stem concomitant with wilting (Genin and Boucher, 2002) which is of the same order as observed in wilted eggplant in this study. Latent infection by *R. solanacearum* in tomato plants has been reported (Schönfeld *et al.*, 2003) and it can reach population of 9 Log CFU.g⁻¹ in stem tissues of symptomless geranium plants (Swanson *et al.*, 2005). Similarly Pradhanang *et al.* (2000) have reported a high population of *R. solanacearum* in stem and roots ranging from 7 Log to 8 log CFU.g⁻¹ tissue in weeds namely *Drymaria cordata* and *Cerastium glomeratum*, with no wilting symptoms, whereas the population of *R. solanacearum* in wilted weed namely *Tropaeolum majus* was higher than 9 Log CFU.g⁻¹ tissue.

These studies show that a population greater than 9 Log CFU.g⁻¹ tissue is necessary for appearance of wilt symptoms in plants. In this study, endophytic colonization by XB177 controlled increase in population levels of *R. solanacearum* beyond 7 Log CFU.g⁻¹ of stem leading to suppression of BW symptoms but not the entry of pathogen and its colonization in rhizosphere of eggplant cv. *Agassaim*. Tan *et al.* (2013) studied population dynamics of two antagonistic strains *Bacillus amyloliquefaciens* and *R. solanacearum* and found that these biocontrol strains colonized tomato rhizosphere with a population density ranging from 4.0 to 8.0 Log CFU.g⁻¹ rhizosphere soil until five weeks and restricted population of *R. solanacearum* below 8.0

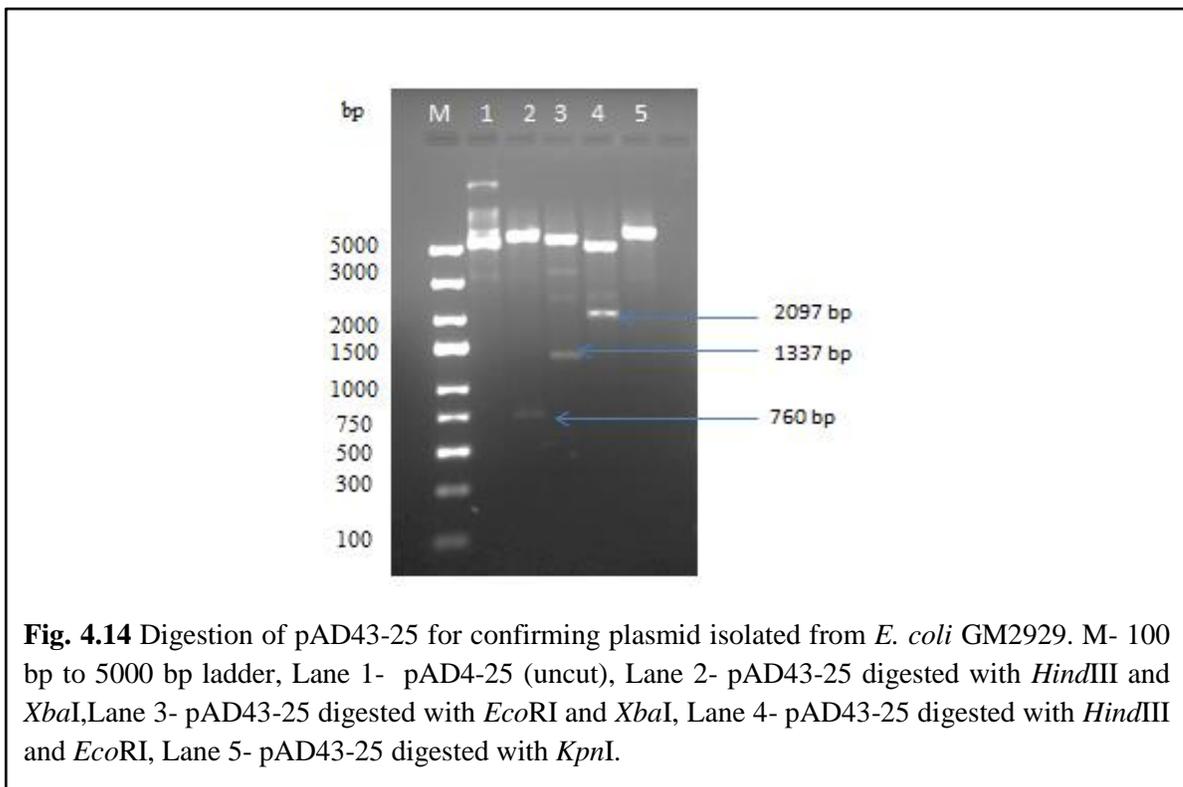
Log CFU.g⁻¹ in rhizosphere soil, but did not prevent colonization of rhizosphere by *R. solanacearum*.

Inoculation of biocontrol strain before and simultaneously with *B. subtilis* controlled wilt incidence, however treatment after challenge with *R. solanacearum* was of little use in preventing wilt (Ji *et al.*, 2008). In the present study, treatment 20 days before challenge of pathogen was the only useful method for preventing wilt in eggplant using XB177. Similar observations on success of pre-treatment of plants before challenging with *R. solanacearum* have been reported (Das and Bora, 2000). However, when co-inoculation of XB177 and Rs-09-100 was done, Rs-09-100 was found to be a faster colonizer and caused wilting symptoms within 4 days after inoculation. Competitive fitness of *R. solanacearum* in plant colonization is governed multiple factors including swimming motility, virulence factors, Type III secretion system (T3SS) and quorum sensing (Yao and Allen, 2006; Coll and Valls, 2013; Meng, 2013) which leads to rapid ingress and colonization of plants and development of disease in a susceptible host.

Systemic spread of endophytic bacteria through xylem vessels has been shown to be mediated by production of cell wall degrading enzymes namely endoglucanase through the type II secretion system and thus it is speculated that endophytes evolved from parasites (Preston *et al.*, 1998). XB177 is motile and also produces cellulase and pectinase (Table 3.12 and Table 3.14 in chapter III) which may aid in its endophytic colonization and systemic spread in eggplant. Involvement and importance of systemic colonization of mulberry by *B. subtilis* in prevention of BW has been reported earlier (Ji *et al.*, 2008). Endophytic and rhizosphere colonization of cotton by antagonistic strain of *B. cereus* was found to be effective in biocontrol of diseases under greenhouse conditions (Pleban *et al.*, 1995).

4.3.6. Visualization of pattern of colonization of XB177 by GFP tagging

In order to confirm endophytic lifestyle after isolation from surface sterilized plants, microscopic observations to affirm re-colonization by putative endophytes is very essential. *Bacillus-E.coli* shuttle vector pAD43-29 with GFP $mut3a$ gene was successfully transformed into XB177 cells by electroporation to study its pattern of endophytic colonization. Vector obtained from a dam^-dcm^- *E. coli* GM2929 was necessary because no transformants were obtained on using vector DNA isolated from *E. coli* DH5 α , indicating that XB177 restricts methylated DNA. Strains of *Bacillus* sp. have been reported to restrict methylated foreign DNA transformed in cells (Macaluso and Mettus, 1991; Quinn *et al.*, 1991; Dunn *et al.*, 2003). pAD43-25 isolated from *E. coli* DH5 α was transformed in *E. coli* GM2929 and unmethylated plasmid was obtained after isolation. Restriction digestion of re-isolated plasmid was a confirmatory test before transforming in XB177 (Fig. 4.14).



After a successful transformation using unmethylated pAD43-25, GFP expression in XB177G cells (XB177 tagged with pAD43-29) was found to be strong owing to the constitutive expression of GFP from a *B. cereus* promoter and useful for observation of GFP positive cells and colony directly under fluorescence microscope (Fig. 4.15a to c). Counter staining of stem tissues with 0.05% methyl violet reduced strong green-yellow auto-fluorescence from plant phenolic compounds and enhanced the contrast of GFP tagged XB177G cells. XB177G colonized root and stem of eggplant when tested at 7 DAI, 20 DAI and 30 DAI by microscopic observations under fluorescence microscope. XB177G cells colonized the root tip (Fig. 4.16 d) and appeared as clusters or singlets on root hair, rhizoplane and sites of lateral root emergence at 7DAI (Fig. 4.15 e to h). Later at 20 DAI XB177G was observed in cortical cells and endodermal cells (Fig. 4.15 i and j). However in the xylem vessels of eggplant, chilli and tomato stems, XB177G cells were observed mainly as singlets (Fig. 4.15 k-n).

No biofilm or macro-colonies of XB177G were observed in rhizoplane and endophytic tissues of eggplant, chilli and tomato after 7 DAI. Production of endoglucanase and pectinase by endophytic bacteria has been shown to be involved in colonization (Compant *et al.*, 2005b). XB177 produces cell wall degrading enzymes which may have enabled it to colonize endophytic tissues after initial colonization of rhizosphere, cortical and endodermal cells. XB177R was re-isolated from leaf samples, however, colonization of XB177G in leaf sections could not be detected. Endophytic bacilli have diverse patterns of colonization. GFP tagged *Bacillus subtilis* cells were found as singlets in endophytic tissues of mulberry and no biofilm formation was reported (Ji *et al.*, 2008). Similarly *Bacillus cereus* strain X5 was mainly seen as single cells when studied for colonization of tomato rhizosphere (Xiao *et al.*, 2012). However a

close relative, *Paenibacillus polymyxa* formed robust biofilms on wheat and soybean roots (Timmusk *et al.*, 2005; Annapurna *et al.*, 2013).

Mean generation time of XB177G (69.89 min) was slightly longer than XB177 (59.36 min) as observed in KB broth without chloramphenicol ($10 \mu\text{g.mL}^{-1}$) (Table 4.7). However, at the end of 10h without antibiotic 55.56% cells of XB177G were chloramphenicol resistant (Cm^{R}) and GFP positive. At the end of 21 h without antibiotic 49.79% cells were Cm^{R} GFP positive and after 30.68 generations (34 h) the culture broth of XB177G contained only 28.08% GFP positive cells.

This suggests that without antibiotic pressure 71.92% cells of XB177G would lose the plasmid pAD43-25 by 34 h in culture broth. Loss of pAD43-25 from XB177 (*Bacillus cereus*) is slightly higher than earlier study which reports loss of pAD43-25 from 70.67 % of *B. cereus* cells after 35 generations (Dunn and Handelsman, 1999). Rapid loss of plasmid by XB177G may have left few GFP positive cells visible when observed under fluorescent microscope over a period of 30 days. However when population of XB177G was assessed by plating at 30 DAI, ten-fold higher population of XB177G was present on KB agar as compared to that on KBC agar (KB agar with chloramphenicol $10 \mu\text{g.mL}^{-1}$) in eggplant and chilli and five-fold higher in tomato (Table 4.8).

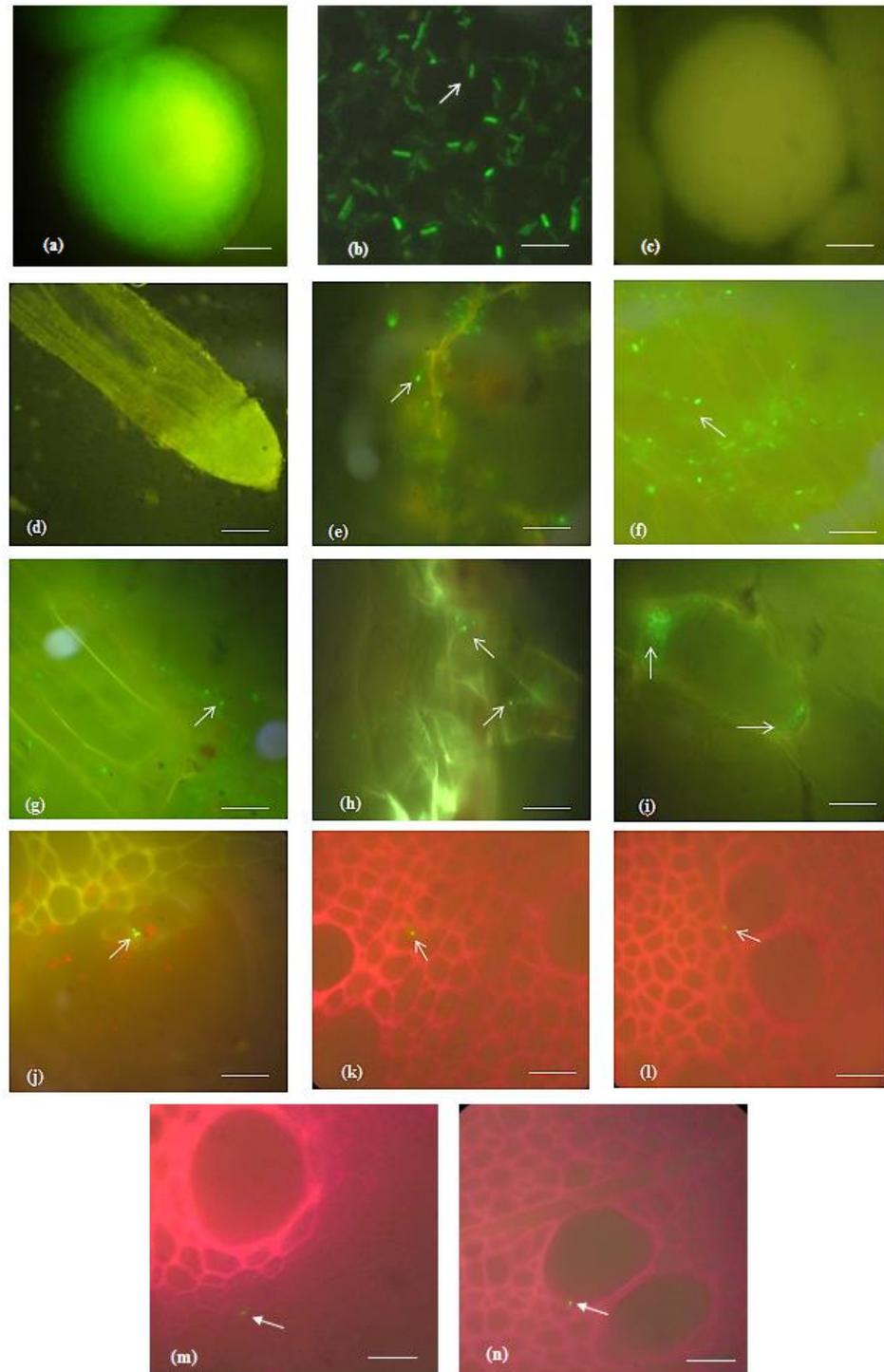


Fig. 4.15 Visualization of XB177G colonizing eggplant seedlings at 7-30 days after inoculation under fluorescence microscope. (a) Colony of XB177G observed, (b) cells of XB177G, (c) colony of XB177 (untagged), (d) cells of XB177G expressing GFP were observed on root tip, 7 DAI, (e) root hair, 7DAI, (f-g) rhizoplane, (h) 7DAI, sites of lateral root emergence, 7DAI, (i) cortical cells, 20 DAI, (j) endodermis cells, 20 DAI, (k-l) xylem vessels of stem of eggplant, 30 DAI, (m) xylem vessels of tomato stem, (n) xylem vessels of chilli stem (n). Scale bars: (a-c) 50 mm, (d) 500 μ m, (e-n): 10 μ m.

This shows that there is loss in plasmid during in planta colonization by XB177G. Integration vectors are a superior choice over other GFP recombinant vectors because they are stably maintained in the chromosome. Homologous recombination based integration vector for tagging of *Bacillus subtilis* with GFP at the *amyE* locus (alpha-amylase), *LacA* (beta-galactosidase) and *bglS* (beta-glucosidase) have been reported (Hartl *et al.*, 2001; Bisicchia *et al.*, 2010).

Use of these vectors for integration at similar sites in genome of *Bacillus cereus* could be useful in determining its colonization in various plant micro-niches over a long period. In addition, differential GFP emission and failure to detect fluorescence in bacteria tagged with GFP even in presence of an active promoter has been observed earlier due to occasional incorrect folding of GFP and differences in physiological state of cells in natural environment (Timmusk *et al.*, 2005). In addition, it has been reported that *Bacillus* sp. form endospores *in planta* and in rhizosphere (Melnick *et al.*, 2008; Huang *et al.*, 2012) which cannot express GFP.

Increased number of colonies of XB177G on KB medium might be due to the presence of XB177 without the GFP plasmid. However spore count by heating and plating the samples as described by Melnick *et al.* (2008) could give a measure of additional GFP positive XB177 present colonizing eggplant.

Table 4.7 Generation time of XB177 and XB177G and plasmid stability in XB177G in absence of chloramphenicol.

Transfer	XB177			XB177G		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd
Incubation period	10 h	11 h	13 h	10 h	11 h	13 h
Ln (N ₀)	15.83	16.46	15.08	15.66	16.44	15.62
Ln (N _t)	23.35	23.69	24.10	21.31	23.17	24.50
No. of generations (n)	10.85	10.43	13.01	8.15	9.72	12.81
n per hour	1.09	0.95	1.00	0.81	0.88	0.89
Generation time (min)	55.29	63.27	59.54	74.1	68.18	67.41
Percentage of GFP positive (Cm ^R) cells before each transfer	NA	NA	NA	93.70 %	64.98 %	38.52 %
Percentage of GFP positive (Cm ^R) cells at the end of incubation	NA	NA	NA	55.56 %	49.79 %	28.08 %

In vitro assessment of population was done on KB and KBC agar at $28 \pm 2^\circ\text{C}$. Mean generation time of XB177 is 59.36 min whereas that of XB177G is 69.89 min. At the end of third transfer and growth of 30.68 generations in 34 h the culture broth of XB177G contained 28.08% GFP positive cells in absence of chloramphenicol ($10 \mu\text{g.mL}^{-1}$). Formula for calculating number of generations (n) = $[\text{Ln} (N_t) - \text{Ln} (N_0)] / \text{Ln} 2$, where n is the number of generations, N_t is the number of colonies before each successive dilution, N₀ is the number of colonies after each successive dilution, Ln 2 = 0.69 (Ramos *et al.*, 2002).

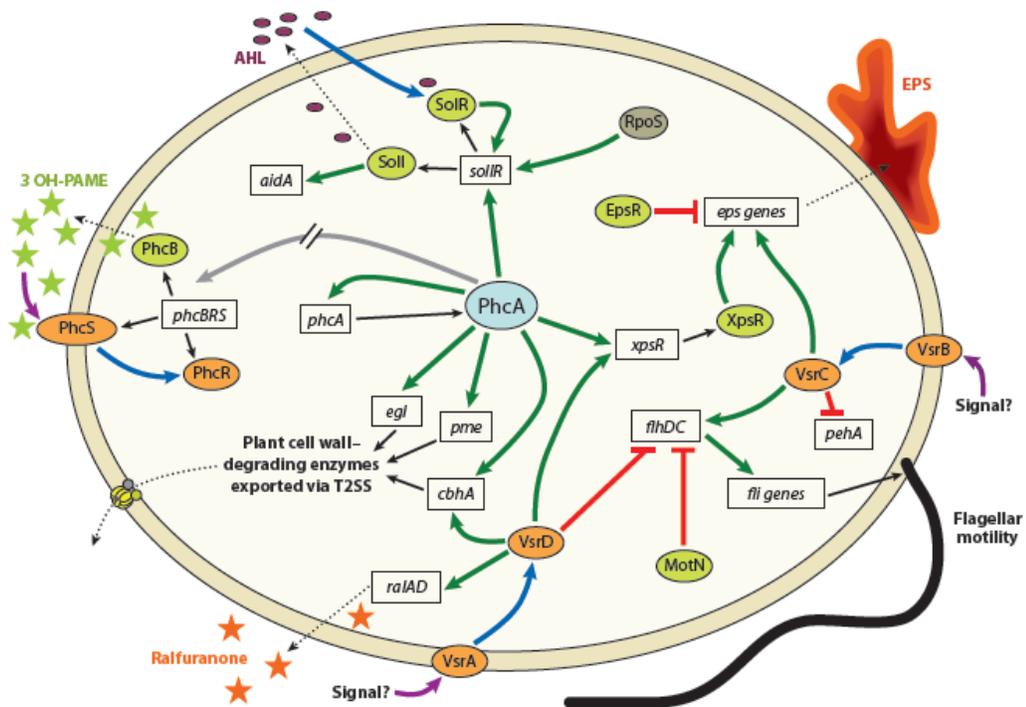
Table 4.8 Population of XB177G re-isolated from eggplant, tomato and chilli at 30 DAI.

	Eggplant		Tomato		Chilli	
	KBC	KB	KBC	KB	KBC	KB
Stem	3.30 ± 0.17	4.51 ± 0.00	3.10 ± 0.10	3.59 ± 0.13	2.86 ± 0.14	3.10 ± 0.08
Rhizosphere	6.20 ± 0.23	7.04 ± 0.48	6.19 ± 0.03	6.47 ± 0.34	5.80 ± 0.03	6.17 ± 0.02

Values are mean of population in Log CFU.g⁻¹ fresh weight from two plants with two replicates per dilution and showing standard deviation, DAI: days after inoculation, ND: not determined. All experiments were performed using eggplant cv. *Agassaim* under greenhouse conditions. *In vitro* assessment of population was done on KB and KBC agar at 28 ± 2°C. KB: population on King's B agar; KBC: population on King's B agar with chloramphenicol (10 µg.mL⁻¹).

CHAPTER V

Mutagenesis of *phcB* gene of quorum sensing system involved in biosynthesis of signaling molecule (3OH-PAME) in *R. solanacearum*



Quorum sensing regulated traits in *R. solanacearum* (Genin and Denny, 2012).

5.1. INTRODUCTION

Quorum sensing plays an important role in the expression of virulence factors of *R. solanacearum* mainly EPS and Egl. The quorum sensing is regulated by Phc (**Ph**enotype **c**onversion) system and consists of *phcA* gene and the *phcBSRQ* operon. *phcA* gene encodes PhcA a LysR type transcriptional regulator, *phcB* encodes SAM dependent methyl transferase which synthesizes 3OH-PAME, *phcS* encodes the membrane bound sensor kinase, *phcR* and *phcQ* encode a response regulators (Schell, 2000). Mutagenesis is a powerful tool to study gene function. Mutagenesis of genes expressing virulence factors in *R. solanacearum* by transposon mutagenesis, insertional mutagenesis, deletion mutagenesis and allelic exchange have been performed for studying the effect on virulence and *in planta* colonization (Allen *et al.*, 1991; Yao and Allen 2006). *phcA* and *phcB* mutants are non-fluidal and avirulent whereas mutations in *phcS* and *phcR* had no obvious change in phenotype or virulence of *R. solanacearum* (Schell, 2000). Though colonization of plants by spontaneous *phcA* mutants and their reversion to wild types is recently reported, ability of *phcB* mutants to colonize plants is unclear. Studies on mutagenesis of *phcB* gene were undertaken with an aim to determine its role colonization and its suitability as a target for quorum quenching in *R. solanacearum*.

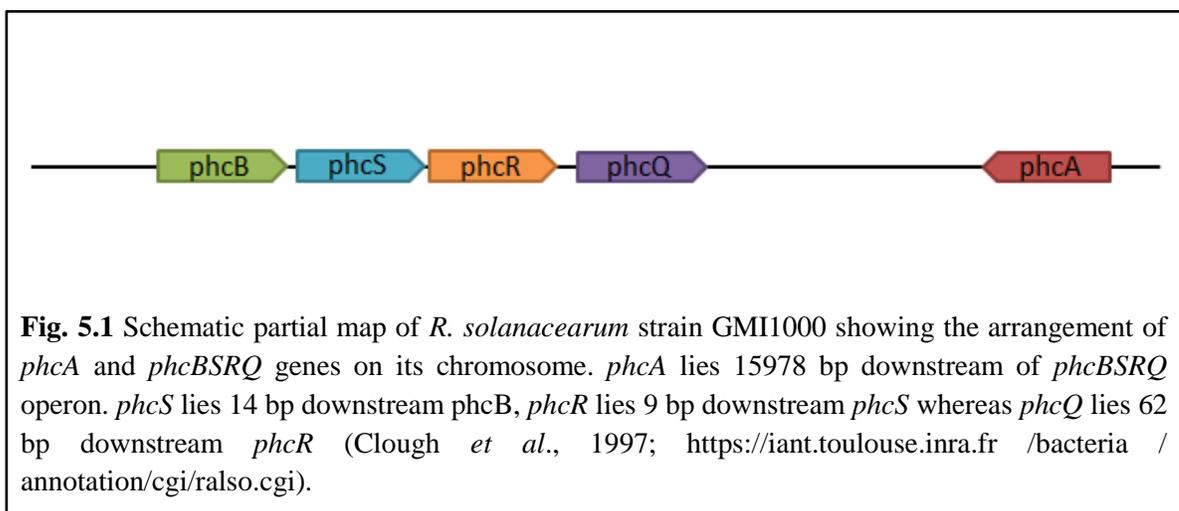
The work presented in this chapter was done in the labs of Dr. Christopher Taylor and Dr. Sally Miller at the department of Plant Pathology, Ohio State University-Ohio Agricultural Research Development Centre (OARDC), Wooster, OH 44691, USA under the Fulbright-Nehru Fellowship. The results presented in this chapter are pertaining to the objective ‘**To study quorum sensing pathway of *R. solanacearum*.**’

5.2. MATERIALS AND METHODS

5.2.1. PCR amplification for obtaining sequences for *phcA* and *phcB* genes for mutagenesis

5.2.1.1. Primer designing for amplification of *phc* genes of *R. solanacearum*

The *phcA* gene (RSc2748) of *R. solanacearum* strain GMI1000 is 1043 bp and lies approximately 16 kb downstream of the *phcBSRQ* operon on the chromosome (Fig. 5.1). *phcB* gene (RSc2735) is 1403 bp, *phcS* gene (RSc2736) is 1328 bp, *phcR* gene (RSc2737) is 1124 bp and *phcQ* gene (RSc2738) is 1010 bp in *R. solanacearum* strain GMI1000.



Partial sequences of *phcB* and *phcA* genes of following strains of *R. solanacearum* were obtained from the NCBI Database. *R. solanacearum* strains **GMI1000** (Phylotype I strain isolated from tomato from French Guyana), **CFBP2957** (Phylotype IIA strain isolated from tomato from French West Indies), **PO82** (Phylotype IIB strain isolated from potato from Mexico), **CMR15** (Phylotype III strain isolated from tomato from Cameroon), **PSI07** (Phylotype IV strain isolated from tomato from Indonesia).

Sequences of *phcB* and *phcA* genes from above strains were aligned using Vector NTI Advance version 11.5 (Life Technologies Inc., USA) to detect conserved regions. Primer sequences for amplification of *phcA* and *phcB* genes were chosen from the regions which were conserved amongst all the strains. Newly designed primers given in Table 5.1 were used for amplification of internal regions of *phcA* and *phcB* genes from two strains of *R. solanacearum* namely FIRs37 and GB3 respectively available at the department of Plant Pathology, Ohio State University-OARDC, Wooster, USA. For PCR amplification template genomic DNA from GB3 and FIRs37 was isolated using Ultra Clean Microbial DNA isolation kit (MoBio Laboratories Inc., USA). Quality and quantity of the DNA was measured using Nanodrop 1000 (Thermo Scientific, USA) as per manufacturer's instructions. In addition, quality of genomic DNA was determined by agarose gel electrophoresis using 0.8% SeaKem agarose (Lonza Group, USA) gel in 1 X SB buffer (Appendix C). Gels were stained in 1 $\mu\text{g.mL}^{-1}$ ethidium bromide solution for 10 min and documented using Alpha Imager (Alpha Innotech Inc., USA) as per manufacturer's instructions. The DNA samples were diluted to a concentration of 50 $\text{ng.}\mu\text{L}^{-1}$, stored at -20°C and used for subsequent PCR amplifications.

Table 5.1 Primers for amplification of internal regions of *phc* genes of *R. solanacearum*.

Strain	Primers	Primer sequence (5' to 3')
GB3	EOA (F)	GTGTTGACCAGGCTGACCACCACCGCCTTGCCG
	EOB (R)	CAGCTTCCGCAACTCCCAGGGTCAGCAGGTTCG
FIRs37	EOC (F)	CGATGCCCTGTTCGACCGCTTCCTGCTGCG
	EOD (R)	TGCATGCACAGTCATGGTGGAGTGGCGCTTCAG

GB3 is phylotype I strain of *R. solanacearum*, EOA and EOB amplify ~300 bp internal region of *phcB* gene from GB3. FIRs37 is phylotype II strain of *R. solanacearum*, EOC and EOD amplify ~350 bp internal region of *phcA* gene from FIRs37. F- forward primer, R- reverse primer.

5.2.1.2. Amplification of *phc* genes of *R. solanacearum*

For PCR amplification of internal fragment of *phcA* and *phcB* genes, 25 μ L reaction mix contained 1X PCR buffer, 1.5 mM MgSO₄, 250 μ M each dNTP, 0.25 μ M each primer, 2 Units of Taq DNA polymerase (New England Biolabs, USA) and 50 ng template DNA. PCR was performed on PTC-200 Peltier thermal cycler (MJ Research, USA) and the cycle consisted of initial denaturation at 95°C for 9 min followed by 30 cycles of denaturation at 95°C for 30s, annealing at 60°C for 30 s and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The amplification of the PCR products was determined by running PCR product on 1% agarose gel in 1 X SB buffer. Gels were stained in 1 μ g.mL⁻¹ ethidium bromide solution for 10 min and documented using Alpha Imager (Alpha Innotech Inc., USA). PCR products were purified from the gel using Wizard SV gel and PCR clean up system (Promega, USA) as per manufacturer's instructions and sequenced using forward primers (Table 5.1) at the Molecular and Cellular Imaging Centre (MCIC) at OARDC. Partial gene sequences were matched against the sequences available in the nucleotide database from National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) using the BLASTn (Basic Local Alignment Search Tool) program to confirm amplification of *phcA* and *phcB* gene fragments from *R. solanacearum* strains FIRs37 and GB3 respectively.

5.2.1.3. Self-formed Adaptor (SEFA) PCR for amplification of unknown sequences flanking *phc* genes

Based on the nucleotide sequences of internal regions of *phcA* and *phcB* genes of FIRs37 and GB3, new set of primers were designed for self-formed adaptor (SEFA) PCR (Wang *et al.*, 2007) (Table 5.2). SEFA PCR was performed to determine the unknown sequences flanking the known region of *phc* genes. It involves the use of three specific primers

(SP1, SP2 and SP4) designed based the known region of the gene and have a relatively high annealing temperatures. One partially degenerate primer (SP3) which has 18 bp sequence from specific parts from the known region of DNA followed by the sequence NNNNNNNNGTGAAA. Primer SP4 is optional and was not used in SEFA PCR in the present study. Schematic representation of SEFA PCR is given in Fig. 5.2. SEFA PCR was carried out in four steps. Reaction mix (30 μ L) of step 1 consisted of 15 μ L of 2X GC buffer I (TaKaRa Biotechnology Co.), 5 μ L of 2.5 mM dNTPs, 1.5 Units of Long and Accurate Taq (TaKaRa Biotechnology Co., USA), 50 ng template DNA and 1 μ L of 5 μ M SP3 primer. PCR step 1 was performed as given in Table 5.3. After step 1 was complete, three μ L of 5 μ M SP1 was added in each tube and cycle 2 was run. After cycle 2 was complete cycle 3 was started immediately with no additional primers. After cycle 3 was complete, product was diluted ten-fold and one μ L was used as template for cycle 4 using primer SP2. Reaction mix of cycle 4 consisted of 1 X Taq buffer, 1.5mM MgSO₄, 250 μ M dNTPs, 3 μ L of 5 μ M primer SP2, one μ L ten-fold diluted reaction mix from cycle 3 and 2 units of Taq DNA polymerase (New England Biolabs, USA). All cycles of SEFA PCR were performed on PTC-200 Peltier thermal cycler (MJ Research, USA).

The SEFA amplicons were purified using Wizard SV gel and PCR clean up system (Promega, USA) as per manufacturer's instructions, cloned in TA cloning vector PCR2.1 (Invitrogen Inc. USA) and transformed in *E. coli* INVF' cells as per the manufacturer's instructions. The vectors with downstream region and upstream region of *phcA*, and upstream region of *phcB* were designated CGT12026 (a), CGT12026 (b) and CGT12028 respectively. Plasmid DNA from the clones was isolated using Wizard plus Plasmid Midiprep System (Promega, USA) as per the manufacturer's instructions and sequenced using M13F and M13R primers at MCIC, OARDC.

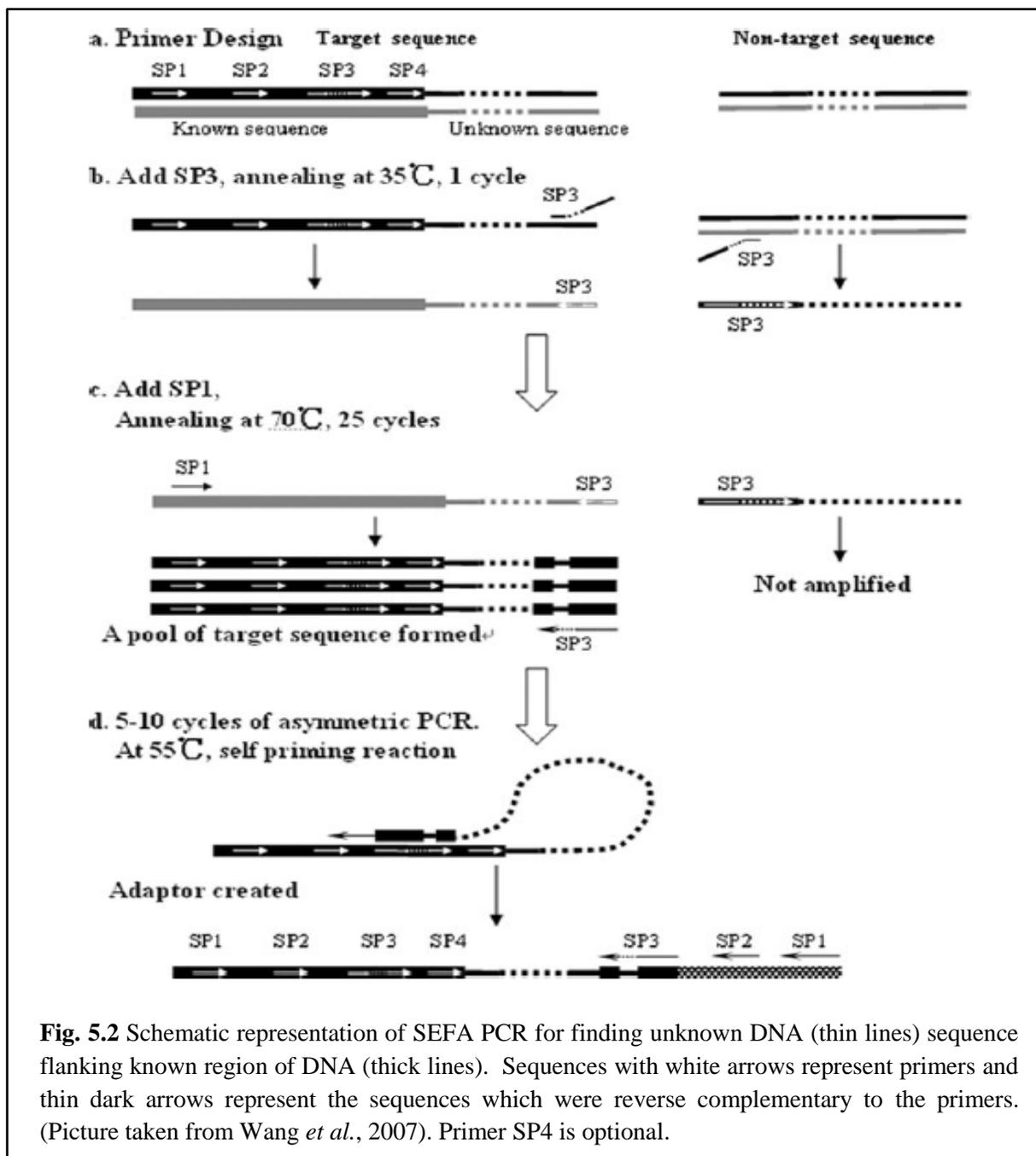


Fig. 5.2 Schematic representation of SEFA PCR for finding unknown DNA (thin lines) sequence flanking known region of DNA (thick lines). Sequences with white arrows represent primers and thin dark arrows represent the sequences which were reverse complementary to the primers. (Picture taken from Wang *et al.*, 2007). Primer SP4 is optional.

Table 5.2 SEFA PCR primers for amplification of unknown upstream and downstream regions of *phc* of *R. solanacearum*.

Primer	Primer (5'-3')	Size of amplicon
EOL ¹	TGGCGCCGGGTCTGCAGGTGCGTCTGACCGA	900
EOM ²	TTCCCGACGCGGCATCCGGAAATCGTGG	
EON ³	GATGGTCCTTGCGCGCGCANNNNNNNNGTGAAA	
EON ³	GATGGTCCTTGCGCGCGCANNNNNNNNGTGAAA	2000
EOO ⁴	GACAGTCATGGTGGAGTGGCGCTTCAGGCC	
EOP ⁵	GGTACTGATCGAGCGAGCAGCGGTTCCTTCA	
EOS ⁶	CAGCTTCCGCAACTCCCAGGGTCAGCAGGTT	750
EOQ ⁷	GACCTCGCTGACCTGCACGATGGAATACGGG	
EOR ⁸	GATGCAGCGGCGCGCCCTGNNNNNNNNGTGAAA	

EOL-EOP: Primers for *phcA* gene of FIRs37, EOS-EOR Primers for *phcB* gene of GB3. ¹ SP1 for downstream of *phcA* of FIRs37, 2 SP2 for downstream of *phcA* FIRs37, 3 common SP3 degenerate primer for amplification of upstream and downstream regions of *phcA* of FIRs37, 4 SP1 for upstream of *phcA* FIRs37, 5 SP2 for upstream of *phcA* FIRs37, 6 SP1 for upstream of *phcB* of GB3, 7 SP2 for upstream of *phcB* of GB3, 8 SP3 for *phcB* of GB3, see plate 5.1 for target binding regions of primers.

Table 5.3 Cycling conditions using SEFA PCR.

Cycle	No. of cycles	Details
1	1	(i) Denaturation at 94°C for 90 sec (ii) annealing at 35°C for 3 mins (iii) ramping to 70°C (iv) extension at 70°C for 5 min (v) 4°C for 5 min
2	25	(i) Denaturing at 94°C for 30 s and (ii) annealing and extension at 70°C for 5.5 min
3	10	(i) Two cycles of denaturing at 94°C for 30 s and annealing and extension at 70°C for 5 min (ii) one cycle of denaturing at 94°C for 30 s, annealing at 55°C to 60°C for 30 s, and extension at 70°C for 5 min
4	20	(i) denaturing at 94°C for 30 s and (ii) annealing and extension at 70°C for 5.5 min.

5.2.2. Designing of homologous recombination (HR) vector for mutagenesis of *phc* genes of *R. solanacearum*.

5.2.2.1. PCR amplification and addition of restriction enzyme sites to flanks of *phc* genes.

Based on the upstream and downstream nucleotide sequences of *phcA* gene of FIRs37 and upstream region of *phcB* gene of GB3 obtained by SEFA PCR, new set of primers were designed (Table 5.4). For amplification of downstream region of *phcB* gene of GB3, standard PCR with primers firing outward from known regions of *phcB* gene and its downstream gene *phcS* were used. All primers were designed with restriction enzyme sites compatible with the MCS of plasmid cloning vectors to be used. For amplification of *phcA* and *phcB* genes 25 μ L reaction mix consisted of 1 X Taq buffer, 1.5mM MgSO₄, 250 μ M dNTPs, 0.25 μ M each primer and 2 units of Taq DNA polymerase (New England Biolabs, USA). Template DNA for PCR for upstream and downstream regions of *phcA* gene was 50 ng plasmid DNA from CGT12026 (b) and CGT12026 (a) respectively. Template for amplification of upstream region of *phcB* was 50 ng plasmid DNA from CGT12028 while 50 ng genomic DNA of GB3 served as template for downstream region of *phcB* gene. For amplification of upstream and downstream regions of *phcA* gene (using primer pair EQI-EQJ and EQG-EQH) cycle consisted of initial denaturation of 95°C for 9 min, followed by 35 cycles of denaturation of 95°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. For amplification of upstream and downstream regions of *phcB* gene (using primer pair EPW-EPX and EPY-EPZ) cycle consisted of initial denaturation of 95°C for 9 min, followed by 30 cycles of denaturation of 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min followed by final extension at 72°C for 10 min.

The amplification was determined by running PCR products on 1 % agarose gel in 1 X SB buffer. Gels were stained in 1 $\mu\text{g.mL}^{-1}$ ethidium bromide solution for 10 min and documented using Alpha Imager (Alpha Innotech Inc., USA). PCR products were purified from the gel using Wizard SV gel and PCR clean up system (Promega, USA) as per manufacturer's instructions, quantified using nanodrop 1000 and stored at -20°C for further cloning.

5.2.2.2. Cloning of amplified flanks with restriction enzyme sites in plasmid vector

5.2.2.2.1. Restriction digestion of PCR products and plasmid vectors

PCR products: Purified upstream and downstream regions of *phcA* (600 bp and 800 bp respectively) and *phcB* (550 bp and 480 bp respectively) genes were used for designing a homologous recombination vector for knockout mutagenesis of *phc* genes. Restriction digests of the purified PCR fragments (inserts) was performed using appropriate restriction enzyme as given in Table 5.5. Reaction mix (100 μL) contained 1X restriction buffer (NEB cut smart), 20 units of each restriction enzyme and approximately 200 $\text{ng.}\mu\text{L}^{-1}$ of purified PCR product. For restriction digestion tubes were incubated at 37°C for 3 h. After incubation equal volumes (100 μL) of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma Aldrich, USA) were added to each tube and mixed by inverting several times. The tubes were centrifuged at 20000 g for 10 min. Upper aqueous layer was taken in a fresh tube and ten μL of 3 M sodium acetate (Appendix C) and 300 μL of 100% ethanol were added. The contents were mixed thoroughly and centrifuged at 20000 g for 10 min. Supernatant was discarded and the pellet was washed with 500 μL of 100% ethanol and spinned at 20000 g for 5 min. Supernatant was discarded and the pellet was dried at 55°C water bath to allow evaporation of residual ethanol. The DNA precipitate

was then dissolved in 20 μL sterile distilled water (55°C) and quantified using Nanodrop 1000.

Plasmid vectors: List of plasmid cloning vectors used for designing a HR vector and their characters are given in Table 5.6. Plasmid vectors pCR2.1 (4340 bp) was provided with the TA Cloning kit (Invitrogen, USA). *E. coli* clones containing plasmid vectors AKK1412 (2959 bp), CGT4437A (4604 bp, donor for Kanamycin resistance gene) and CGT4441 (7399 bp, donor for *SacB-SacR* negative selection marker) were available at OARDC. Plasmid DNA from the *E. coli* clones was isolated using Wizard plus Plasmid Midiprep System (Promega, USA) and stored at -20°C until use. Plasmid vectors were digested with appropriate restriction enzymes (Table 5.5). Reaction mix (100 μL) contained 1X restriction buffer (NEB cut smart buffer), 20 units of each restriction enzyme and approximately $1000 \text{ ng} \cdot \mu\text{L}^{-1}$ of plasmid DNA. For restriction digestion tubes were incubated at 37°C for 3 h. After incubation, one μL ($1 \text{ U} \cdot \mu\text{L}^{-1}$) of Calf intestinal Alkaline phosphatase (CIP) (New England Biolabs, USA) was added to tubes and incubated further at $37 \pm 2^{\circ}\text{C}$ for 2 h. After incubation equal volumes (100 μl) of phenol: chloroform:isoamyl alcohol (25:24:1) (Sigma Aldrich, USA) were added to each tube and mixed by inverting several times. The tubes were centrifuged at 20000 g for 10 min. Upper aqueous layer was taken in a fresh tube and ten μL of 3 M sodium acetate (Appendix C) and 300 μL of 100% ethanol were added. The contents were mixed thoroughly and centrifuged at 20000 g for 10 min. Supernatant was discarded and the pellet was washed with 500 μL of 100% ethanol and spinned at 20000 g for 5 min. Supernatant was discarded and the pellet was dried at 55°C waterbath to allow evaporation of residual ethanol. The DNA precipitate was then dissolved in 20 μL sterile distilled water (55°C) and quantified using Nanodrop 1000.

Table 5.4 Primers with restriction enzyme sites for amplification of flanking regions of *phcA* and *phcB* genes of FIRs37 and GB3 respectively.

Primer	Primer sequence 5'-3'	Region amplified
EQI (F)	GAGCTCGAGGAAGCTGCATGCCACGCTGGA	<i>phcA</i> upstream ¹
EQJ (R)	GAGCAATTGCTGAGAGAGGTCTGTGGCGAA	
EQG (F)	GAGTCTAGAGACAGTCATGGTGGAGTGGCG	<i>phcA</i> downstream ²
EQH (R)	GAGGGATCCCAAGCTGACCATCCTGAAATC	
EPW (F)	GAGCTCGAGGACCTCGCTGACCTGCACGATGG	<i>phcB</i> upstream ³
EPX (R)	GAGGAATTGGCTTGACCTCGCTGACCTGCAC	
EPY (F)	GAGTCTAGATTACTTGATGTAGCACACCGAGA	<i>phcB</i> downstream ⁴
EPZ (R)	GAGGGATCCTCAAGCTTCCGCAAGTCCCAGGG	

F-Forward primer, R- reverse primer, Letters in bold represent restriction enzyme sites: TCTAGA (*Xba*I), GGATCC (*Bam*HI), CTCGAG (*Xho*I), CAATTG (*Mfe*I, used in place of *Eco*RI, because the *phcA* gene had internal *Eco*RI sites and because the two enzymes produce compatible sticky ends), GAATTC (*Eco*RI). ¹ length of upstream flank of *phcA* is ~600 bp, ² length of downstream flank of *phcA* is ~800 bp, ³ length of upstream flank of *phcB* is ~550 bp, ⁴ length of downstream flank of *phcA* is ~480 bp.

Table 5.5 Restriction enzymes used for digestion of amplified upstream and downstream flanks of *phc* genes for cloning.

Insert/ Vector	Restriction enzymes used
<i>phcA</i> upstream	<i>Xho</i> I and <i>Mfe</i> I
<i>phcA</i> downstream	<i>Xba</i> I and <i>Bam</i> HI
<i>phcB</i> upstream	<i>Xho</i> I and <i>Eco</i> RI
<i>phcB</i> downstream	<i>Xba</i> I and <i>Bam</i> HI
AKK1412	As per the fragment to be cloned
CGT4437A	<i>Bam</i> HI and <i>Hinc</i> II
CGT4441	<i>Pac</i> I

5.2.2.2.2. Ligation and transformation

Molar ratio of insert: vector for ligation reactions were maintained as 3:1. Moles of ends of insert and vector required for ligations were calculated using the formula moles of ends of DNA = $(2 \times 10^{-6} \text{ g of DNA}) / (\text{No. of bp} \times 660 \text{ g.mol}^{-1}.\text{bp}^{-1})$. Ligation reaction consisted of 1X T4 DNA ligase buffer, one μL ($400 \text{ U}.\mu\text{L}^{-1}$) of T4 DNA ligase and appropriate volumes of insert and vector DNA in a total volume of 10 μL . Tubes were incubated at 15°C overnight. Chemically competent *E. coli* DH5 α cells were prepared as described in section 4.2.6.2. Five μL of ligated DNA was mixed with 100 μL of competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 2 min and immediately one mL LB broth was added to the tubes. The tubes were incubated at $37 \pm 2^\circ \text{C}$ for 1 h with constant shaking at 200 rpm and centrifuged at 20000 g for 1 min. Supernatant was discarded, pellet was re-suspended in 100 μL LB broth and plated on LB agar plates with appropriate antibiotics as given in Table 5.6 and incubated at $37 \pm 2^\circ \text{C}$ for overnight. Colonies appearing on plates were picked up and grown in LB broth with appropriate antibiotics at 37°C with constant shaking at 200 rpm for 18h. Plasmid DNA was isolated by using Wizard SV mini-prep kit (Promega, USA) as per the manufacturer's instructions. Restriction digestion of using plasmid DNA performed as described above in section 5.2.2.2.1. Restriction digests were run on 1% agarose gel to re-confirm the size of the insert.

5.2.2.2.3. Cloning for designing homologous recombination vectors

Cloning steps involved in designing a homologous recombination (HR) vector for *phcB* gene and *phcA* gene are depicted in Fig. 5.3 and Fig. 5.4 respectively.

5.2.2.2.3.1. Cloning steps for HR vector designing for mutagenesis of *phcB* gene

Step 1 in Fig. 5.3 shows the cloning of the upstream flank of *phcB* gene with added *EcoRI* and *XhoI* restriction sites and cloned in the *EcoRI-XhoI* site of vector AKK1412. The resulting vector is designated CGT12031(3550 bp). Step 2 shows the cloning of the downstream flank of *phcB* gene with added *BamHI* and *XbaI* restriction sites and cloned in the *XbaI-BamHI* site of vector AKK1412. The resulting vector is designated CGT12034 (3448 bp). Step 3 shows the cloning of *XbaI-BamHI* fragment (containing the downstream flank of *phcB* gene) from vector CGT12034 cloned into vector CGT12031 at *XbaI-BamHI* site. The resulting vector is designated CGT12035 (3998 bp). In step 4, neomycin phosphotransferase gene (confers kanamycin resistance, KanR) is released from the *BamHI-HincII* site of vector CGT4437A and cloned in *BamHI-HincII* site of vector CGT12035. The resulting 5631 bp vector is designated CGT12037. Step 5 shows cloning of 4589 bp *PacI* fragment (consisting of *SacB-SacR* negative selection marker and chloramphenicol acetyl transferase gene-*camR* conferring chloramphenicol resistance) from vector CGT4441 at the *PacI* site of vector CGT12037. The resulting vector CGT12038 (10228 bp) Fig. 5.5a is the final HR vector for mutagenesis of *phcB* gene of *R. solanacearum*.

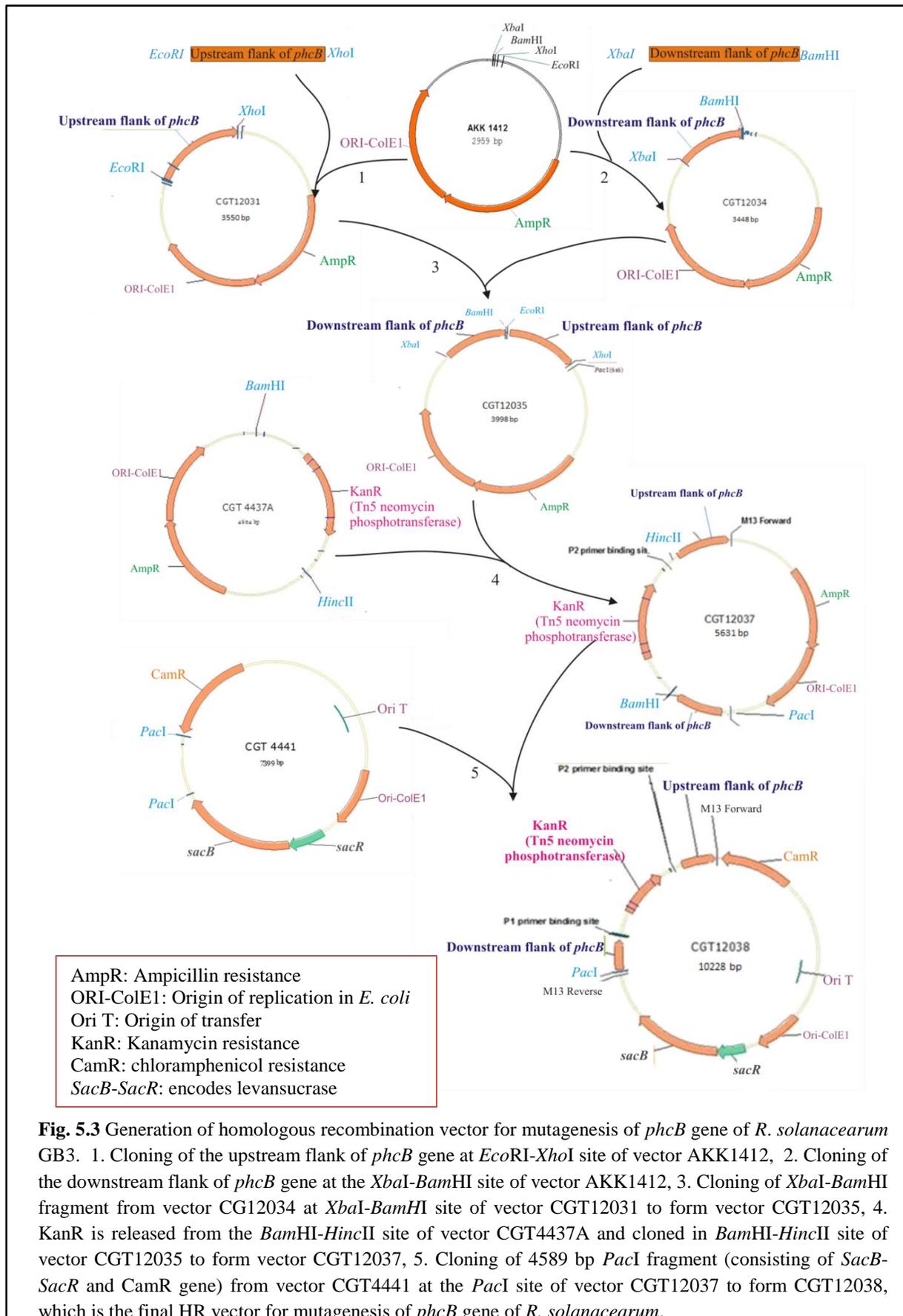
Table 5.6 List of plasmid vectors used for designing HR vector.

Vector name	Antibiotic selection*	Fragment cloned
CGT12026(a)	Ampicillin 100 µg.mL ⁻¹ Kanamycin 50 µg.mL ⁻¹	Downstream flank of <i>phcA</i> gene of FIRs37
CGT12026(b)	Ampicillin 100 µg.mL ⁻¹ Kanamycin 50 µg.mL ⁻¹	Upstream flank of <i>phcA</i> gene of FIRs37
CGT12028	Ampicillin 100 µg.mL ⁻¹ Kanamycin 50 µg.mL ⁻¹	Upstream flank of <i>phcB</i> gene of GB3
CGT12031	Ampicillin 100 µg.mL ⁻¹	Upstream flank of <i>phcB</i> gene of GB3 amplified from CGT12028 and cloned at <i>XhoI-EcoRI</i> site of AKK1412
CGT12034	Ampicillin 100 µg.mL ⁻¹	Downstream flank of <i>phcB</i> gene of GB3 amplified using EPY-EPZ primers using genomic DNA and cloned at <i>XbaI-BamHI</i> site of AKK1412
CGT12035	Ampicillin 100 µg.mL ⁻¹	CGT12031 plus <i>XbaI-BamHI</i> fragment from CGT12034
CGT12037	Ampicillin 100 µg.mL ⁻¹ Kanamycin 50 µg.mL ⁻¹	Tn5 neomycin phosphotransferease (KanR) gene from CGT4437A cloned into <i>BamHI-HincII</i> sites of CGT12035
CGT12038	Kanamycin 50 µg.mL⁻¹ Chloramphenicol 10 µg.mL⁻¹	Fragment from CGT12037 released using <i>PacI</i> and cloned in <i>PacI</i> sites of CGT4441
CGT12039	Ampicillin 100 µg.mL ⁻¹	Downstream flank of <i>phcA</i> gene of FIRs37 amplified using EQG-EQH primers from CGT12026(a) and cloned at <i>XbaI-BamHI</i> site of AKK1412
CGT12040	Ampicillin 100 µg.mL ⁻¹	Upstream flank of <i>phcA</i> gene of FIRs37 amplified using EQI-EQJ primers from CGT12026(b) and cloned at <i>XhoI-EcoRI</i> ** site of AKK1412
CGT12043	Ampicillin 100 µg.mL ⁻¹	CGT12040 plus <i>XbaI-BamHI</i> fragment from CGT12039
CGT12046	Ampicillin 100 µg.mL ⁻¹ Kanamycin 50 µg.mL ⁻¹	Tn5 neomycin phosphotransferease (KanR) gene from CGT4437A cloned into <i>BamHI-HincII</i> sites of CGT12043
CGT12047	Kanamycin 50 µg.mL⁻¹ Chloramphenicol 10 µg.mL⁻¹	Fragment from CGT12046 released using <i>PacI</i> and cloned in <i>PacI</i> sites of CGT4441

AKK1412, CGT4437A and CGT4441 were available at Department of Plant Pathology, OSU. Cloning vector is pCR2.1 obtained along with the TA Cloning kit (Invitrogen Inc. USA). *LB agar containing given concentration of antibiotics was used. **Fragment amplified using EQI-EQJ primers (*XhoI-MfeI* sites) to was cloned in AKK1412 at sites *XhoI-EcoRI*. Fragments digested with *MfeI* and *EcoRI* enzymes have compatible sticky ends. CGT12047 and CGT12038 are final HR vectors for mutagenesis of *phcA* and *phcB* genes of FIRs37 and GB3 respectively.

5.2.2.2.3.2. Cloning steps for HR vector designing for mutagenesis of *phcA* gene

Step 1 in Fig. 5.4 shows the cloning of the downstream flank of *phcA* gene with added *Bam*HI and *Xba*I restriction sites and cloned in the *Xba*I-*Bam*HI site of vector AKK1412. The resulting vector is designated CGT12039 (3759 bp). Step 2 in shows the cloning of the upstream flank of *phcA* gene with added *Mfe*I and *Xho*I restriction sites and cloned in the *Eco*RI-*Xho*I site of vector AKK1412. In this step, the *phcA* fragment was amplified with primers adding *Mfe*I and *Xho*I sites and digested with *Mfe*I which has internal *Eco*RI site. *Mfe*I was used because it produces compatible sticky ends for cloning in a vector digested with *Eco*RI enzyme. The resulting vector is designated CGT12040 (3559 bp). Step 3 shows the cloning of *Xba*I-*Bam*HI fragment containing the downstream flank of *phcA* gene from vector CG12039 cloned into vector CGT12040 at *Xba*I-*Bam*HI site. The resulting vector is designated CGT12043 (4393 bp). In step 4, neomycin phosphotransferase gene (confers kanamycin resistance, KanR) is released from the *Bam*HI-*Hinc*II site of vector CGT4437A and cloned in *Bam*HI-*Hinc*II site of vector CGT12043. The resulting 6034 bp vector is designated CGT12046. Step 5 shows cloning of 4589 bp *Pac*I fragment (consisting of *SacB-SacR* negative selection marker and chloramphenicol acetyl transferase gene-*camR*) from vector CGT4441 at the *Pac*I site of vector CGT12046. The resulting vector CGT12047 (10623 bp) Fig. 5.5b is the final HR vector for mutagenesis of *phcA* gene of *R. solanacearum*.



The final HR vectors for mutagenesis of *phc* genes consist essentially the ColE1 origin for replication in *E. coli* (but not in *R. solanacearum*), the KanR gene inserted between the homologous upstream and downstream flanks of the cloned *phc* genes which disrupts the chromosomal *phc* genes after homologous recombination and aids in selection of primary recombinants, *SacB-SacR* negative selection marker for selection of secondary recombinants after double crossover event.

5.2.3. Mutagenesis of *R. solanacearum*

5.2.3.1. Preparation of electrocompetent cells

Single colony of GB3 and FIRs37 from BG agar plate was inoculated in five mL BG broth (Appendix A) and grown for 18 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 150 rpm. Culture (500 μL) was inoculated in 50 mL BG broth and incubated at $28 \pm 2^\circ\text{C}$ with constant shaking at 150 rpm until the OD_{580} reached 0.8. Culture broth was centrifuged at 8000 rpm at 4°C for 10 min. Supernatant was discarded and pellet was re-suspended in 50 mL sterile ice cold 10% glycerol (v/v in distilled water) and centrifuged at 8000 rpm at 4°C for 10 min. This procedure was repeated successively by re-suspending in reduced volumes of 10% glycerol (first 25 mL, followed by 5 mL and then 2.5 mL) and the pellet was finally suspended in one mL ice cold 10% Glycerol. Fifty μL aliquots of the electrocompetent cells were frozen immediately in chilled tubes on dry ice and stored at -80°C .

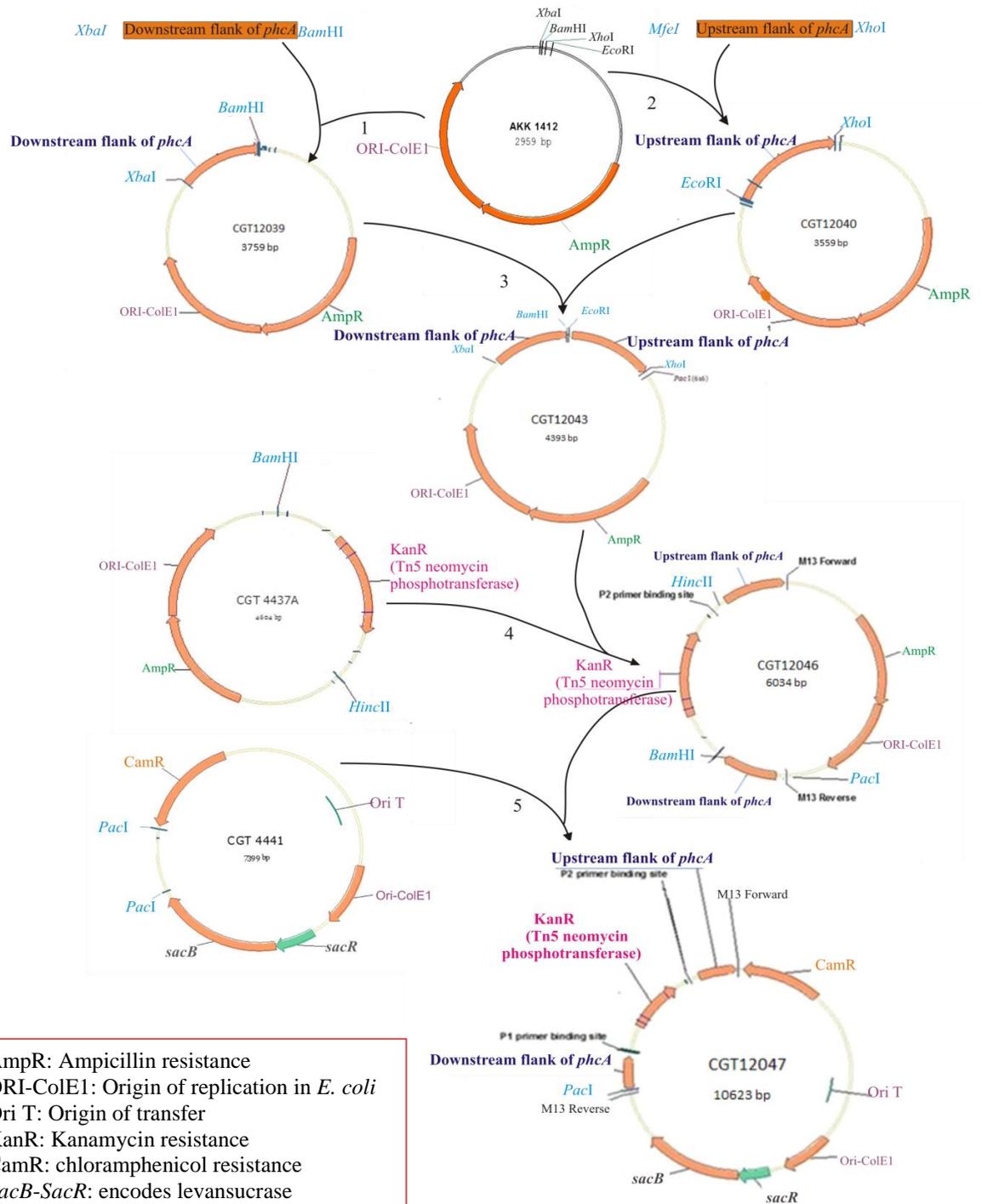
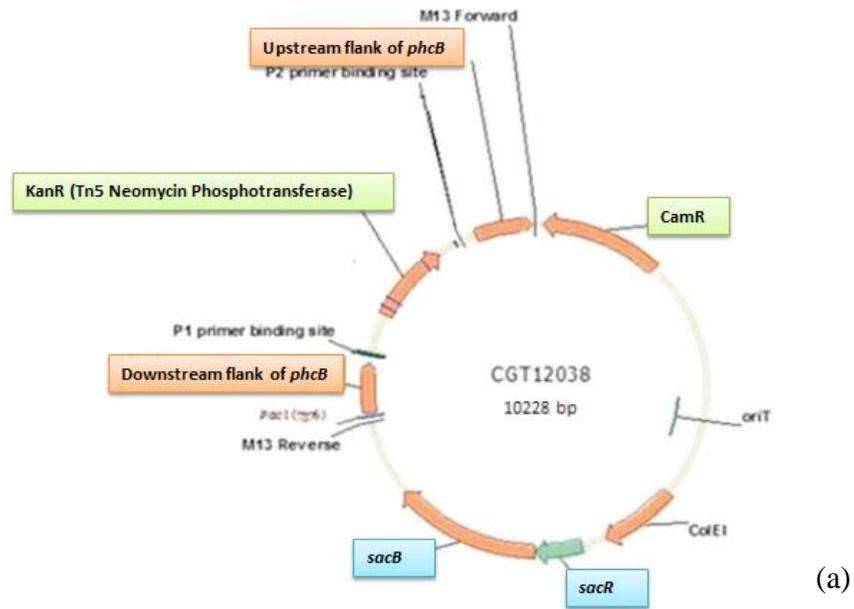
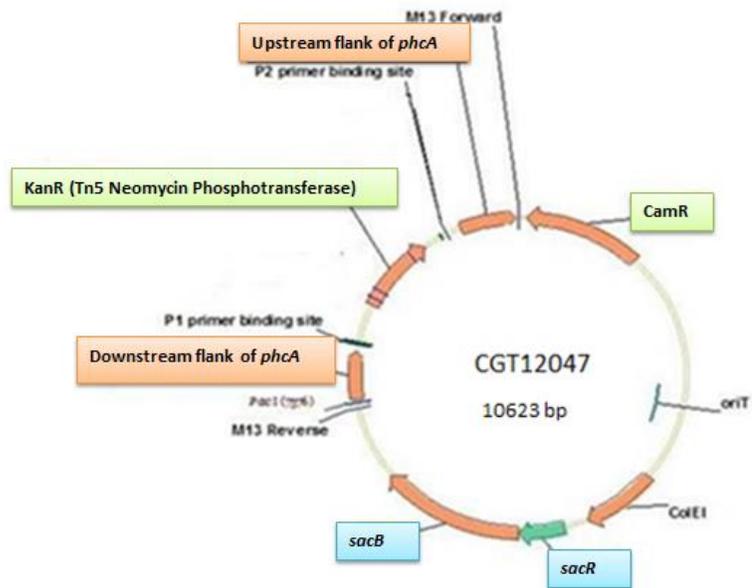


Fig. 5.4 Generation of homologous recombination vector for mutagenesis of *phcA* gene of *R. solanacearum* FIRs37. 1. Cloning of the downstream flank of *phcA* gene at *EcoRI*-*XhoI* site of vector AKK1412, 2. Cloning of the upstream flank of *phcA* gene at the *XbaI*-*BamHI* site of vector AKK1412, 3. Cloning of *XbaI*-*BamHI* fragment from vector CG12039 at *XbaI*-*BamHI* site of vector CGT12040 to form CGT12043, 4. *KanR* is released from the *BamHI*-*HincII* site of vector CGT4437A and cloned in *BamHI*-*HincII* site of vector CGT12043 to form vector CGT12046, 5. Cloning of 4589 bp *PacI* fragment (consisting of *SacB*-*SacR* negative selection marker and *CamR* gene) from vector CGT4441 at the *PacI* site of vector CGT12046 to form CGT12047 which is the final HR vector for mutagenesis of *phcA* gene of *R. solanacearum*.



(a)



(b)

Fig. 5.5a and b Final homologous recombination vectors for knockout mutagenesis of *phcB* and *phcA* genes of *R. solanacearum*.

5.2.3.2. Electroporation and selection of mutants

Ten μg of plasmid DNA (CGT12038 or CGT12047) was mixed with 50 μL of the electrocompetent cells and electroporated at 2.5 kV and 25 μF capacitance using a chilled 0.1 cm cuvette in *E. coli* pulser (Bio-Rad Laboratories Inc., USA). One mL BG broth was immediately added after electroporation and shaken overnight at $28 \pm 2^\circ\text{C}$. Cells were pelleted by centrifugation at 12000 g for 5 min, re-suspended in 200 μL BG broth. Suspension was plated on BG agar plates containing kanamycin ($50 \mu\text{g}\cdot\text{mL}^{-1}$) (BG-kan) and incubated at $28 \pm 2^\circ\text{C}$ for 72 h. Primary recombinants were grown in BG broth at $28 \pm 2^\circ\text{C}$ with constant shaking at 150 rpm without antibiotic selection to encourage second crossover event to occur. Culture broth was serially diluted and 10^3 , 10^4 and 10^5 fold dilutions were spread plated on BG-kan and sucrose (6%) for selecting secondary recombinants (mutants). *phcB* mutant of GB3 was designated GB3-2 where as *phcA* mutant of FIRs37 was designated FIRs37-A. Mutants were routinely cultured on BG-Kan agar.

5.2.4. Characterization of *phcB* mutant of *R. solanacearum*

5.2.4.1. Phenotypic characterization

Phenotypic characters of GB3-2 and FIRs37-A mainly fluidal nature of colony was compared to the wild type parent strains GB3 and FIRs37. Fluidal nature of colony due to EPS production of was determined by visual comparison of 48 to 72 h old colonies of mutants and wild type *R. solanacearum* on BG agar plates. Cellulase production and motility of only GB3-2 was assessed and compared to the wild type parent strain GB3. Qualitative cellulase test was performed using CMC agar as described in section 3.2.3.4.3. Motility was observed by hanging drop method under microscope as described in Appendix B.

5.2.4.2. Pathogenicity of *phcB* mutants on eggplant and tomato

GB3-2 was studied for its pathogenicity and ability to cause wilt in eggplant and tomato under greenhouse conditions. Thirty day old seedlings of susceptible eggplant and tomato were raised in non-sterile soil in greenhouse were transplanted in pots filled with standard non sterile soil mixture. Single colony of GB3-2 and its wild type GB3 was grown in five mL BG broth for 36 h at $28 \pm 2^{\circ}\text{C}$ with constant shaking at 150 rpm. Culture broth (200 μL) was transferred to 20 mL BG broth in flasks and further incubated at $28 \pm 2^{\circ}\text{C}$ with constant shaking at 140 rpm for 48 h. Culture was centrifuged at 8000 rpm for 10 min at 4°C . The pellet was washed once with sterile 1X PBS and centrifuged at 8000 rpm for 10 min at 4°C . The pellet was then dissolved in 20 mL sterile 1X PBS and diluted to 200 mL with sterile 1X PBS. Ten mL suspension (containing approximately $8.0 \text{ Log CFU.mL}^{-1}$) was applied per seedling by soil drenching as described in section 3.2.4.3 except growth parameters were not measured. Each treatment consisted of two replicates with two pots per replication and five seedlings per pot. Control consisted of virulent wild type *R. solanacearum* strain GB3. Plants were maintained with suitable watering and appearance of wilt symptoms was noted until 23 days post inoculation.

5.2.4.3. Invasiveness and endophytic colonization by GB3-2 in eggplant

5.2.4.3.1. Plant inoculation and re-isolation of GB3-2

Plants which remained healthy after 23 DAI after inoculation (from the pathogenicity experiment) of GB3-2 were collected to assess colonization of eggplant rhizosphere and stem by GB3-2 at 23 DAI by plating on semi selective medium SMSA agar (Appendix A) containing Kanamycin ($50\mu\text{g.mL}^{-1}$) (SMSA-Kan). SMSA is a semi-selective medium for isolation of *R. solanacearum* (Kelman, 1954; Pradhanang *et al.*, 2000). Wild type *R.*

solanacearum is sensitive to kanamycin whereas its *phcB* mutant GB3-2 is resistant to kanamycin because its internal region is disrupted with functional KanR gene for mutagenesis. Colonization of endophytic tissue was assessed by macerating the surface sterilized stem piece followed by serial dilution and plating on (SMSA-Kan) as described in section 4.2.5 in chapter IV. Isolation of bacteria from rhizosphere was performed as described by Buyer (1995) as described in section 4.2.4.4 in chapter IV. Serially diluted samples were plated on SMSA-Kan. Plates were incubated at $28 \pm 2^{\circ}\text{C}$ for 48 h and non-fluidal red colonies of GB3-2 were counted.

5.2.4.3.2. Confirmation of re-isolated mutants by *R. solanacearum* species specific colony PCR

Typical non-fluidal red colonies obtained on SMSA-Kan were subjected to colony PCR (Sun *et al.*, 2008) using *R. solanacearum* species specific 759f-760r primers (Opina *et al.*, 1997) as described in section 4.2.5 in chapter IV. Single colony was dissolved in 20 μL sterile distilled water and one μL was used as a template for colony PCR.

5.3. RESULTS AND DISCUSSION

5.3.1. PCR amplification for obtaining sequences for *phcA* and *phcB* genes for mutagenesis

R. solanacearum is a species complex of genetically diverse strains across the world. Based on the ITS region, strains from Asia are clustered in Phylotype I (except GMI1000 which is from French Guyana), strains from America in Phylotype II, strains from Africa and Madagascar in Phylotype III and strains from Indonesia in Phylotype IV. Conserved regions of *phcA* and *phcB* genes of select strains from all four phlotypes were useful in

designing universal primers for amplification from *R. solanacearum*. Regions of *phcA* and *phcB* genes amplified from FIRs37 and GB3-2 were confirmed by sequencing and BLAST search. Sequences of *phcB* and *phcA* genes obtained by amplification with newly designed primers and used for designing primers for SEFA PCR are given below:

Sequence of internal region of *phc* genes used for designing primers for SEFA PCR

>FIRs37-*phcA* (348 bp)

```
CGTCAAGCGCCTGGCGCCGGGTTTGCAGGTGCGCCTGACCGAGTCCTCCTCGTGGGAAGCCGA
CGTGCCGCGGATGCGTTCCAATGAGCTGGACCTGGCGTTCTCCCCGTTCCCGACGCGGCATCG
GAAATCGTGGAAGAGGTCGTGACCTCCTTCAACATGTGGGTCTGCGCGCGCAAGGACCTCCG
GTCCTGAAGGATCACTGCTCGCTCGATCAGTACCTCGAATGCGAGCATATCTTCATCGCGGGG
CAACCCCGGCACCCGCGCGGGCGCCGTCGCTGATTCCGCTCGATTACGCGCTGCAGCAGCGTGG
CCTGAAGCGCCACTCCACCATGACTGTGCATGCA
```

>GB3-*phcB* (303 bp)

```
AATGTAGGCCTTGGCGCGTGCCCATCTTGATGGCCAGGTCTGGAGAGGACCTCGCTGACCTGCA
CGATGGAATAGGGGTTGTAGACCTCCATCACCAGGGCGCGCCGCTGCAGCGTGATGATGGTG
CCGCGAACCTGCTGACCCTGGGAGTTGCGGAAGCTGACCATCGCGTGCTCTGATGCACGTGGT
GATGGAGGATAAACCCCTATTCATGGTGAAGTAGCAAGGTCTATGGTCCTGGCCATCAAGAT
GGGCCACGCCAGGCCTACATTGGTAGGCGGTGGTGGTAGCCTGGTAACACAG
```

Upstream and downstream flanking sequences of *phcB* and *phcA* genes obtained by SEFA PCR and used for designing new set of primers with restriction enzyme sites for cloning.

>FIRs37-*phcA*-Upstream flank (614 bp)

```
CGACGCCAGTGATGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGCATGCTCGA
GCGGCCCGCAGTGTGATGGATATCTGCAGAATTCGGCTTGACAGTCATGGTGGAGTGGCGCTT
CAGGCCAGGTGGTGTGCTGCTGGA AAACTGCCGCGTCAACAAGGGCGAGAAGAAGAACAGCGA
CGAACTGGCCCAGAAGATGGCCAAGCTGTGCGACGTCTACGTCAACGACGCCTTCGGCACCG
CCCACCGCGCCGAGGCCACCACGCACGGCATCGCCAAGTTTGCCCCATCGCCTGCGCCGGCC
CGCTGTGGGCGCCGAGCTGGACGCGCTGGGCAAGGCGCTGGGCCAACCGGCCCGTCCGCTG
GTCGCCATCGTGGCCGGCTCCAAGGTGTCGACCAAGCTGACCATCCTGAAATCGCTGGCCCGC
AAGGTGGACAACCTGATCGTCGGCGGGCGGCATCGCCAACACGTTTCATGCTGGCCCGGGCCCT
GAAGATCGGCAAGTCGCTGGCCGAGGCGGACCTGATTGGTGTGCTCGCGCCATCATCGATCT
GATGGCCGCGCGCGGCGCTTCGGTGCCGATCCCGGTTCGATGTGGTGTGCGCC
```

>FIRs37-*phcA*-Downstream flank (819 bp)

```
GTAAAGCCGGGGCAGTGATTGTAATACGACTCACTATAGGGCGAATTGGGCCCTCTAGATGC
ATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTTGGCGCCGGGTCTGCA
GGTGCGTCTGACCGAAACCAATACGCAGTCAACTGGAAGCCGTACGCCAAAGCCTGCAGCAG
CAAGCCCAGCAGGCCCCGCGAGGAGCAGGGCAGCGCGCTGCGCACGCTTCGGCGAGTTCGATGC
AGCAACAGCTCGCGCTATTGGCCGAGGGCGACGAGCGGGCAGCTGGCCGAAGTGGCGCGC
CGACGCTGGAGCAGAAGCTCAAGGACATCGAGGCCAACACGCGACCAAGCTCGACGAGAT
GCGGCGCACCGTTCGACGAGAAGCTGCATGCCACGCTGGAGCAGCGTCTGGGCAGAGTCTGTT
```

CAAGCTGGTGTCCGACCGGCTGGAGCAGGTGCATCGCGGCCTGGGCGAAATGCAGGCACTGG
CGCAGGGCGTCCGGTGTCTGAAGAGGGTGTGACCAACGTGAAGACGCGCAGGCACCTGGGG
CGAAGTCCAGCTGGACGATGCTGCTGGAACAGATGCTGACGCCCCGAGCAGTACGACAAGAAC
GTCGAGACCGTCCGCGTGCACCGGGGCCGCGGGTTCGAGTTCGCCATCCGCCTGCCCGGGACC
AAGACCGATGCGGACCGCGAAGCGACCGTCTGGTTGCCGATCGATGCCAAGTTCCCCAAGGA
GCAGTACGAGCGTCTGCTGGATGCGCAGGAGCGCGCGGACGCGGAGGGCCGGGCCACCGTAG
GCCAGCCGCGAGC

>GB3-*phcB*-Upstream flank (556 bp)

GACCTCGCTGACCTGCACGATGGAATACGGGTTGCGACAGCGGGGCGCGGGAATAGGCGTG
CGCCATGGATATCAGGGGAGTGCATCGCTCCAGTCTGTTTTCGGTTCACGCGACGCCCTTGACC
ACGCTGTCGGTCCGGTAACGGTGGACGCCGTCTGCCGTGGCTCCGACCGGGCCTCCTCCAA
TCATCTCGAAGCGGGTTCGGGCGCGAGGCGCCAGGCCGTTCGCGCGCCGCGCGCGGGCCGGCT
AACGCCGGACACGCTTTTACGACTGATGCAGCGGCATCCCCTGCCCGACGGGCAGCAGGCCG
CCAGACCGGGACCGCGGTGCGGTTCCGGGTGCATGGTAGTGGAGTGAGCTTCCCGCTGGATG
GACGTTTCTCCTGAAGTCTCCGGCAAATTCGCACCGTGTACTCTCCCAACCAAATCGATCCGG
CGGTCAGCTTCCGCAACTCCAGGGTCAGCAGGTTTCGCGGCACCATCATCACGCTGCACCGGC
GCGCCCTGGTGATGGAGGTCTACAACCCGTATTCCATCGTGCAGGTCAGCGAGGTC

>GB3-*phcB*-Downstream flank (487 bp)

TTACTTGATGTAGCACACCGAGATGGCGGCCTCGTCGCCGGGCACCAGCGTGTTGGGCGGCAA
CTGGCGATTACCTCCACGCCGCGCAGTTCGTGGCTGGTCAGGCGGATGGCCGAATCGATGGCC
TTTTCCGCGAGGAAGGGCGTGTGCTCTCGCCTTTCTCGGGGCTGCGGTAGGCGAAGGTCTTC
AGGTCCGAGACGATGTGCTGGACGCGCTGCATGCCTTCTTGGCGTTCGGCCAGGCATTCGAGC
AGCACTCGTCTCCACTGAAGACTTCGCCTACCGCAGCCCCGAGAAAGGCGAGAGCGACACGC
CCTTCTCGCGAAAAGGCCATCGATTCCGCCATCCGCCTGACCAGCCACGAACTGCGCGGGC
TGGAGAAAATCGTCAGTTGCCGCCGACACGCTGGTGCGCGGCGACCAGGCCGCCATCATC
GGTGTGCTGATCAACCTGCCTTACCCCTGGGACTTGCGGAAGCTTGA

5.3.2. Designing homologous recombination (HR) vector and knockout mutagenesis of *phcA* and *phcB* genes of *R. solanacearum*

Sequences of fragments of *phcA* and *phcB* genes amplified by PCR using newly designed primers were confirmed by sequencing and BLAST search and used in steps 1 and 2 of cloning shown in Fig. 5.3 and 5.4 for constructing HR vectors. In addition, presence of right sized inserts after cloning at in steps 1 to 5 of cloning shown in Fig. 5.3 and 5.4 was confirmed by performing restriction digests using plasmid DNA isolated from *E. coli* clones.

Electroporation of HR vectors CGT12038 (10288 bp) and CGT12047 (10623 bp) in *R. solanacearum* strains GB3 and FIRs37, and final selection on sucrose (6%) and kanamycin (50 $\mu\text{g.mL}^{-1}$) resulted in selection of double crossover knockout mutants with disruption of *phcB* and *phcA* genes due to replacement of ~300-350 bp internal region with 1641 bp kanamycin resistance gene (Tn5 neomycin phosphotransferase) (Fig. 5.6). Both mutants (GB3-2 and FIRs37-A) produced less EPS on BG agar after incubation at $28 \pm 2^\circ\text{C}$ for 48 to 72 h as compared to their respective wild type parents which produced more EPS and were highly fluidal (Fig. 5.7 and Table 5.7).

phcA gene encodes PhcA a LysR type transcriptional regulator which up-regulates the expression of EPS through a cascade which involves additional genes (Schell, 2000). The expression of *phcA* is however governed by a quorum sensing through 3OH-PAME synthesized by a product of *phcB* gene (Schell, 2000). 3OH-PAME represses *phcA* at low cell density whereas increases its expression at cell density greater than 10^7 cells.mL⁻¹ in a confined environment like xylem vessels or culture broth (Schell, 2000, Mole *et al.*, 2007). Mutagenesis has been employed for generating mutants of *phcA* and *phcB* genes to study their involvement in pathogenicity of *R. solanacearum* (Boucher *et al.*, 1985, Clough *et al.*, 1997, Liu *et al.*, 2005). Spontaneous and site-directed mutations in the *phcA* and *phcB* genes of *R. solanacearum* have essentially resulted in mutants producing less amounts quorum sensing regulated virulence factors (Brumbley and Denny, 1990; Clough *et al.*, 1997; Poussier *et al.*, 2003). Thus mutagenesis of *phcA* gene results in non-fluidal and avirulent mutants of *R. solanacearum* (Poussier *et al.*, 2003).

Mutants of *phcB* gene have a phenotype similar to the *phcA* mutants and are also non-fluidal in appearance (Clough *et al.*, 1997; Lin *et al.*, 2008). In the present study,

congruent to earlier reports, mutagenesis of *phcA* and *phcB* genes of *R. solanacearum* GB3 and FIRs37 using HR vectors resulted in mutants with a non-fluidal phenotype.

Additionally, both *phcA* and *phcB* mutants are known to be deficient in production of endoglucanase and are more motile than the wild type parents (Clough *et al.* 1997; Tans-Kersten *et al.*, 2001). This indicates that higher expression of *phcA* and *phcB* genes represses expression of genes involved in flagellar and twitching motility but increase expression of endoglucanase (Schell, 2000; Liu *et al.*, 2001; Kang *et al.*, 2002). Congruent to these reports, the *phcA* and *phcB* mutants generated in the present study were found to produce less endoglucanase and were more motile in comparison to their wild type parents.

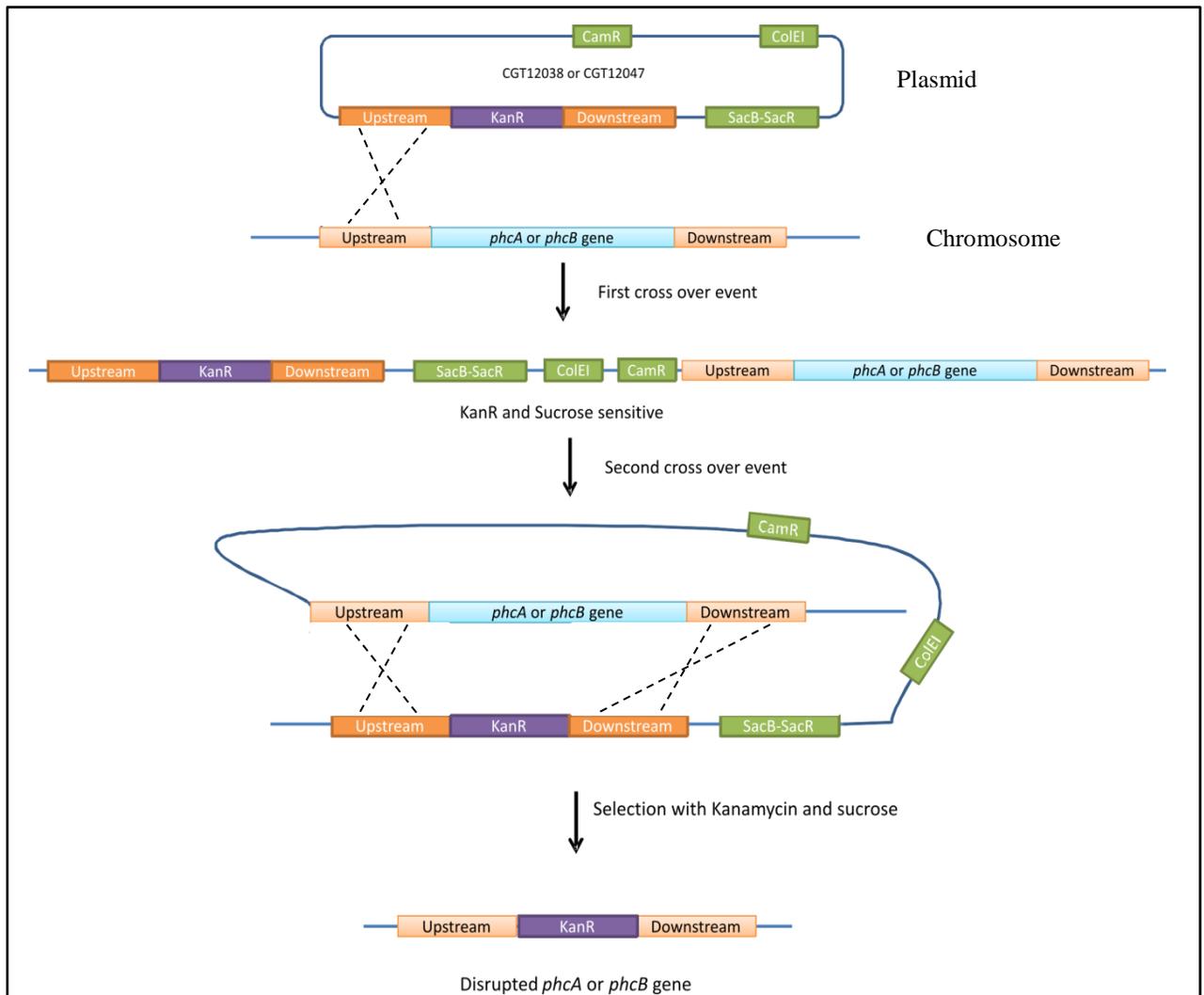


Fig. 5.6 Schematic representation of mechanism of gene disruption by homologous recombination for mutagenesis of *phcA* and *phcB* genes of *R. solanacearum*. The plasmid vectors CGT12038 or CGT12047 have kanamycin resistance gene cloned between homologous sequences flanking each side of internal regions of *phcA* and *phcB* genes. After electroporation and after first crossover event, selection for kanamycin resistance (KanR) in absence of sucrose yields primary recombinants in which the plasmid has integrated on one side or the other of the wild-type gene in the chromosome. Because the plasmid does not have an origin of replication for *R. solanacearum*, kanamycin resistant clones are a result of a recombination event. All kanamycin resistant clones will also be sensitive to sucrose because of the *sacB-sacR* negative selection marker inserted in the chromosome. A primary recombinant grown in BG broth without kanamycin and sucrose undergoes a secondary recombinational event in which the intervening plasmid DNA (containing *sacB-sacR*, ColEI, OriT and CamR) loops out of the genome. On selection of the secondary recombinants on medium with kanamycin and sucrose, clones that have not undergone a secondary recombinational event will be killed whereas the secondary recombinants that lost the integrated suicide vector and the *sacB-sacR* gene but having the kanamycin gene inserted in between the target gene will survive. Sucrose resistant population may also have wild-type gene, but the mutants can be selected based on change in phenotype due to disruption of *phcA* or *phcB* genes.

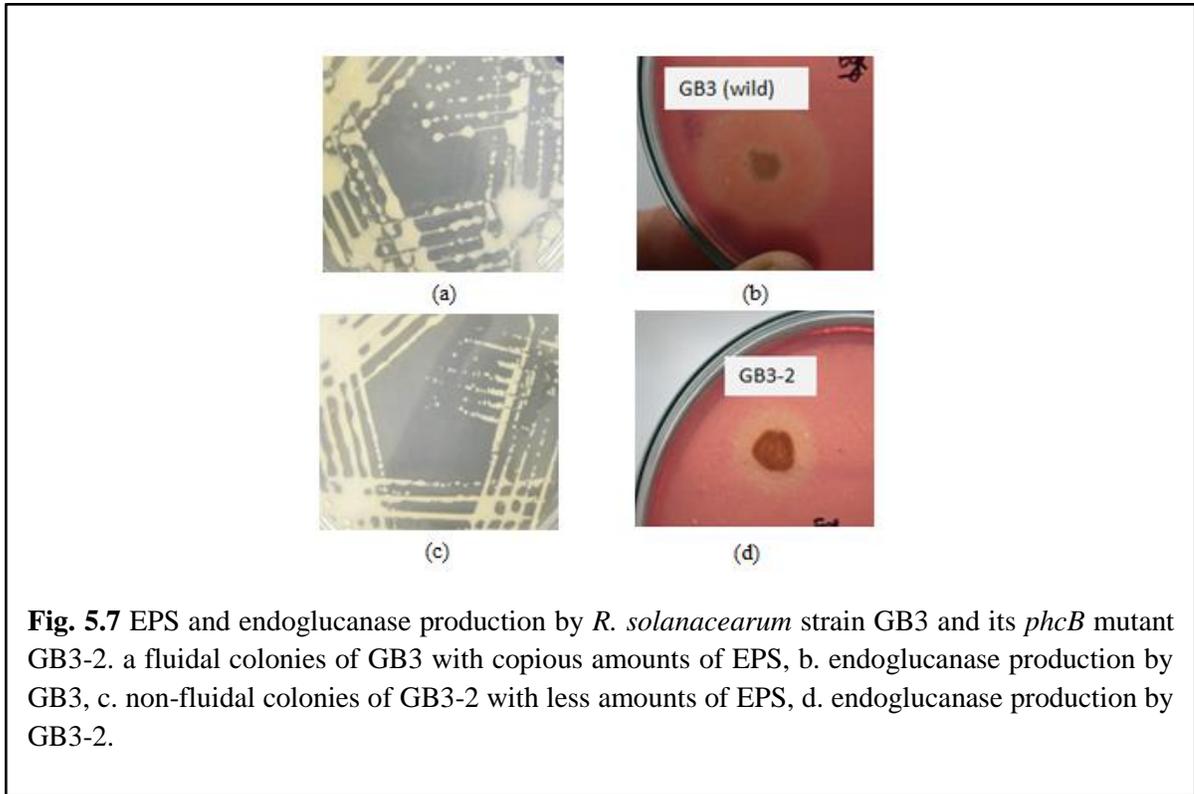


Table 5.7 Mutants obtained by mutagenesis using HR vectors and their phenotypic characters.

<i>R. solanacearum</i> strain	Phenotypic characters			Pathogenicity 23DAI	
	EPS	Endoglucanase	Motility	Eggplant	Tomato
GB3 (Wild type)	+++	+++	+	P	P
GB3-2 (<i>phcB</i> mutant)	+	+	+++	-	-
FIRs37 (Wild type)	+++	ND	ND	P	P
FIRs37-A (<i>phcA</i> mutant)	+	ND	ND	ND	ND

Levels of phenotypic characters +++: high, +: low, P: pathogenic, -: non-pathogenic
 ND: Not determined, DAI: days after inoculation.

5.3.3. Phenotypic characterization of mutant

Pathogenicity of *R. solanacearum* is attributed to production of virulence factors mainly EPS and endoglucanase. Wild type strains GB3 and F1Rs37 were pathogenic on eggplant and tomato. However as expected, GB3-2 lost its ability to cause wilt in eggplant and tomato (Table 5.7, Fig. 5.8) since it produced less amounts of virulence factors EPS and endoglucanase. However GB3-2 was still able to colonize eggplant when tested at 23 DAI with a population of $4.44 \pm 0.18 \text{ Log CFU.g}^{-1}$ and $6.43 \pm 0.01 \text{ Log CFU.g}^{-1}$ stem and rhizosphere respectively. Re-isolated GB3-2 were resistant to kanamycin ($50 \mu\text{g.mL}^{-1}$) and confirmed by species specific PCR (Fig. 5.9). Mutants of *R. solanacearum* of several genes including *phcA* (Poussier *et al.*, 2003) and *phcB* (Lin *et al.*, 2008), Hrp gene cluster (Frey *et al.*, 1994), EPS and endoglucanase genes (Saile *et al.* 1997) have been reported to lose pathogenicity but still able to colonize stem and rhizosphere of susceptible host without apparent wilt symptoms. However, it is a common observation that the quorum sensing mutants colonize plants less efficiently and with a lower population as compared to the wild type strains (Trigalet and Demery 1986; Poussier *et al.*, 2003). Interestingly, when tested for its biocontrol activity against *R. solanacearum* by the method described in section 4.2.3, GB3-2 prevented 50.0% wilt in eggplant cv. *Agassaim* after challenging with Rs-09-100. Production of bacteriocins, niche exclusion and induction of tylose formation are known to be involved in biocontrol by *R. solanacearum* mutants against BW (Frey *et al.*, 1994). From the present study it is clear those mutations in *phcB* gene of the quorum sensing pathway of *R. solanacearum* causes reduction in its virulence and pathogenicity. Thus interference in quorum sensing signal of the BW pathogen may also lead to reduction in virulence and pathogenicity. Quorum

quenching (signal interference) against *R. solanacearum* can be used as an alternate strategy for BW management.



Fig. 5.8 Pathogenicity of GB3 and its *phcB* mutant GB3-2 in tomato. a. tomato seedlings inoculated with GB3 completely wilted at 7 DAI. b. tomato seedlings inoculated with GB3-2 remained healthy up to 23 DAI.

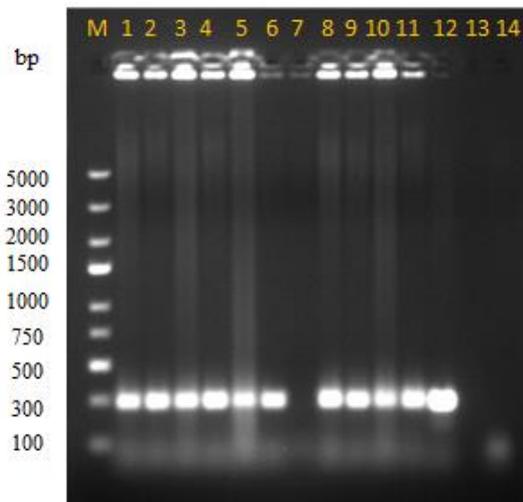
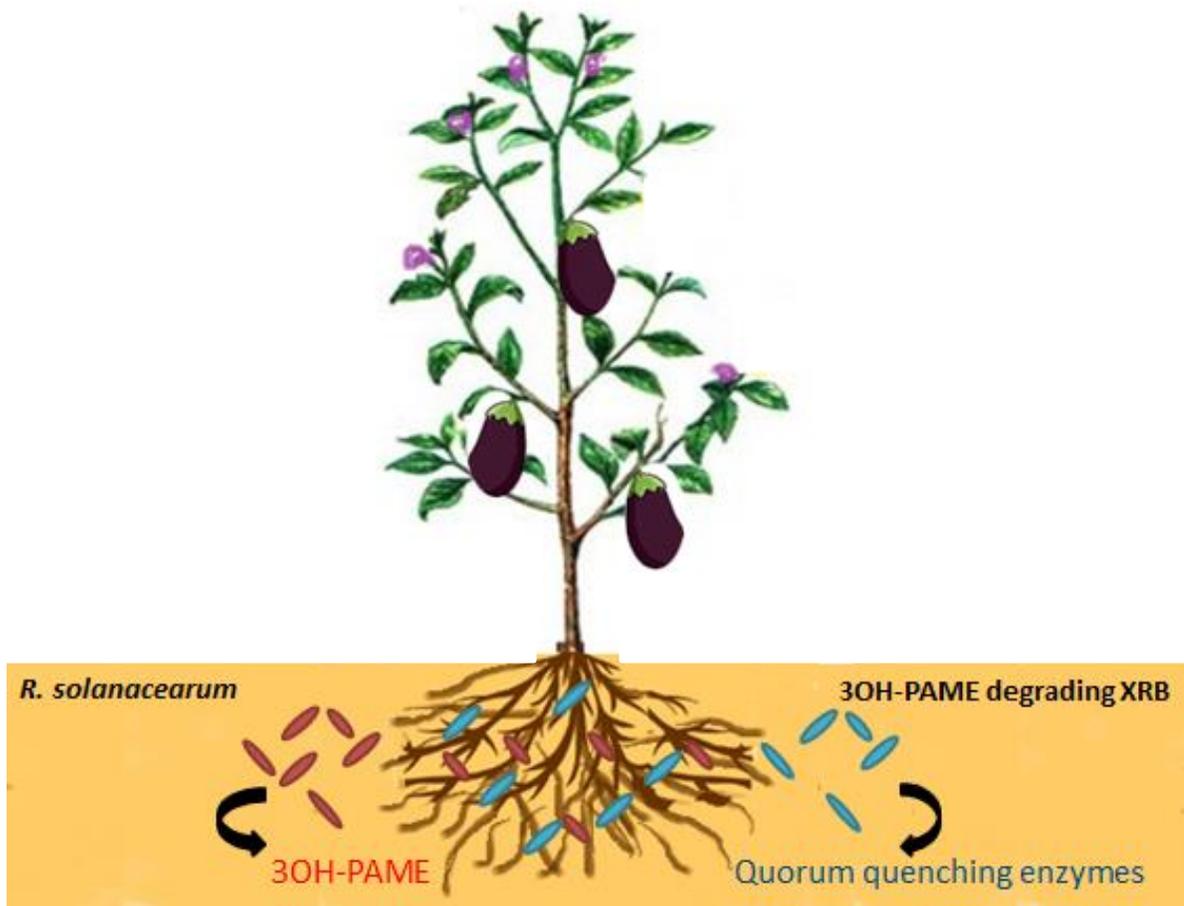


Fig. 5.9 Confirmation of re-isolated GB3-2 by colony PCR. M- Marker, Lanes 1 to 6: different colonies of GB3-2 re-isolated from endophytic tissues of eggplant, Lane 7: colony with similar morphology as GB3-2, Lanes 8 to 12: different colonies of GB3-2 re-isolated from rhizosphere of eggplant. Lane 13: unloaded, Lane 14: PCR negative control.

CHAPTER VI

Characterization of 3OH-PAME degrading XRB and quorum quenching molecules



6.1. INTRODUCTION

Interference in the quorum sensing (QS) by bacterial quorum quenching (QQ) molecules is lately reported to be an effective anti-virulence strategy for plant disease suppression. Enzymes degrading the main signaling molecules of phytopathogens are common QQ molecules and widely distributed in bacteria from diverse ecological niches. Enrichment of bacteria using QS molecules as source of C and/or N or screening bacteria from various sources for degradation of QS molecule are the two basic methodologies used for screening plant associated bacteria for QQ activities (Dong *et al.*, 2000; Shinohara *et al.*, 2007). QS indicator bacteria and analytical techniques have made quantification of QQ possible to select efficient QQ bacteria (Cirou *et al.*, 2012; Barbey *et al.*, 2013).

Plant associated bacteria are well studied for QQ activities against phytopathogens having acyl-HSL as their main signaling molecules. *R. solanacearum* possesses acyl-HSL based QS system but it plays no role in expression of virulence factors necessary to cause wilt. The 3OH-PAME dependent QS system is the main virulence regulatory system in *R. solanacearum* (Flavier *et al.*, 1997b). Recently, tomato rhizosphere inhabitant *Ideonella* sp. is reported to produce an ester hydrolase enzyme that degrades 3OH-PAME of *R. solanacearum* and renders it ineffective as a QS molecule (Shinohara *et al.*, 2007). In addition, antagonistic properties of QQ bacteria are reported to enhance their disease suppression activities (Zhao *et al.*, 2008). Extracellular 3OH-PAME degrading QQ enzyme from *Ideonella* sp. was effective in preventing expression of QS regulated genes in *R. solanacearum in vitro* (Shinohara *et al.*, 2007). Plants treated with QQ bacteria degrading acyl-HSL are reported to resist infection by phytopathogenic bacteria (Uroz *et al.*, 2009). The current literature supports that QQ is an effective strategy for management of plant diseases. However, efficacy of 3OH-PAME degrading

XRB (3PD-XRB) or their QQ enzymes (QQE) against management of BW has remained unexplored. This chapter presents results pertaining to the objective ‘**to screen the xylem residing antagonistic bacteria for production of quorum quenching molecules**’. The present study was undertaken to develop QQ based biocontrol strategies for management of BW.

6.2. MATERIALS AND METHODS

6.2.1. Synthesis of 3OH-PAME in the laboratory and its biological confirmation

6.2.1.1. Synthesis of 3OH-PAME

3OH-PAME was synthesized by acid catalyzed esterification of 3-hydroxy palmitic acid (3OH-PA) (Sigma Aldrich, USA) with methanol in the lab because 3OH-PAME is not available commercially. Ten mg 3OH-PA was mixed with 200 μ L of 2% H_2SO_4 in methanol (Appendix E) and allowed to react at RT for 20 h in the amber colored glass tube. 600 μ L of dichloromethane (DCM) (Merck, Germany) was added to the above tube and mixed well by inverting several times and transferred to a pre-weighed 1.5 mL centrifuge tube. Immediately, 200 μ L saturated NaCl solution (Appendix E) was added and mixed well by inverting several times. Tube was centrifuged at 2000 g for 5 min. Upper aqueous layer was carefully pipetted and discarded. The lower DCM layer was washed twice with saturated NaCl as described before and aqueous layer was discarded. Later equal volumes of saturated $NaHCO_3$ solution (Appendix E) was added to the DCM layer and mixed well by inverting several times. Tube was centrifuged at 2000 g for 5 min. Upper aqueous layer was carefully pipetted and discarded. DCM layer washed twice with equal volumes of saturated $NaHCO_3$ solution as described before. Finally the organic layer was washed with equal volumes of distilled water to remove traces of

unreacted 3OH-PA. The lower organic DCM layer was dried in vacufuge vacuum concentrator (Eppendorf, Germany) at 45°C until all the DCM evaporated. Weight of 3OH-PAME was determined by weighing the tube and subtracting the tube weight that was determined earlier. The residue was dissolved in appropriate volume of absolute ethanol to a final concentration of 0.1 M and preserved at 4°C as a master stock. For using in plate based bioassays 100 µM stock of 3OH-PAME was prepared and used at the concentration of 10 µM in agar medium.

6.2.1.2. Confirmation of quorum sensing activity laboratory synthesized 3OH-PAME

Determination of quorum sensing activity of laboratory synthesized 3OH-PAME was carried out using quorum sensing indicator *Ralstonia solanacearum* strain AW1-3 (Flavier *et al.*, 1997b). Strain AW1-3 (*phcB83::Tn5, eps-130::Tn3lacZ*) does not produce its own 3OH-PAME and is engineered to express *lacZ* encoding a functional *beta*-galactosidase in response to exogenous 3OH-PAME (5.0 nM) (Flavier *et al.*, 1997b). AW1-3 was routinely cultured on BG medium with kanamycin (50 µg.mL⁻¹) (BG-Kan). For determining quorum sensing activity, BG-Kan agar medium with X-Gal (20 µg.mL⁻¹) was seeded with AW1-3 (two µL of overnight culture containing approximately 10 Log CFU.mL⁻¹ was added per mL medium) (BG-Kan-X-Aw), plates were poured and allowed to solidify. Ten µL of 1 nM, 2 nM, 4 nM, 6 nM, 8 nM and 10 nM 3OH-PAME was spotted onto sterile filter paper discs (10 mm diameter) and placed on BG-Kan-X-Aw agar. Plates were incubated at 28 ± 2°C for 72 h and observed for blue coloration around the disc. Appearance of blue colour is a positive response indicating induction of expression of *beta*-galactosidase in AW1-3 by 3OH-PAME present in the disc. Two

replications per concentration of 3OH-PAME were maintained and the screening was repeated twice as independent experiments.

6.2.2 Screening of XRB for degradation of 3OH-PAME

A rapid bioassay was developed for determining the degradation of 3OH-PAME by XRB. All the XRB in the collection (n=167) were screened for 3OH-PAME degradation. XRB were grown in five mL BG broth at $28 \pm 2^\circ\text{C}$ for 48 h with shaking at 140 strokes/min. Equal volumes (25 μL) of XRB culture and fresh BG medium containing 10 μM 3OH-PAME were mixed and incubated at $28 \pm 2^\circ\text{C}$ with gentle agitation for 18 h. The reaction was terminated by adding 2.5 μL of concentrated HCl. The reaction mixture was extracted with equal volumes of DCM as described by Shinohara *et al.* (2007). The tubes were centrifuged at 2000 g for 5 min and the DCM layer was recovered by careful pipetting and spotted onto sterile paper discs (10 mm), evaporated to dryness and placed on BG-Kan-X-Aw plates. Un-inoculated BG broth incubated with 10 μM 3OH-PAME served as control. The plates were incubated at $28 \pm 2^\circ\text{C}$ for 72 h to determine whether 3OH-PAME was degraded based on absence of blue coloration. Three replications were maintained per XRB and the screening was repeated thrice as independent experiments. DCM extracts containing un-degraded 3OH-PAME induced expression of *lacZ* in AW1-3, resulting blue colour around the disc. XRB that prevented expression of *lacZ* in AW1-3 causing no blue pigmentation were positive for 3OH-PAME degradation.

In order to determine whether the 3OH-PAME degrading molecule is extracellular, cell free supernatant (CFS) of 3OH-PAME degrading XRB (3PD-XRB) was tested for 3OH-PAME degradation. Single colony of 3PD-XRB was grown in 5 mL BG broth for 24-48 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. One mL culture was

centrifuged at 8000 rpm for 5 min at 4°C. CFS was obtained by membrane filtration (0.22 µm) of supernatant. Fifty µL CFS was assayed for 3OH-PAME degrading activity as described above. To determine whether degradation of 3OH-PAME was enzymatic, CFS of 3PD-XRB was boiled for 10 min prior to assay in a separate test. Each test was performed in three replications per 3PD-XRB screened and the screening was repeated twice as independent experiments.

6.2.3. Characterization of 3OH-PAME degrading bacteria

6.2.3.1. Hydrolysis of tweens and tributyrin by 3OH-PAME degrading XRB

Since 3OH-PAME is a fatty acid ester, it is most likely that 3PD-XRB may hydrolyze additional esters and lipid substrates. Hydrolytic activities of eight 3PD-XRB *viz.* XB7, XB19, XB102, XB109, XB115, XB122, XB174 and XB179 towards additional esters and triglycerides were tested. Tween 20 and Tween 80 were used as esterase substrates whereas tributyrin was used as a lipase substrate. 3PD-XRB were spot inoculated on Tween 20, Tween 80 and tributyrin agar (Appendix A) (Kumar *et al.*, 2012). Plates were incubated at $28 \pm 2^\circ\text{C}$ for 24-48 h and observed for appearance of white hazy haloes for tween degradation and clear haloes for tributyrin degradation around the point of inoculation of 3D-XRB.

6.2.3.2. Production of antagonistic compounds by 3OH-PAME degrading XRB

Production of HCN and siderophores by XB7 and XB122 was studied in chapter IV. HCN and siderophore production by 3PD-XRB was studied as given in sections 4.2.1.2 and 4.2.2.1 respectively.

6.2.3.3. Identification of 3OH-PAME degrading XRB

XB102, XB115 and XB174 were identified by 16S rRNA gene sequencing. 1500 bp fragment of 16S rRNA gene was amplified as described in section 3.2.2.1.3 and sequence read was obtained, analyzed and the strains were identified as described in section 3.2.3.3. XB7, XB122 and XB109 are identified in chapter III. XB19 and XB179 were not identified.

6.2.4. Biocontrol efficacy and eggplant colonization by 3OH-PAME degrading XRB

6.2.4.1. Biocontrol efficacy of 3OH-PAME degrading XRB

Biocontrol efficacy (BCE) and colonization of XB7 and XB122 was studied earlier in chapter IV. BCE and colonization of additional six 3D-XRB (XB19, XB102, XB109, XB115, XB174 and XB179) was performed as described in section 4.2.3 of chapter IV.

6.2.4.2. Colonization by 3OH-PAME degrading XRB

6.2.4.2.1. Generation of rifampicin resistant mutants of 3OH-PAME degrading XRB

Generation of rifampicin mutants of XB7 and XB122 and their characterization is presented in section 4.2.4 of chapter IV. Rifampicin mutants of XB19, XB102, XB109, XB115, XB174 and XB179 were generated colonization of eggplant rhizosphere and stem was studied as described in section and 4.2.4 of chapter IV. Rifampicin resistant mutant (RR mutant) was designated with parent name followed by suffix letter 'R'. Stability of rifampicin resistance, growth curve and BOX-PCR fingerprinting to confirm genotypic similarity to parent strain of 3PD-XRB was assessed as given in section 4.2.4.2 of chapter IV.

6.2.4.2.2. Inoculation of rifampicin mutants in plants and re-isolation

Plant inoculation in eggplant under in vitro and greenhouse conditions and re-isolation was performed as given in section 4.2.4.3 and section 4.2.4.4 in chapter IV

6.2.5. Analysis of 3OH-PAME degradation by quorum quenching enzymes

6.2.5.1. Partial purification of extracellular quorum quenching enzymes from XRB by Ammonium sulphate precipitation and dialysis

3OH-PAME degrading enzyme/ quorum quenching enzyme (QQE) from the XRB (n=8) was partially purified using ammonium sulphate $[(\text{NH}_4)_2\text{SO}_4]$ and dialysis. XRB were grown in 100 mL BG broth at $28 \pm 2^\circ\text{C}$ for 48 h at 140 rpm. Supernatant was collected by centrifugation at 8000 g for 8 min at 4°C . To 100 mL supernatant, 25 mL of saturated $(\text{NH}_4)_2\text{SO}_4$ (Appendix D) was added for a final concentration of 20% and contents were mixed thoroughly. Volume of saturated $(\text{NH}_4)_2\text{SO}_4$ required to obtain the desired final concentration was calculated as described by Scopes (1987) (Appendix D). Precipitation was allowed to proceed for 30 min at 4°C . Solution was centrifuged at 14000 g for 30 min at 4°C . Supernatant was collected and 300 mL of saturated $(\text{NH}_4)_2\text{SO}_4$ was added for a final concentration of 80%. Precipitation was allowed to proceed at 4°C for 3-4 h. Solution was then centrifuged at 14000 g for 30 min at 4°C . Supernatant was discarded and the pellet was re-suspended in 5 mL of 20 mM phosphate buffer pH 6.8. Dialysis membrane with a molecular weight cut off 12000 to 14000 Daltons (Hi-Media Laboratories, Mumbai) was activated as given in appendix D. Dialysis was allowed to proceed for a period of 18 h. During dialysis, the buffer was replaced with fresh buffer at interval of 4 h, 8 h and finally 3 h before stopping the dialysis. Partially purified enzyme was sterilized by passing through 0.22 μm filter (low protein binding polyvinyl

difluoride). The sterilized protein solution was stored at 4°C upto a few weeks or mixed with equal volumes of 50% sterile glycerol and stored at -20°C for three months.

6.2.5.2. Estimation protein in crude quorum quenching enzyme

The protein concentration of crude QQE was measured using the Bradford's protein assay (Bradford, 1976). Twenty μL of culture supernatant or dialysate was mixed with one mL of 1X Bradford's dye (Appendix D) in a 1.5 mL microfuge tube. Tubes were incubated at room temperature for 10 min and absorbance was read at 595 nm using Nanodrop 1000 (Thermo Scientific, USA) as per manufacturer's instructions. Protein concentration in the samples was determined using standard curve extrapolated using BSA in the liner range of 0.1 -1.0 mg.mL^{-1} (Fig. E1 in appendix E). Concentration of protein in the sample was expressed in mg.mL^{-1} .

6.2.5.3. Analysis of 3OH-PAME degradation by quorum quenching enzymes by High Performance Liquid Chromatography Mass Spectrometry (HPLC-MS)

It has been reported earlier that bacterial ester hydrolases are involved in degradation of 3OH-PAME to 3OH-PA and methanol (Shinohara *et al.*, 2007). To confirm degradation of 3OH-PAME by 3D-XRB in a similar fashion, HPLC-MS was performed to detect formation of 3OH-PA after incubation of 3OH-PAME with the crude enzymes from 3PD-XRB. Assays for 3OH-PAME degradation were carried out as described by Shinohara *et al.* (2007) with slight modifications. Reaction contained 0.1 mg 3OH-PAME, 20 μL DMSO in 20 mM phosphate buffer (pH 6.8) and 10 μL crude enzyme (100 $\mu\text{g protein.mL}^{-1}$). The reactions were performed at $28 \pm 2^\circ\text{C}$ with constant shaking at 200 strokes/min for 2 h. Reactions were terminated by adding 20 μL conc. HCl and extracted with 400 μL DCM and analyzed for the presence of 3OH-PA by HPLC-MS. HPLC (1200

series, Agilent Technologies, USA) was coupled to Triple Quadrupole Mass Spectrometer (6410 series, Agilent Technologies, USA) with Xterra MS C18 column (4.6 mm x 30 mm, 3.5 μm particle size, Waters Corporation, MA, USA). Column temperature was maintained at room temperature. 3OH-PA was eluted using a flow rate of 1.8 $\text{mL}\cdot\text{min}^{-1}$. Mobile phase solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile. Gradient program was solvent B, 10% (0 min), 100% (2.0 min), 100% (3.0 min), 10% (3.2 min), 10% (4.0 min). Ionisation was carried out using electrospray ionization, mass detection was in the negative ionization mode, capillary voltage was set at 4 kV and fragmentor voltage was 100 V. Nebulizing gas was nitrogen and flow rate was 11 $\text{L}\cdot\text{min}^{-1}$, at a temperature of 350°C and pressure of 35 pounds per square inch sample analysis time was 4 min. Pure 3OH-PA in a linear range of 0 - 250 $\text{ng}\cdot\mu\text{L}^{-1}$ was used as standard. From the standard curve prepared by using peak area vs known concentration of 3OH-PA (Fig. E2 in appendix E), amount of 3OH-PA released from esterase degradation of 3OH-PAME by was estimated. One unit (U) of the enzyme was defined as the amount that catalyzed the release of one μM 3OH-PA per min under standard conditions. Specific activity of crude enzyme of XRB towards 3OH-PAME as substrate was expressed as units of enzyme per milligram of protein ($\text{U}\cdot\text{mg}^{-1}$).

6.2.6. Characterization of quorum quenching enzymes

Crude QQE from five strains of 3PD-XRB were chosen for *in vitro* characterization. The main criteria for selection of the five strains namely XB7, XB102, XB109, XB115 and XB122 was that they re-colonized eggplant and exhibited wilt prevention ability >65%. Their specific activities towards 3OH-PAME ranged from 1.07 to 6.39 $\text{U}\cdot\text{mg}^{-1}$. Though

XB174 exhibited highest activity against 3OH-PAME, it was not included in this study since it did not recolonize eggplant or prevent wilt.

6.2.6.1. Effect of quorum quenching enzymes on expression of EPS and Egl by *R. solanacearum*

To study the effect on EPS production by Rs-09-100, crude enzyme (100 µg protein.mL⁻¹) was added in BG agar. Appropriate dilution (10⁹ fold dilution) of 36 h old culture broth of Rs-09-100 (*c.* 2.0 Log CFU.mL⁻¹) was spread plated and incubated at 28 ± 2°C for 48 h to obtain isolated colonies. Control plates consisted of BG medium spread with suspension of Rs-09-100 without the crude enzyme. Reduction in EPS and fluidity of the Rs-09-100 colonies on BG agar with crude enzyme was observed visually in comparison to control. Three replications were maintained for each XRB and experiments were repeated thrice independently.

To study the effect on Egl production by Rs-09-100, crude enzyme (100 µg protein.mL⁻¹) was added in BG broth prior to inoculation of a colony of Rs-09-100. Control consisted of BG broth without the crude enzyme and inoculated with Rs-09-100. Tubes were incubated at 28 ± 2°C for 18 h with constant shaking at 140 strokes/min. Culture supernatant obtained after centrifugation at 8000 g for 8 min was sterilized by membrane filtration (0.22 µm) and 80 µL was assayed in wells on carboxy methyl cellulose (CMC) agar plates to detect Egl activity as described by Compant *et al.* (2005b) and given in section 3.2.3.4.3 in chapter III. Reduction in Egl production by Rs-09-100 in presence of crude enzyme was observed visually in comparison to control. Three replications were maintained for each XRB and repeated thrice as independent experiments.

6.2.6.2. Determination of lipase and esterase activity of quorum quenching enzymes

True esterases are defined as carboxylesterases (EC 3.1.1.1) that hydrolyze ester substrates with short-chain fatty acids (<C10), whereas lipases (EC 3.1.1.3) are defined as, carboxylesterases that have the ability to hydrolyze long-chain acylglycerols (>C10) (Henne *et al.*, 2000). To determine the nature of the QQE which degraded 3OH-PAME (a long chain fatty acid ester), activity of QQE towards additional substrates was tested. P-Nitrophenyl esters are classically used as substrates to quantitate esterase/lipolytic activity. In the present study they were used as an alternate substrate to 3OH-PAME for rapid characterization of quorum quenching enzymes. Short chain p-Nitrophenol acetate (C2) was used as a substrate to determine esterase activity whereas long chain p-Nitrophenol Palmitate (C15) was used as a lipase substrate. All spectrophotometric readings of p-nitrophenol liberated were measured at 410 nm using Nanodrop 1000 (Thermo Scientific, USA) as per manufacturer's instructions. Recipes for substrates, buffers, reagents and stocks of metal ions used for characterization of QQE are given in appendix D.

6.2.6.2.1. Activity of quorum quenching enzymes against p-Nitrophenyl acetate (C2) and p-Nitrophenyl palmitate as substrates (C15)

Hydrolytic activity QQE produced by XRB towards p-nitrophenyl acetate (PNPA) as an esterase substrate and p-nitrophenyl palmitate (PNPP) as a lipase substrate was determined. Fifty μL reaction contained 5 μL of 2 mM PNPA or PNPP and 35 μL of 20 mM phosphate buffer pH 6.8. Reaction was initiated by adding crude QQE (2 $\mu\text{g}\cdot\text{mL}^{-1}$ protein) and allowed to proceed at 37°C for 10 mins. The reaction was stopped by adding 50 μL of 0.25 M Na_2CO_3 as described by Ozcan *et al.* (2009). The amount of p-

nitrophenol (PNP) released was measured at 410 nm. Blank reactions (no enzyme added) were performed to subtract the background absorbance caused by non-enzymatic hydrolysis of substrate. Each reaction was carried out in triplicates. Standard curve using p-nitrophenol (PNP) was made to determine the concentration of PNP liberated by enzymatic reaction (Fig. E3 in appendix E). One unit (U) of esterase activity was defined as the amount of enzyme liberating 1 μ M of PNP per minute under the above assay conditions. The specific activity was expressed as the units of enzyme activity per milligram of protein.

6.2.6.2.2. Enzyme kinetics of quorum quenching enzymes using PNPA and PNPP

The effect of substrate (PNPA and PNPP) concentrations (0.05–2 mM) on the reaction rates of the crude QQE from 3PD-XRB were assayed by using enzyme assay as described above in section 6.2.6.2.1. The Michaelis–Menten constant (K_m) and the maximum velocity for the reaction (V_{max}) with PNPA and PNPP were calculated by Lineweaver–Burk plot obtained by plotting the 1/Substrate concentration ($1/S$) and 1/reaction velocity ($1/V$). Catalytic efficiency for each enzyme was determined from V_{max}/K_m ratios. Concentration of PNP liberated by enzymatic reaction was calculated from the p-Nitrophenol standard curve to determine the enzyme activity (Fig. E3 in appendix E).

6.2.6.3. Effect of pH, temperature and inhibitors on activity of quorum quenching enzymes

6.2.6.3.1. Effect of pH and temperature

Effect of pH on esterase activity was determined at 37°C at pH 4.0, pH 6.0, pH 6.8, pH 8.0 and pH 10.0. Buffers (20 mM) used were Citrate-Phosphate buffer (pH 4.0 and pH

6.0), phosphate buffer (pH 6.8 and pH 8.0), Glycine-NaOH buffer (pH 10.0). Enzyme was pre-incubated in the appropriate buffer for 2 h, and residual esterase activity was measured using PNPA as substrate. Effect of temperature was assessed by pre-incubating the enzyme at different temperatures (15°C, 28°C, 37°C and 65°C) for 2 h, and the residual esterase activity was measured using PNPA substrate as described above. To determine effect of pH and temperature, each reaction contained 20 µL of appropriate buffer (0.1 M), 5 µL of 2mM PNPA and distilled water to 50 µL. Reaction was initiated by adding 10 µL dialysate (2 µg.mL⁻¹ protein) and allowed to proceed under standard conditions described in section 6.2.6.2.1. Concentration of PNP liberated by enzymatic reaction was calculated from the p-Nitrophenol standard curve to determine the enzyme activity (Fig. E3 in appendix E).

6.2.6.3.2. Effect of chemical reagents and metal ions inhibitors

Effect of several chemical reagents [(1 mM concentration) ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), beta-mercaptoethanol, iodoacetic acid and SDS] and metal ions [(5 mM concentration) Sn⁺², Hg⁺², Ag⁺, Cu⁺², Fe⁺², Mg⁺², Ca⁺², NH₄⁺², Na⁺, K⁺, Mn⁺² and Zn⁺²] on the esterase activity were examined by pre-incubating the enzyme with them at 37°C for 1 h, residual activity was determined using PNPA as a substrate. Esterase activity in the absence of any inhibitor was considered 100%. Each reaction contained 20 µL of 0.1 M phosphate buffer (pH 6.8), 2 mM PNPA and distilled water to 50 µl. Reaction was initiated by adding 10 µL dialysate (2 µg.mL⁻¹ protein) and allowed to proceed under standard conditions described in section 6.2.6.2.1. Concentration of PNP liberated by enzymatic reaction was calculated from the p-Nitrophenol standard curve to determine the enzyme activity (Fig. E3 in appendix E).

6.2.7. Determination of molecular weight of quorum quenching enzymes

Recipes for buffers, reagents and stacking and resolving polyacrylamide gel are given in Appendix D.

6.2.7.1. Acetone precipitation of proteins

Acetone precipitation of extracellular esterases from QQE was carried out as described by Sommer *et al.* (1997). XRB were grown in 5 mL BG broth for 24-48 h at 28°C with constant shaking at 140 rpm. Supernatant of the culture was obtained by centrifugation at 10000 rpm for 10 min at 4°C. Four volumes of chilled (stored at -20°C) 100% acetone was added and contents were mixed thoroughly for a final concentration of 80% of acetone. Tubes were incubated at -20°C for 15 min. Solutions were mixed thoroughly and centrifuged at 13000 rpm for 30 min at 4°C. Supernatant was discarded and protein pellet was allowed to dry at room temperature for 30 min. Pellet was dissolved in 100 µL of 20 mM phosphate buffer (pH 6.8) and 10 µL was used for PAGE analysis.

6.2.7.2. Seminal SDS PAGE

SDS PAGE was performed as described by Sambrook and Russell (2001). When activity staining was performed, no beta-mercaptoethanol was used and sample was not boiled. Vertical gel electrophoresis was performed using SDS PAGE apparatus (Atto Corporation, Japan). The plates were assembled using the clamps and gasket as per the manufacturer's instructions. One cm mark was labelled below the end of the comb. Seven millilitres of 8% resolving gel was prepared and added in between the gap of plates fixed using a gasket using pipette taking care that no air bubble was trapped. Immediately, one mL of 0.1% SDS solution was overlaid over the top of resolving gel to prevent oxygen

penetrating and inhibiting polymerization and also to allow formation of evenly polymerised gel top. Resolving gel was placed vertical and undisturbed to allow polymerization at room temperature for 30 min. The overlay solution was poured off and gel top was rinsed using distilled water. Four percent stacking gel was prepared and 1.5 mL was overlaid on the resolving gel and immediately comb (cleaned with ethanol) was placed carefully avoiding trapping of air bubbles. Stacking gel was allowed to polymerize for 30 min at room temperature. Comb was removed carefully and the wells were washed using a squirt bottle. The gel-plate assembly was fit into the electrophoresis unit as per manufacturer's instructions containing 400 mL of 1X tris-glycine buffer. In addition 100 mL of 1X tris-glycine buffer was added in the top reservoir. Ten μL sample was mixed with five μL of 3 X Laemmli dye (Laemmli , 1970) and loaded in each well. In the unloaded wells 15 μL of 1X Laemmli dye was added. Electrophoresis was performed at 75 Volts/ 30 mA for 2 h. Gel was gently moved to a polypropylene container and stained as desired.

6.2.7.3. Zymography

Zymography (activity staining) for esterase was done as described by Sommer *et al.* (1997). After electrophoresis, the gel was placed in 50 mL of 20% isopropanol for 20 min and incubated on a shaker at 50 rpm to remove SDS. The gel was then washed twice for 10 min in distilled water with constant shaking at 50 rpm at RT. The gel was then placed over agar slab containing 10% tween 20, 5 mM CaCl_2 , 1.3% agar in 20mM phosphate buffer pH 6.8. The gel was incubated at 37°C for 18 h. A white precipitate of Ca-laurate formed near the esterase active protein bands. To visualize the molecular weight of the band the gel was counter stained with coomassie brilliant blue (CBB).

6.2.7.4. Coomassie blue staining

The gel was shaken overnight in 50 mL CBB staining solution overnight with constant shaking at 50 rpm at RT. Destaining was carried out using 50 mL destaining solution for 6 hours 50 rpm at RT with changing of destaining solution after an interval of 2 h. The gel placed on a white light illuminator (Bangalore Genei, India) and photographed for documentation using Canon IXUS 115 HS camera. The relative molecular mass of the enzyme was determined from its mobility relative to those of the standard molecular markers. Molecular mass marker consisted of aprotinin (6.5 kDa), alpha lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), glutamic dehydrogenase (55 kDa), bovine serum albumin (66 kDa), fructose 6-phosphate kinase (84 kDa), phosphorylase *b* (97.4 kDa), beta-galactosidase (116 kDa), and myosin (205 kDa) wide range marker (Sigma Aldrich, USA).

6.3. RESULTS AND DISCUSSION

6.3.1. Biological confirmation of laboratory synthesized 3OH-PAME

Laboratory synthesized 3OH-PAME was found to be biologically active as seen from the response of quorum sensing indicator *R. solanacearum* strain AW1-3 towards concentrations of 3OH-PAME as little as 4 nM (Table 6.1). 3OH-PAME is volatile, water soluble and diffusible quorum sensing molecule of *R. solanacearum*, synthesized by SAM dependent methyl transferase (a product of *phcB* gene) (Clough *et al.*, 1994, Flavier *et al.* 1997b). 3OH-PAME was found to completely restore expression of quorum sensing regulated traits in a *phcB* mutant of *R. solanacearum* at less than 1 nM concentrations (Clough *et al.*, 1994, Flavier *et al.*, 1997b). Recognition of 3OH-PAME by *R.*

solanacearum is highly specific and alterations in the acyl chain or methyl ester reduce its quorum sensing activity over 1000 fold (Schell, 2000). Indicator strain AW1-3 does not produce its own 3OH-PAME however it responds to exogenous 3OH-PAME by expressing the *eps-lacZ* reporter fusion and this property is used throughout the study to detect 3OH-PAME.

6.3.2. Screening of XRB for degradation of 3OH-PAME

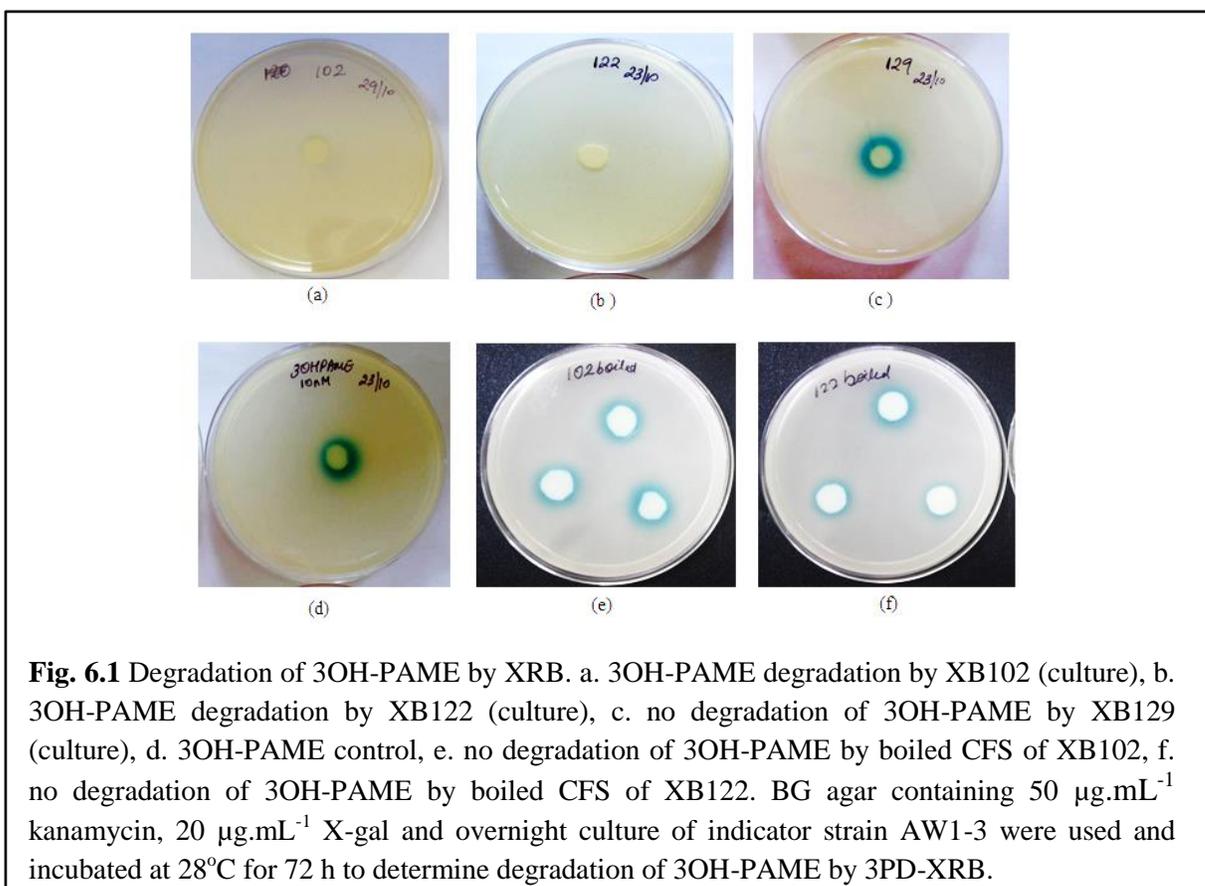
Using the rapid bioassay with indicator AW1-3, eight XRB were found to degrade 10 μ M 3OH-PAME. 3OH-PAME degrading factor was found to be extracellular and proteinaceous in nature when the CFS and boiled CFS of 3PD-XRB was tested respectively in bioassay using AW1-3 (Fig. 6.1, Table 6.2). These results clearly demonstrate the degradation and reduction in concentration of 3OH-PAME below 4.0 nM (minimum concentration of 3OH-PAME to which AW1-3 responded by expressing *lacZ*), by the XRB screened in this study. Further, these results suggest that the 3OH-PAME degradation may be a rare trait amongst the endophytic bacteria from eggplant and chilli as only 4.79 % XRB in the collection were found positive.

Isolation of bacteria able to degrade quorum sensing molecules, most commonly the acyl-HSLs and recently 3OH-PAME has widely relied on enrichment from agricultural or rhizosphere soils (Leadbetter and Greenberg, 2000; Shinohara *et al.*, 2007; Chan *et al.*, 2011). Enrichment-isolation procedure has longer incubation periods of 3-4 days on an average and 5-6 successive transfers of bacterial growth in a defined medium containing quorum sensing molecules.

Table 6.1 Minimum concentration of laboratory synthesized 3OH-PAME required to trigger quorum sensing response of indicator *R. solanacearum* strain AW1-3¹.

Concentration of 3OH-PAME	Quorum sensing response of AW1-3 ¹
1 nM	-
2 nM	-
4 nM	+
6 nM	+
8 nM	+
10 nM	+

¹interpreted based on appearance of blue color on BG-Kan-X-Aw plates due to expression of beta galactosidase, + indicates appearance of blue color (quorum sensing response), - indicates no blue colour (no quorum sensing response).



Enrichment procedure relies on the ability of bacteria to utilize acyl-HSLs as a sole source of carbon and/or nitrogen (Leadbetter and Greenberg, 2000). A faster approach would be use of fast growing isolates from various ecological niches to be used as quorum quenching agents. XRB with 3OH-PAME degrading ability are of particular advantage because they may re-colonize plant xylem and be used in biocontrol of BW. Several researchers have screened pre-grown cultures of bacteria isolated from soil, stem and rhizosphere of plants for quorum quenching activities against phytopathogens colonizing similar ecological niche (Dong *et al.*, 2000; Reimann *et al.*, 2002; Cho *et al.*, 2007). The present study is the first report to use non-enrichment type screening of 3OH-PAME degradation by bacteria. In addition the rapid bioassay makes it possible to screen large number of bacterial isolates at a time using less quantity of 3OH-PAME while the enrichment requires large quantities. Thus the bioassay methodology presented herein is a faster and cheaper alternative for screening of 3OH-PAME degradation by plant associated bacteria.

6.3.3. Characterization of 3OH-PAME degrading bacteria

6.3.3.1. Hydrolysis of tweens and tributyrin by 3OH-PAME degrading XRB

Results of degradation of Tween 20, Tween 80 and tributyrin by 3PD-XRB is presented in Table 6.3 and Fig. 6.2. The results clearly demonstrate that the 3PD-XRB possess esterase and lipase activity which may possibly be involved in degradation of 3OH-PAME which is a long chain fatty acid methyl ester. Traditionally plate based bioassays for screening for microbial extracellular esterases and lipases have used tweens and tributyrin as substrates (Tomioka, 1983; Castro *et al.*, 1992; Plou *et al.*, 1998; Henne *et al.*, 2000; Kudanga *et al.*, 2007; Kumar *et al.*, 2012). Agar based assay using these substrates is a useful test for detection of strains producing esterase/lipases.

Table 6.2 Degradation of 3OH-PAME by XRB and cell free supernatant of XRB.

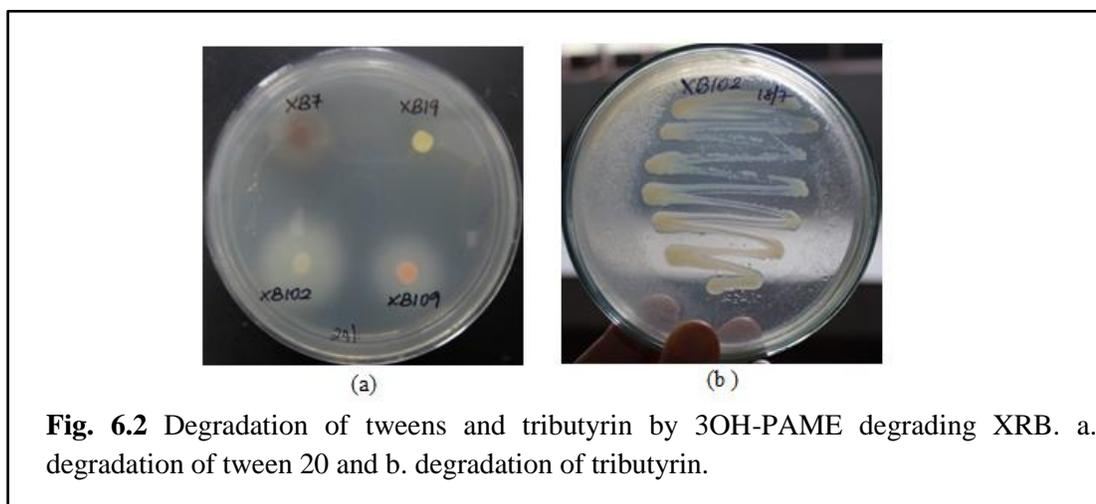
Strain	Induction of <i>lacZ</i> expression in AW1-3 by XRB ¹	Induction of <i>lacZ</i> expression in AW1-3 by CFS of XRB ¹	Induction of <i>lacZ</i> expression in AW1-3 by boiled CFS of XRB ¹
XB7	-	-	+
XB19	-	-	+
XB102	-	-	+
XB109	-	-	+
XB115	-	-	+
XB122	-	-	+
XB174	-	-	+
XB179	-	-	+
BG broth (control)	+	NA	NA

Indicator bacterium used in the bioassay was *R. solanacearum* strain AW1-3 (*phcB83::Tn5*, *eps-130::Tn3lacZ*), ¹Development of blue colour in plate assay (+) and no blue colour (-) (development of blue colour in plate assay indicates no degradation of 3OH-PAME), CFS: cell free supernatant, NA Not applicable.

Table 6.3 Characterization of 3OH-PAME degrading bacteria.

Strain	T20	T80	TB	HCN ^a	Sid ^b	BCE (%) ^c
XB7	+	-	+	+	+	77.5 ± 3.5
XB19	-	-	-	-	+	65.0 ± 21.2
XB102	+	+	+	-	+	77.5 ± 3.5
XB109	+	+	+	-	+	67.5 ± 3.5
XB115	+	+	+	-	+	67.4 ± 10.6
XB122	+	+	+	+	+	87.5 ± 17.6
XB174	+	+	+	-	-	55.0 ± 7.0
XB179	-	+	-	-	-	45.0 ± 7.0

T20- tween 20 degradation, T80- Tween 80 degradation, TB- tributyrin degradation tested by plate based bioassay, + presence of trait, - absence of trait, ^a hydrogen cyanide (HCN) production and ^b Siderophore (Sid) production was determined *in vitro*, ^c BCE-Biocontrol efficacy was determined under greenhouse conditions.



6.3.3.2. Production of antagonistic compounds by 3OH-PAME degrading XRB

Results of production of antagonistic compounds by 3PD-XRB are presented in Table 6.3. Volatile and diffusible molecules may act synergistically with quorum quenching in enhancing BW prevention in eggplant. Ability of the bacteria to produce additional inhibitory compounds simultaneously with quorum quenching ability proved effective in preventing *Erwinia caratovora* infection (Zhao *et al.*, 2008).

6.3.3.3. Identification of 3OH-PAME degrading XRB

3PD-XRB namely XB7 and XB122 were identified as *Pseudomonas aeruginosa*, XB109 and XB115 were identified as *Rhodococcus corynebacterioides*, XB102 was identified as *Stenotrophomonas maltophilia* and XB174 as *Acinetobacter* sp. GenBank accession numbers and tree based on 16S rRNA gene sequences of 3PD-XRB is presented in Fig. 6.3. A collection of endophytic XRB belonging to phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were tested for 3OH-PAME degradation in the present study. Interestingly, the 3OH-PAME degrading XRB were restricted to *Gammaproteobacteria* and *Actinobacteria*. In an earlier study *Ideonella* sp. a

betaproteobacterium isolated from tomato rhizosphere was reported degrade 3OH-PAME (Shinohara *et al.*, 2007). The present study thus increases the known diversity of plant associated bacteria able to degrade 3OH-PAME.

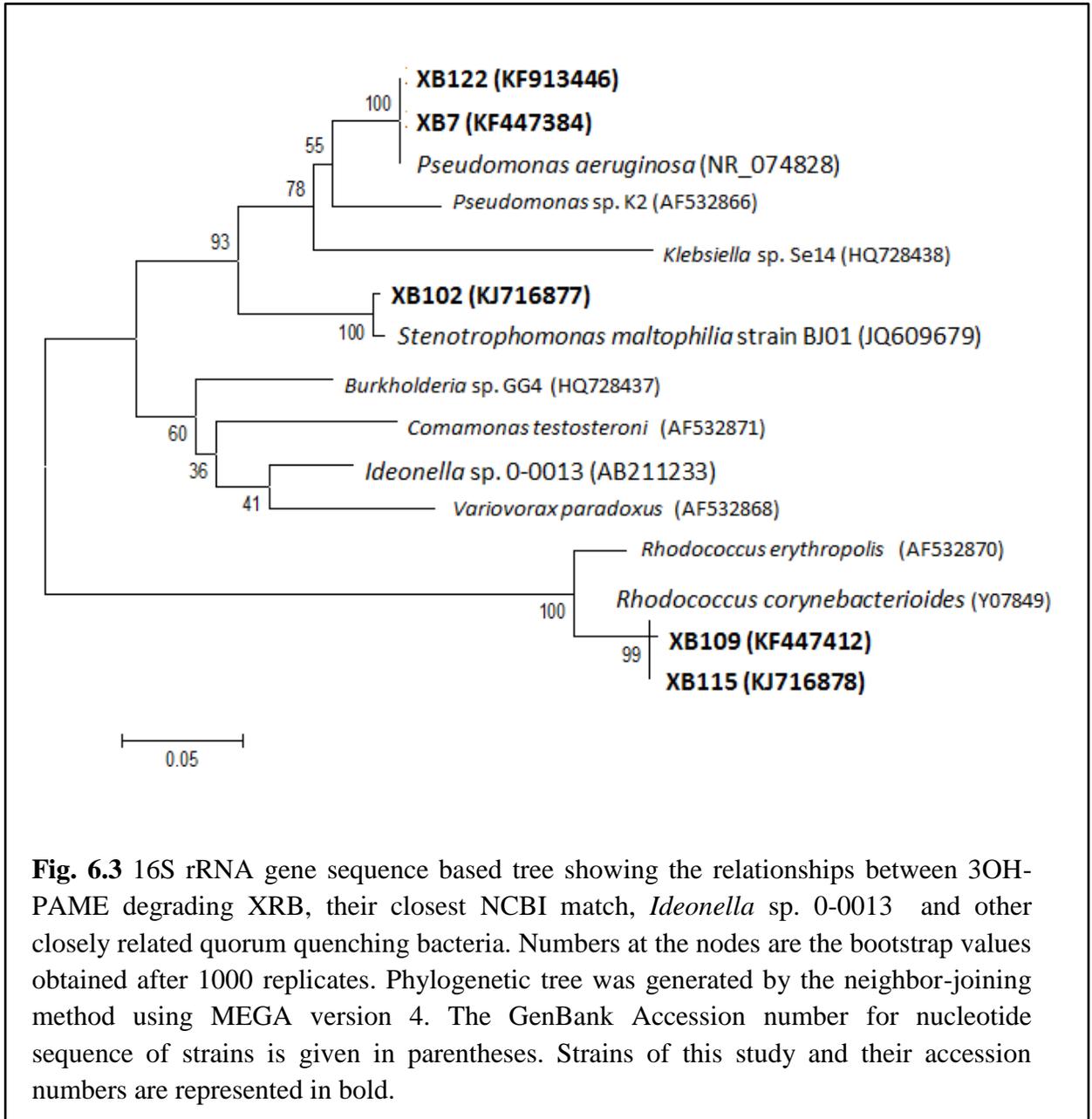


Fig. 6.3 16S rRNA gene sequence based tree showing the relationships between 3OH-PAME degrading XRB, their closest NCBI match, *Ideonella sp.* 0-0013 and other closely related quorum quenching bacteria. Numbers at the nodes are the bootstrap values obtained after 1000 replicates. Phylogenetic tree was generated by the neighbor-joining method using MEGA version 4. The GenBank Accession number for nucleotide sequence of strains is given in parentheses. Strains of this study and their accession numbers are represented in bold.

6.3.4. Biocontrol efficacy and eggplant colonization by 3OH-PAME degrading XRB

6.3.4.1. Biocontrol efficacy of 3OH-PAME degrading XRB

Amongst the 3PD-XRB highest BCE was that of XB122 (87.5%) followed by XB7 and XB102 which recorded a BCE of 77.5%. BCE of XB19, XB109 and XB115 ranged between 65% to 67.5%. XB174 recorded a BCE of 55% whereas XB179 recorded the lowest BCE of 45% (Table 6.3). Bacteria capable of naturally producing quorum quenching molecules have been shown to decrease disease severity in plants. Co-inoculation of quorum quenching bacteria with the soft rot pathogen *Erwinia caratovora* has been shown to be effective biocontrol strategy in potato (Dong *et al.*, 2004; Chan *et al.*, 2011; Cirou *et al.*, 2012). Newman *et al.*, 2008 reported that co-inoculation of strains degrading Diffusible Signal Factor (DSF), a quorum sensing molecule with *Xylella fastidiosa* and *Xanthomonas campestris* decreased the disease severity in grapevine and vegetables of family *Brassicaceae*. Till date there is no study reporting the use of 3OH-PAME degrading endophytic bacteria as biocontrol agents for management of BW in eggplant under greenhouse conditions. In this study, it clearly seen that plants pre-treated with 3OH-PAME degrading strains exhibited reduced wilt incidence upon challenging with *R. solanacearum*. In addition the 3PD-XRB also produced volatile and diffusible antimicrobials which may aid in BW disease suppression.

6.3.4.2. Colonization of eggplant by 3OH-PAME degrading XRB

Pre-treatment of plants before the challenge with pathogen may allow colonization by the 3OH-PAME degrading bacteria and regulate the buildup of quorum required to trigger quorum sensing in *R. solanacearum*. Hence colonization ability of 3PD-XRB was carried out using rifampicin mutants. Growth curve and BOX-PCR fingerprinting of XB7R and XB122R is presented in chapter IV.

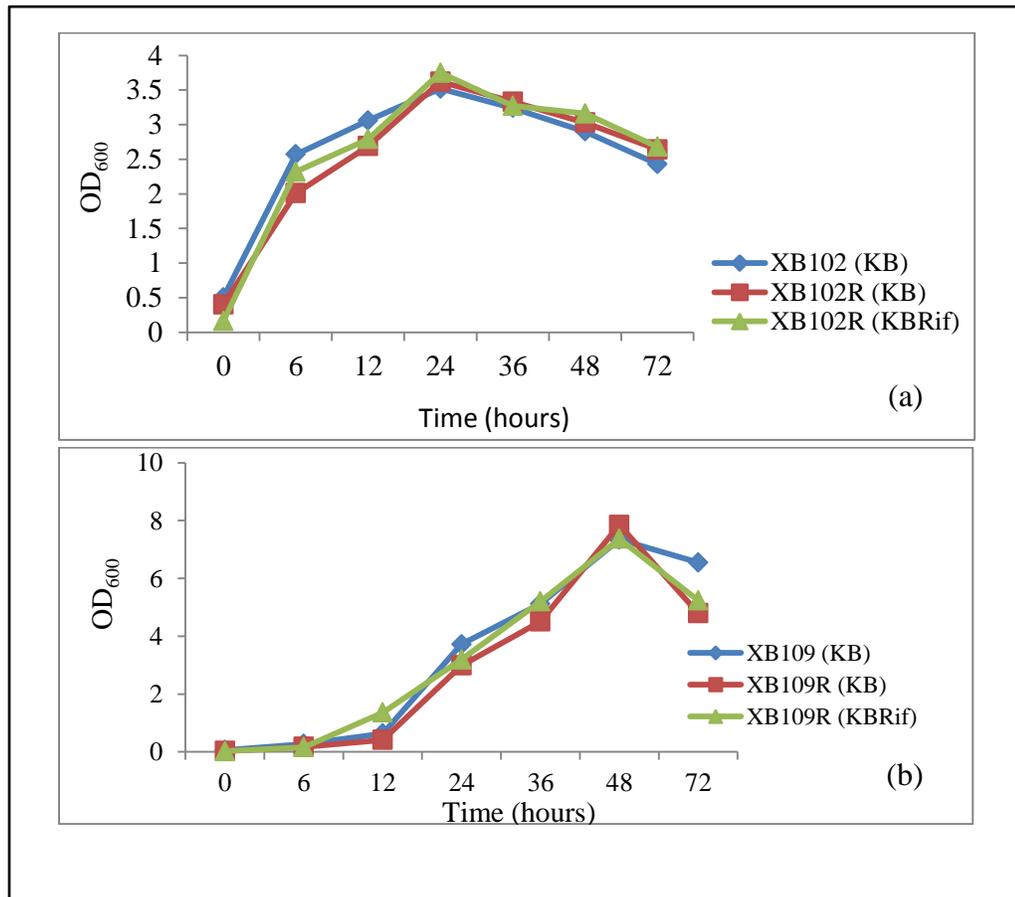
As seen from the results the population of RR colonies obtained on KB and KBRif agar after 15 transfers on medium without rifampicin were the same indicating the resistance is stable (Table 6.4). In addition the growth rates of the RR mutants in KB and KBRif broth was found similar to that of the wild type parent when tested over a period of 72 h (Fig. 6.4 a, b and c below and Fig. 4.6 in chapter IV). BOX PCR results indicated that genetic fingerprints of RR mutants and the parent XRB were the same (Fig. 6.5 below and Fig. 4.9a in chapter IV). Therefore the RR mutants were regarded as representatives of their respective parent wild type XRB for studying their colonization in eggplant cv. *Agassaim* under greenhouse conditions. RR mutants re-isolated from eggplant samples after 15 and 30 DAI were confirmed similar to introduced RR mutants in eggplant based on their similar colony characteristics and BOX PCR fingerprints (Fig. 6.5 below and Fig. 4.9a).

Results of colonization of eggplant by XB7R and XB122R are discussed in section 4.3.4.2 in chapter IV. XB102 is identified as *Stenotrophomonas maltophilia*. Population of XB102R (RR mutant of XB102) immediately after inoculation, 15 DAI and 30 DAI is presented in Fig. 6.6. Rhizosphere population of XB102 increased to 6.33 Log CFU.g⁻¹ at 15 DAI and slightly reduced by the 30th day to 5.20 Log CFU.g⁻¹. However its endophytic population increased from 15 DAI to 30 DAI from 3.67 Log CFU.g⁻¹ to 4.02 Log CFU.g⁻¹. XB109 and XB115 are identified as strains of *Rhodococcus corynebacterioides*. XB109 colonized stem and rhizosphere of eggplant whereas XB115 colonized only the rhizosphere. Rhizosphere population of XB109 and XB115 decreased from 15 DAI to 30 DAI ranging from 6 Log CFU.g⁻¹ to 5 Log CFU.g⁻¹. However, endophytic population of XB109 was stable at 15 DAI and 30 DAI at 4.25-4.34 Log CFU.g⁻¹.

Table 6.4 Population of rifampin resistant cells of 3OH-PAME degrading XRB in a single colony before and after 15 serial transfers on KB and KBRif agar.

Strain	KB (Log CFU.mL ⁻¹)	KBRif (Log CFU.mL ⁻¹)
XB7R (T1)	10.1 ± 0.00	10.05 ± 0.00
XB7R (T15)	10.1 ± 0.00	10.0 ± 0.00
XB122R (T1)	9.9 ± 0.01	9.9 ± 0.01
XB122R (T15)	10.0 ± 0.01	10.0 ± 0.01
XB102R (T1)	10.1 ± 0.0	10.0 ± 0.02
XB102R (T15)	9.7 ± 0.0	9.8 ± 0.01
XB109R (T1)	8.9 ± 0.01	8.9 ± 0.02
XB109R (T15)	8.6 ± 0.01	8.7 ± 0.0
XB115R (T1)	8.7 ± 0.02	8.8 ± 0.04
XB115R (T15)	8.7 ± 0.00	8.6 ± 0.00

T1: colonies obtained after the first transfer, T15: colonies obtained after the 15th transfer, KB: KB agar, KBRif: KB agar with rifampin (100 µg.mL⁻¹). Assessment of population was done on KB and KBRif agar. All experiments were conducted at 28 ± 2°C, values are mean colony counts in Log CFU.mL⁻¹ of two replications per isolate per dilution and showing standard deviations.



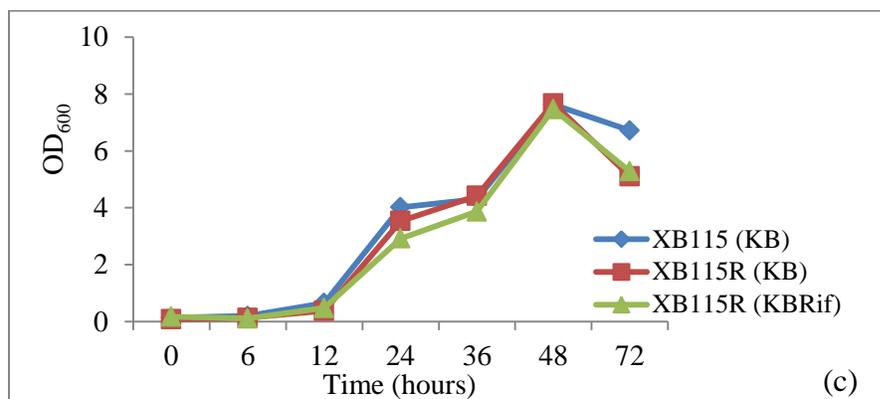


Fig. 6.4 Growth curves of 3OH-PAME degrading XRB and their respective rifampicin resistant mutants grown in KB and KBRif broth. a. growth curve of XB102 and XB102R, b. growth curve of XB109 and XB109R, c. growth curve of XB115 and XB115R.

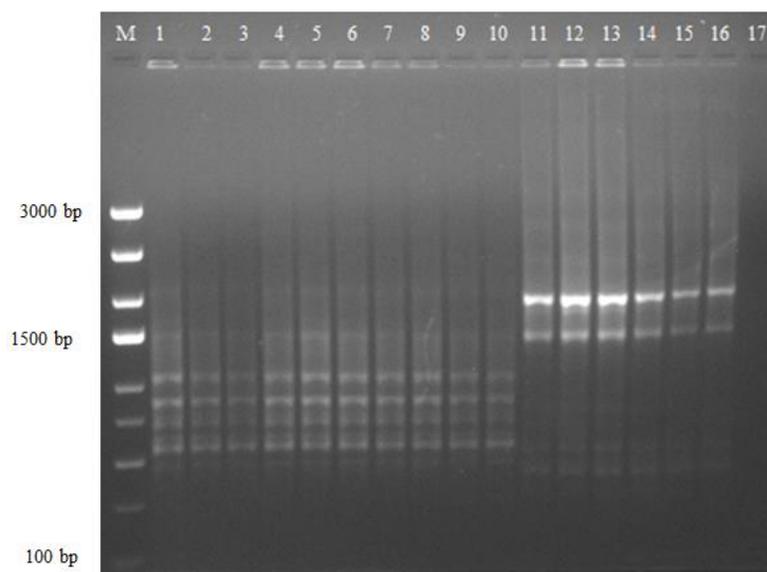


Fig. 6.5 BOX PCR fingerprinting of 3OH-PAME degrading XRB. M- 100 bp to 5 kb ladder, Lane 1-XB109, Lane 2-XB109R, Lane 3-XB109R-15D-E, Lane 4-XB109R-15D-Rh, Lane 5-XB109R-30D-E, Lane 6-XB109R-30D-R, Lane 7-XB115, Lane 8-XB115R, Lane 9-XB115R-15Rh, Lane 10-XB115R-30Rh, Lane 11-XB102, Lane 12-XB102R, Lane 13-XB102R-15D-E, Lane 14-XB102R-15D-Rh, Lane 15-XB102R-30D-E, Lane 16-XB102R-30D-Rh, Lane 17-PCR negative control. D-days after inoculation, R- Rifampicin resistant mutant, Rh-Rhizosphere, E- endophytic.

Strains of *Stenotrophomonas maltophilia*, *Pseudomonas* sp. and *Rhodococcus* sp. produce quorum quenching molecules (acyl-HSL degrading enzymes) and colonize rhizosphere and endophytic tissues of several plants including Indian mustard, potato and *Cyperus laevigatus* (Pandey *et al.*, 2005; Huang *et al.*, 2006; Cirou *et al.*, 2012; Singh *et al.*, 2013). This study reports re-colonization of eggplant by 3OH-PAME degrading bacteria. Orthologues of *phcB* gene (involved in synthesis of 3OH-PAME) are shown to be present in endophytic *Burkholderia* sp., *Ralstonia* sp. and soil bacterium *Cupriavidus* sp. (Genin and Denny, 2012; Mitter *et al.*, 2013). Certain strains of *Agrobacterium* sp. were found to induce expression of EPS a trait governed by *phcB* gene, in a *R. solanacearum* mutant lacking *phcB* gene (Clough *et al.*, 1994). If involvement of 3OH-PAME in plant colonization by other bacteria is ascertained, then 3PD-XRB may be postulated to have competitive advantage against other soil and plant associated bacteria for faster colonization of plant rhizosphere and stem tissues. It may be concluded that the re-colonization ability of XB7, XB122, XB102, XB109 and XB115 may have contributed to efficient delivery of QQE *in planta* and led to the decrease in wilt incidence in plants treated with 3PD-XRB. Though the 3PD-XRB produced HCN and siderophores, no direct antagonism towards *R. solanacearum* was exhibited by XB102, XB109 and XB115 when tested by agar cup bioassay (Chapter III).

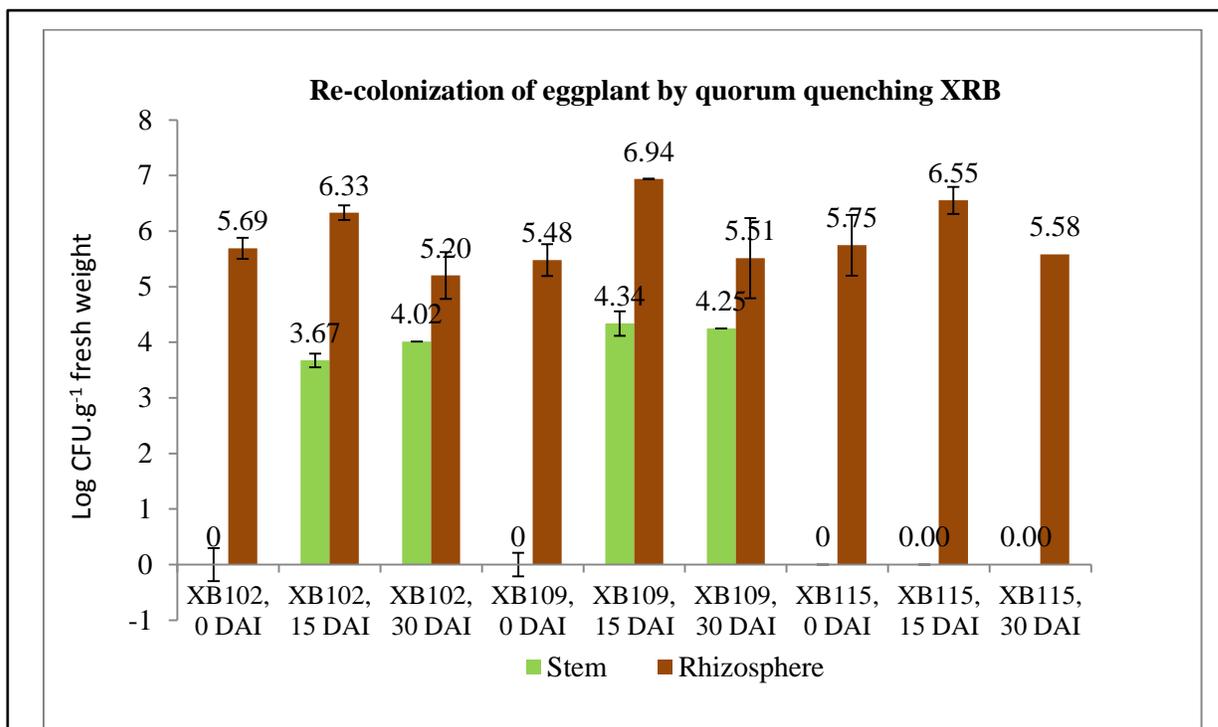


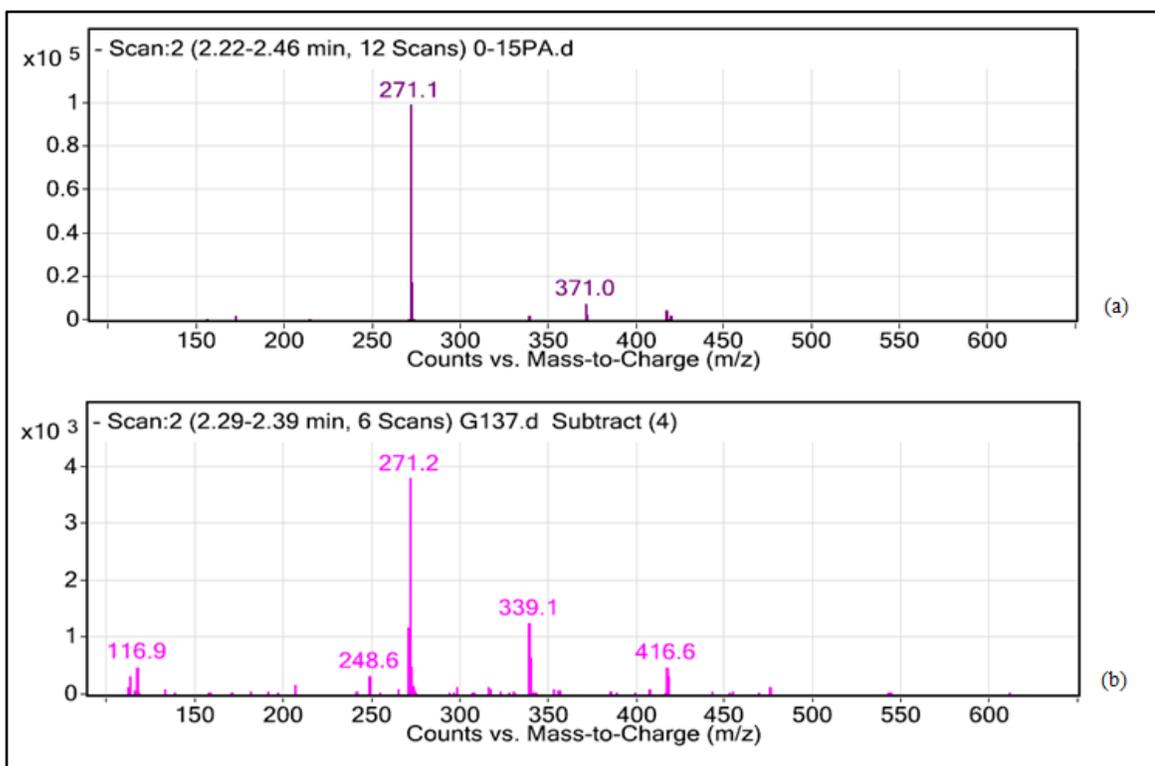
Fig. 6.6 Re-colonization of eggplant by 3OH-PAME degrading XRB. Values indicate population in Log CFU.g⁻¹ fresh weight of two replicate samples and error bars indicate standard deviation. Recolonization of eggplant cv. *Agassaim* was studied under greenhouse conditions and plating on KBRif plates. DAI: Days after inoculation.

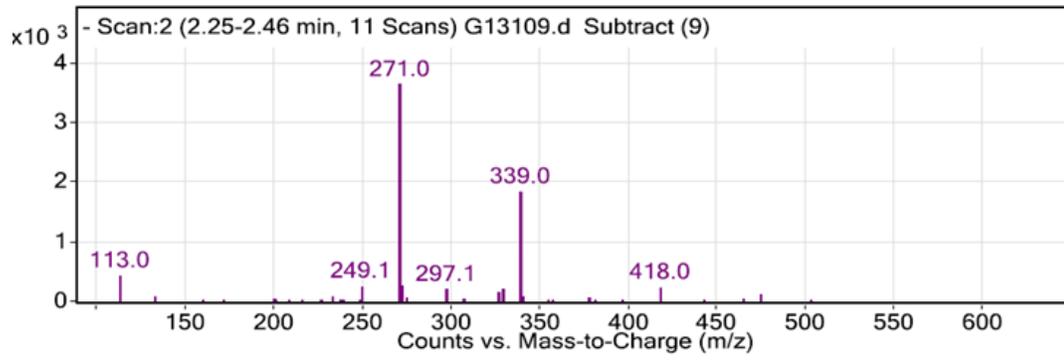
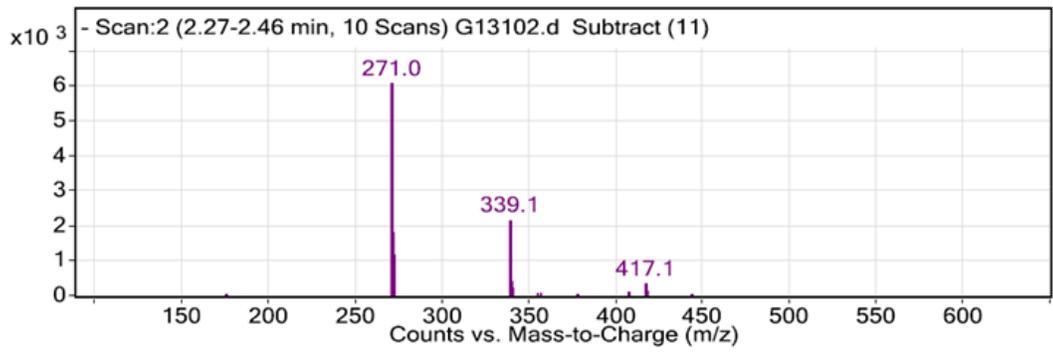
6.3.5. Analysis of 3OH-PAME degradation by quorum quenching enzymes by HPLC-MS

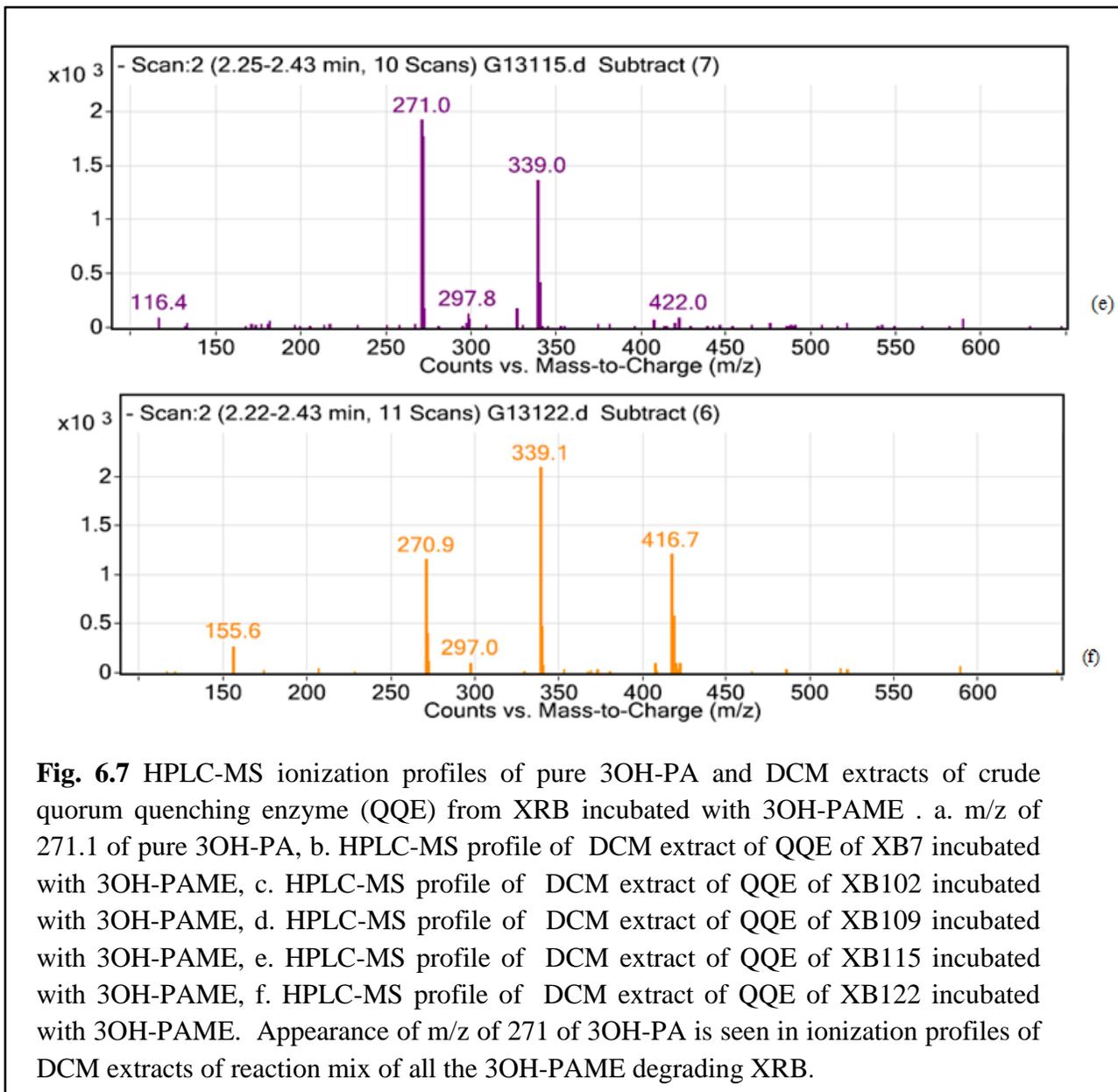
Quantification of QQ has been classically done by measuring decrease in concentration QS molecules using indicator strains in plate based bioassays. Several QS indicator strains with diverse responses such as pigment production (*Chromobacterium violaceum* CV026, McClean *et al.*, 1997), GFP expression (*Escherichia coli* JB523 Medina-Martínez *et al.*, 2007), luminescence (*E. coli*-pSB401, Chan *et al.*, 2011) and *lacZ* expression (*Agrobacterium tumefaciens* NT1, Barbey *et al.*, 2013) were designed for quantification. However, use of indicator strain (AW1-3) to quantify decrease in concentration 3OH-PAME would be inaccurate because 3OH-PAME is volatile and diffuses throughout the plate (Shinohara *et al.*, 2007) and response of AW1-3 to 3OH-

PAME concentrations less than 4 nM was not observed in this study. Therefore HPLC-MS was used for quantification of degradation of 3OH-PAME.

Preliminary analysis of 3OH-PAME degradation by crude QQE using HPLC-MS revealed that product of degradation was 3OH-PA as seen by appearance of a peak of 271 m/z in the mass spectra of reactions using crude QQE (Fig. 6.7a to 6.7e). Similar fashion of 3OH-PAME degradation to 3OH-PA by bacterial esterase is been reported (Shinohara *et al.*, 2007). No peak with m/z of 271 was seen when reaction mixture without 3OH-PAME was analyzed. However, when control reaction containing 3OH-PAME and no enzyme was analyzed by HPLC-MS, a small peak at m/z of 271 was seen, most likely due to auto-degradation of 3OH-PAME at high temperature and acidic conditions used in HPLC-MS. For background subtraction, the peak area that appeared in control mix with only 3OH-PAME (no enzyme) was deducted from the peak areas obtained for the test reactions.







A standard curve of peak area vs. concentration of authentic 3OH-PA (Fig. E2 in appendix E) was plotted to determine the amount of 3OH-PA released from the enzymatic reaction. Specific activities (μM 3OH-PA released per min per mg protein) of crude QQE towards 3OH-PAME are presented in Table 6.5. Highest specific activity towards 3OH-PAME was that of XB174 ($17.78 \text{ U}\cdot\text{mg}^{-1}$), followed by XB102 ($6.39 \text{ U}\cdot\text{mg}^{-1}$) and XB122 ($3.56 \text{ U}\cdot\text{mg}^{-1}$). HPLC and HPLC-MS have been reported as superior techniques for quantification of degradation of several types of acyl-HSL molecules (Chan *et al.*, 2011; Cirou *et al.*, 2012). Quantification of 3OH-PAME degradation has earlier been carried out using GC-MS (Shinohara *et al.*, 2007). In the present study HPLC-MS served for confirmation that 3OH-PAME is degraded to 3OH-PA by QQE which is presumed to be either an esterase or a lipase.

6.3.6. Characterization of quorum quenching enzyme

6.3.6.1. Effect of quorum quenching enzymes on expression of EPS and Egl by *R. solanacearum*

Addition of purified QQE to growth media caused reduction in EPS and Egl expressed by wild and virulent *R. solanacearum* in the *in vitro* experiments. Colonies of *R. solanacearum* strain Rs-09-100 produced less EPS and appeared less fluidal in presence of crude QQE from XB7, XB102, XB109, XB115 and XB122 as compared to control without the QQE. However, based on visual comparison of the fluidity of the colonies, markedly reduced EPS production was seen in presence crude QQE from XB102 and XB122 in the medium (Fig. 6.8 a to c). Similar results on reduction in EPS production by *R. solanacearum* in presence of 3OH-PAME hydrolase was observed by Shinohara *et al.* (2007). In the present study, decrease in extracellular Egl produced by Rs-09-100 in presence of the crude enzyme was also noticed (Fig. 6.8 d to f).

Table 6.5 Characterization of 3OH-PAME degrading enzyme.

Strain	Protein conc. mg.mL ⁻¹	Specific activity		
		3OH-PAME	PNPA	PNPP
XB7	0.22	1.07	21.54 ± 1.03	7.01 ± 1.07
XB19	0.03	-0.92	ND	ND
XB102	0.47	6.39	13.02 ± 1.07	7.86 ± 0.71
XB109	0.25	1.17	28.15 ± 1.12	11.56 ± 1.18
XB115	0.22	1.07	31.45 ± 1.65	12.31 ± 1.36
XB122	0.16	3.56	26.20 ± 0.81	17.08 ± 1.62
XB174	0.04	17.78	ND	ND
XB179	0.15	0.75	ND	ND

Values of specific activity are given as U.mg⁻¹ under the standard conditions mentioned in materials and methods section 6.2.4. ND not determined.

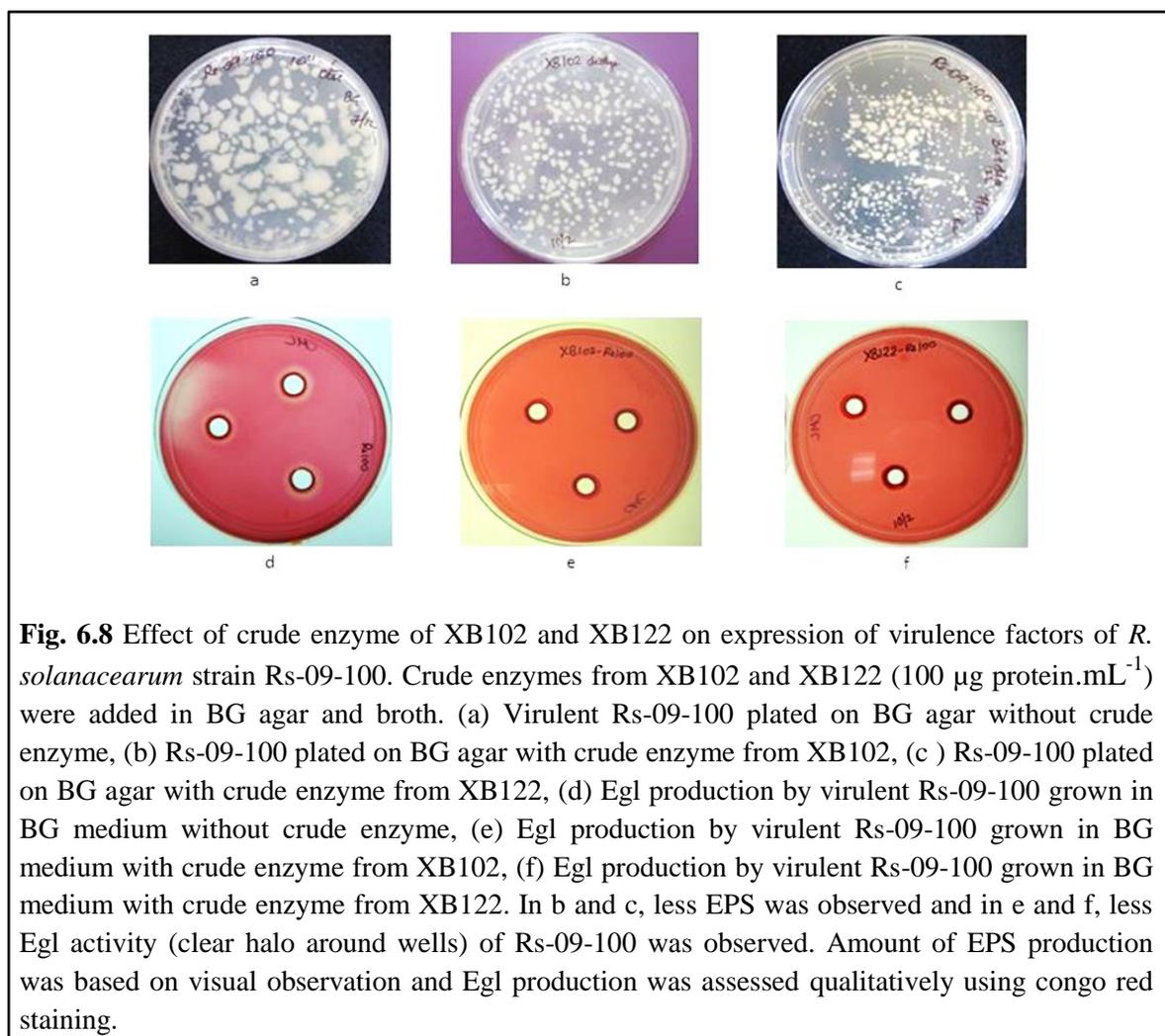


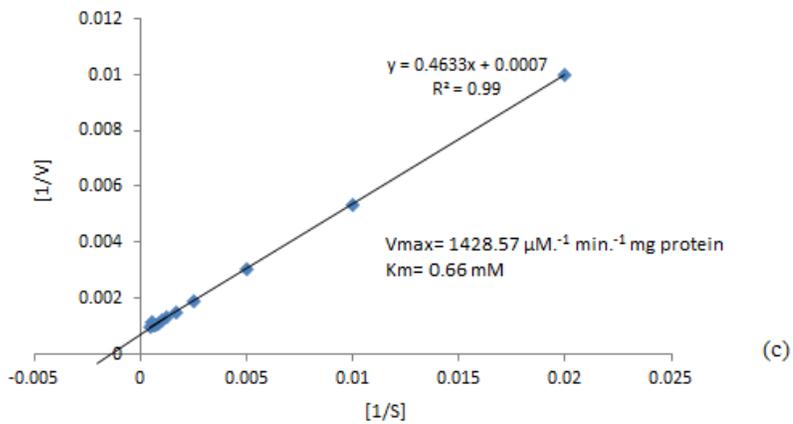
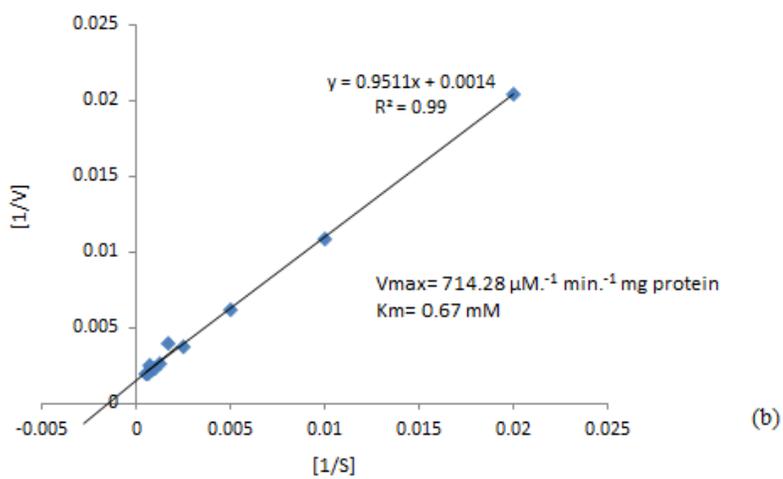
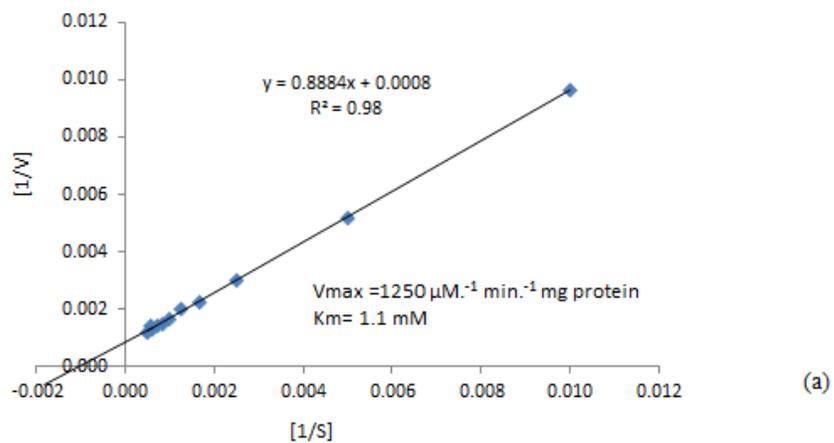
Fig. 6.8 Effect of crude enzyme of XB102 and XB122 on expression of virulence factors of *R. solanacearum* strain Rs-09-100. Crude enzymes from XB102 and XB122 (100 µg protein.mL⁻¹) were added in BG agar and broth. (a) Virulent Rs-09-100 plated on BG agar without crude enzyme, (b) Rs-09-100 plated on BG agar with crude enzyme from XB102, (c) Rs-09-100 plated on BG agar with crude enzyme from XB122, (d) Egl production by virulent Rs-09-100 grown in BG medium without crude enzyme, (e) Egl production by virulent Rs-09-100 grown in BG medium with crude enzyme from XB102, (f) Egl production by virulent Rs-09-100 grown in BG medium with crude enzyme from XB122. In b and c, less EPS was observed and in e and f, less Egl activity (clear halo around wells) of Rs-09-100 was observed. Amount of EPS production was based on visual observation and Egl production was assessed qualitatively using congo red staining.

Reduction in expression of EPS and Egl in presence of QQE indicate that there is interference in the QS regulated expression of virulence factors by *R. solanacearum*. The results suggest that the QQE from 3PD-XRB degrades natural 3OH-PAME produced by Rs-09-100 and prevents build-up of its concentration beyond the threshold necessary for QS. Earlier studies have shown that co-culturing with QQ bacteria or addition of QQ molecules in the medium reduces expression of multiple quorum sensing regulated traits in bacterial pathogens (Shinohara *et al.*, 2007; Medina-Martínez *et al.*, 2007; Uroz *et al.*, 2009; Chu *et al.*, 2013; Rajesh and Ravishankar, 2013; Tay and Yew, 2013). Advanced and detailed studies are necessary to determine the other traits which are up-regulated or down regulated in presence of QQE. However based on the current results it is clear that QQE of 3PD-XRB reduce the expression of virulence factors of *R. solanacearum*, which can lead to control of BW symptoms.

6.3.6.2. Lipase and esterase activity of quorum quenching enzymes

6.3.6.2.1. Specific activity and enzyme kinetics of quorum quenching enzymes

QQE from 3PD-XRB were presumed to be either an esterase or a lipase, therefore its activity against alternate esterase substrates mainly p-Nitrophenyl esters (p-Nitrophenyl acetate and p-Nitrophenyl palmitate) was tested to determine the nature of enzyme. Specific activities of the QQE from 3PD-XRB towards PNPA and PNPP are presented in table 6.5. Kinetic parameters (K_m and V_{max}) of QQE were determined from Lineweaver-Burk plot using different substrate concentrations of PNPA and PNPP (Fig. 6.9 a to e and 6.10 a to e). The values of V_{max} , K_m and catalytic efficiency (V_{max}/K_m) for QQE are given in Table 6.6.



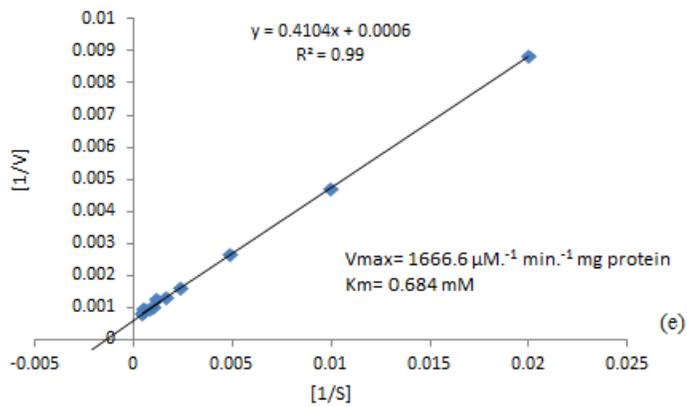
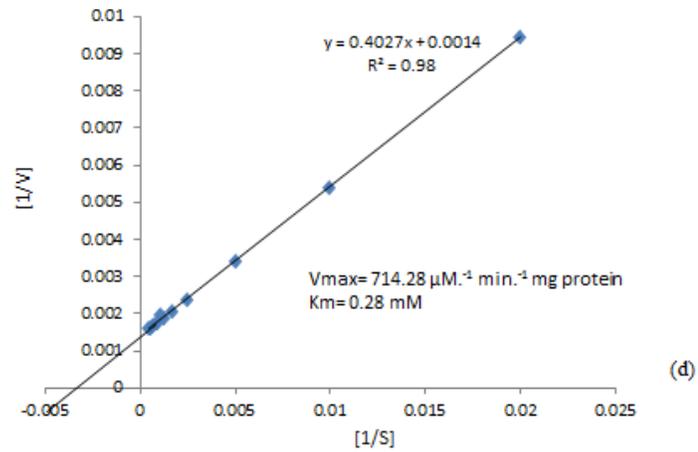
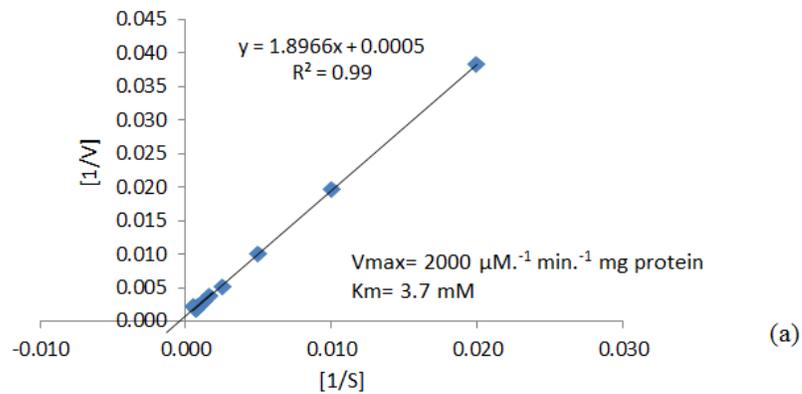
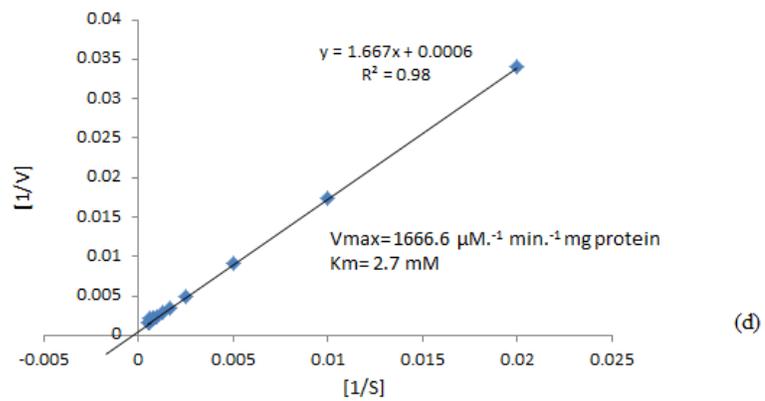
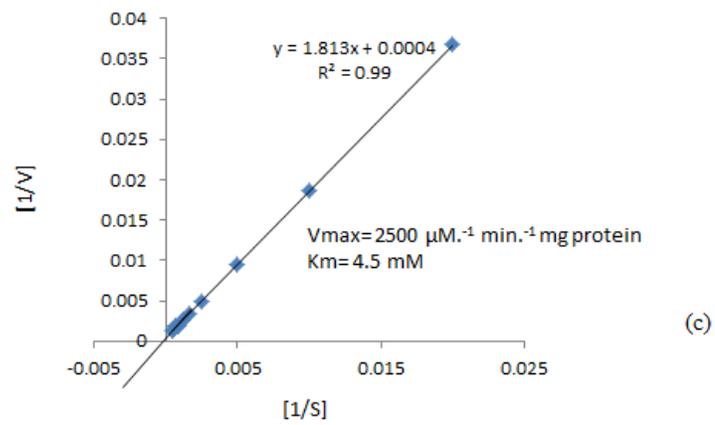
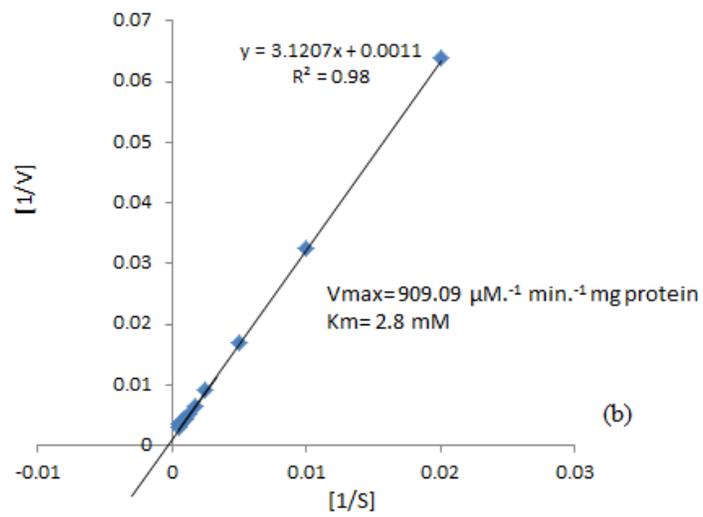


Fig. 6.9 Lineweaver-Burk plot using p-Nitrophenyl acetate for determination of K_m and V_{max} of QQE a. XB7, b. XB102, c. XB109, d. XB115, e. XB122. The y intercept of the line is equal to $1/V_{max}$ whereas the slope of the line is equal to K_m/V_{max} . These values are used for calculating V_{max} and K_m .





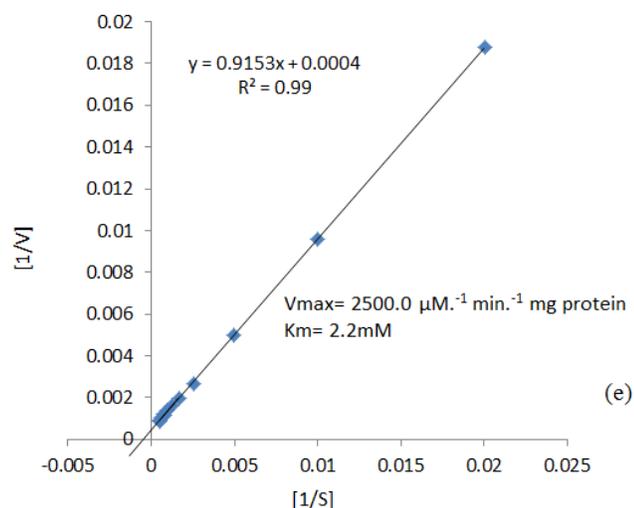


Fig. 6.10 Lineweaver-Burk plot using p-Nitrophenyl palmitate for determination of K_m and V_{max} of QQE a. XB7, b. XB102, c. XB109, d. XB115, e. XB122. The y intercept of the line is equal to $1/V_{max}$ whereas the slope of the line is equal to K_m/V_{max} . These values are used for calculating V_{max} and K_m .

Table 6.6 Kinetic parameters of quorum quenching enzyme from 3OH-PAME degrading XRB based on hydrolysis of p-Nitrophenyl acetate and p-Nitrophenyl palmitate.

Strain	p-Nitrophenyl Acetate			p-Nitrophenyl Palmitate		
	V_{max} (mM.min ⁻¹ . mg ⁻¹ protein)	K_m (mM)	V_{max}/K_m	V_{max} (mM.min ⁻¹ . mg ⁻¹ protein)	K_m (mM)	V_{max}/K_m
XB7	1.25	1.11	1.13	2.00	3.79	0.53
XB102	0.71	0.68	1.05	0.91	2.84	0.32
XB109	1.43	0.66	2.16	2.50	4.53	0.55
XB115	0.71	0.29	2.48	1.67	2.78	0.60
XB122	1.67	0.68	2.44	2.50	2.29	1.09

K_m and V_{max} values of enzymes vary depending upon substrates, thus V_{max}/K_m ratios are useful for comparison of activity of same enzyme towards different substrates (Ozcan *et al.*, 2009). From the results it is clear that 3PD-XRB have higher activity towards PNPA and have higher V_{max}/K_m ratios as compared to PNPP. This further confirms that the QQE produced by 3PD-XRB are enzymes with higher affinity towards the short chain fatty acid esters. The values of V_{max} and K_m of the 3PD-XRB fall within the range described for other diverse bacterial esterases (Tomioka, 1983; Politino *et al.*, 1997; Fernandez *et al.*, 2000; Ozcan *et al.*, 2009; Peng *et al.*, 2011). Till date there is only one report that confirms that 3OH-PAME degrading enzyme from *Ideonella* sp. is a serine esterase (Shinohara *et al.*, 2007). Interestingly bacterial acyl-HSL lactonases and mammalian paraoxonases are a class of esterases famous for their quorum quenching activities against several Gram negative bacteria (Dong *et al.*, 2007; Camps *et al.*, 2011; Fan *et al.*, 2012; Chen *et al.*, 2013). Amino acid sequencing to detect the presence of Gly-X-Ser-X-Gly motif (where X represents any type of amino acid residue) at the catalytic site of QQE could confirm whether it belongs to the serine esterase/ lipase family (Henne *et al.*, 2000; Kim *et al.*, 2006; Shinohara *et al.*, 2007; Jiang *et al.*, 2012; Fang *et al.*, 2014). In addition activity of the QQE of 3PD-XRB as acyl HSL lactonases against Gram negative phytopathogens can be tested (Fan *et al.*, 2012; Faure and Dessaux, 2007).

6.3.6.3. Effect of pH, temperature and inhibitors on activity of QQE

6.3.6.3.1. Effect of pH and temperature

Results of effect of temperature and pH on the activity is presented in Fig. 6.11a and 6.11b respectively. Optimum temperature for the activity of QQE of XB7, XB122, XB109 and XB115 was found to be 37°C with only slight decrease in activity at 28°C. XB102 had an optimum temperature of 28°C with only slightly less activity at 37°C.

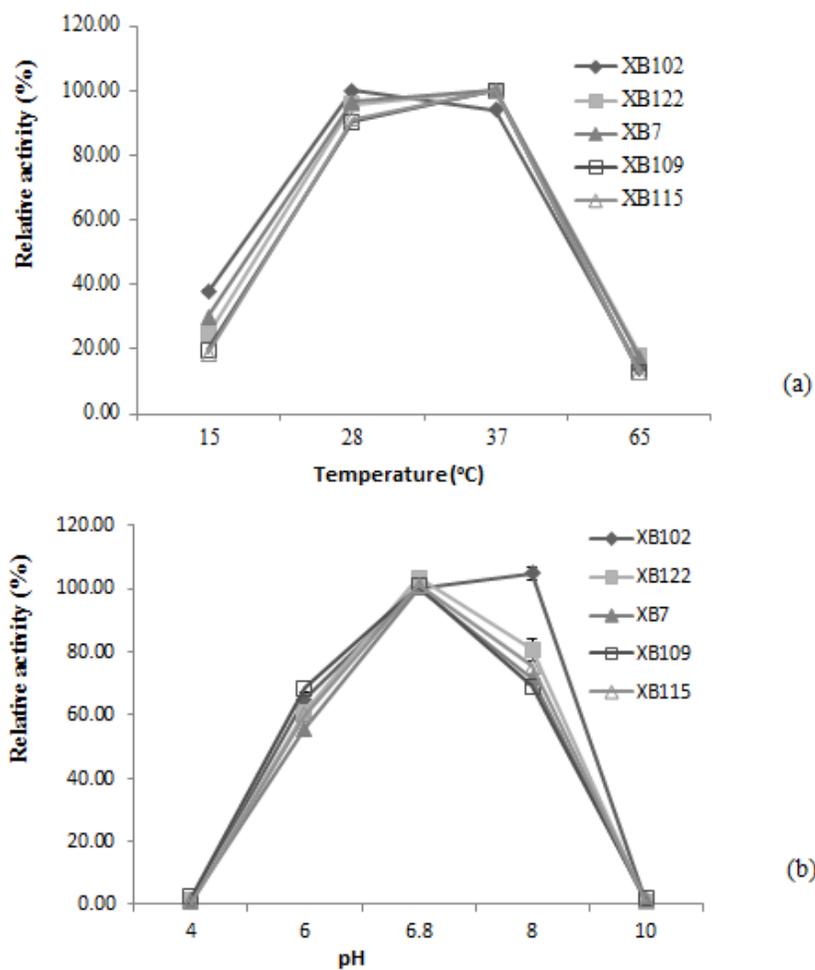


Fig. 6.11 Effect of temperature and pH on the hydrolysis of p-Nitrophenyl acetate by quorum quenching enzymes. a. effect of different temperatures on the quorum quenching enzymes of 3OH-PAME degrading XRB b. effect of pH on the quorum quenching enzymes of 3OH-PAME degrading XRB. Values indicate relative activity of hydrolysis of p-Nitrophenyl acetate. Filled diamond- XB102, filled square XB122, filled triangle XB7, Open square XB109, open triangle XB115.

More than 80% of relative activity was present in QQE from all 3PD-XRB at temperatures 28°C and 37°C. Optimum pH for the activity of QQE of XB7, XB122, XB109 and XB115 was found to be pH 6.8 whereas pH 8.0 was optimal for XB102. More than 60% of relative activity was present in QQE from all 3PD-XRB at pH 6.8 and 8.0. Bacterial esterases have been known to be active over a wide temperature and pH

ranges based on its bacterial source. Esterases from a metagenomic library of soil samples had an optimal temperature and pH of 25°C and pH 7.0 (Kim *et al.*, 2006) whereas a temperature of 55°C and pH 9.0 was optimal for esterase from obtained a compost sample library (Kang *et al.*, 2011). Fu *et al.*, 2011 have reported a cold active esterase functional at temperatures of 0-60°C and pH of 7-9. In addition, Ozcan *et al.* (2009) have reported archaeobacterial esterases active at a temperature of over 40°C and pH range of 7-8. Fang *et al.*, 2014 have reported esterases with temperature optima of 65°C and pH 8.

6.3.6.3.2. Effect of chemical reagents and metal ion inhibitors

Cu^{2+} and Ca^{2+} enhanced activity of all QQE whereas large decrease in the activity in presence of Fe^{2+} , Sn^{2+} , Hg^{2+} , Ag^+ and beta-mercaptoethanol was observed in QQE of all the 3PD-XRB tested (Table 6.7). Majority of enzyme inhibitors reduced the activity of QQE below 80%. 3OH-PAME hydrolase of *Ideonella* sp. was inhibited by Fe^{2+} and Sr^{2+} ions whereas its activity was promoted by Na^+ and K^+ ions (Shinohara *et al.*, 2007). Metal ions can act as competitive inhibitors of enzymes by reducing to the sulphahydryl (SH) groups of cysteine residues of proteins which may have resulted in loss of activity of QQE in presence of metal ions. PMSF and iodoacetic acid are known inhibitors of serine esterases. They link covalently to the hydroxyl groups of serine in the active site and inhibit binding of substrates. EDTA which is a chelator of metal ion reduces the activity of esterases. SDS breaks non-covalent inter and intramolecular protein interactions and beta mercaptoethanol breaks covalent bonds causing proteins to lose its enzymatic activity (Hoskins *et al.*, 1992; Rhee *et al.*, 2005; Fu *et al.*, 2011).

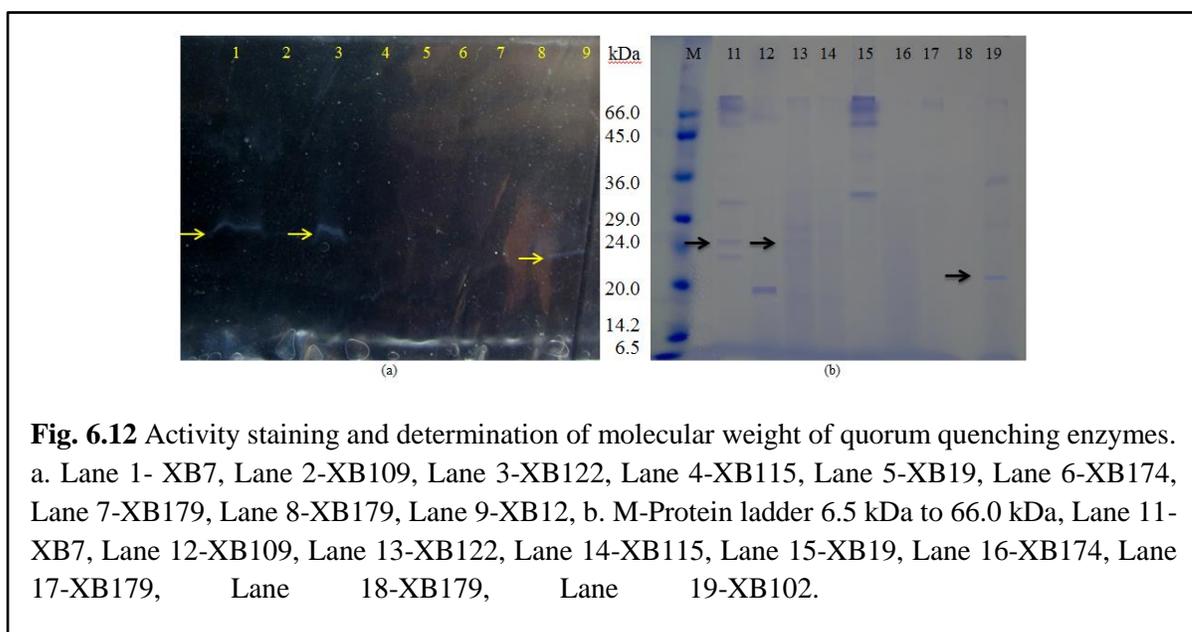
6.3.7. Molecular weight of quorum quenching enzymes

One of the widely used methodology for detection of molecular weight of enzymes is zymography (Sommer *et al.*, 1997; Singh *et al.*, 2006; Kwon *et al.*, 2011). Molecular weight of QQE produced by 3PD-XRB was determined by semi-native SDS-PAGE followed by zymography using Tween 20 as a substrate and counter staining with Coomassie brilliant blue (Fig. 6.12 a and b respectively). Esterase activity of XB7, XB102 and XB122 in zymography with Tween 20 was seen as a white band of calcium laurate precipitate. When the same gel was counterstained, the molecular mass of the active esterase of XB7 and XB122 was found to be approximately 24 kDa whereas that of XB102 was found to be 20.0 kDa (Fig. 6.12b). Determination of molecular weight of esterases has been done by screening of clones from genomic DNA library of a particular bacterial strain for esterase activities followed by sequencing of the positive clone (Petersen *et al.*, 2001; Ro *et al.*, 2004; Ewis *et al.*, 2004; Shinohara *et al.*, 2007; Sumbly *et al.*, 2009). Metagenomic libraries of environmental samples have also been screened for esterase and lipases (Kim *et al.*, 2006; Peng *et al.*, 2011; Fang *et al.*, 2014). These techniques are useful in determining gene sequence, amino acid sequence as well as molecular weight of esterases. Using zymography only the molecular weight of the enzyme can be determined, however, the technique is cheaper and faster. Molecular weights of XB109, XB115 and XB174 could not be determined by zymography. PNPA can be used as an alternate substrate for these QQE followed by staining with Fast blue RR (Kim and Lee, 2004; McGoldrick *et al.*, 2014).

Table 6.7 Effect of inhibitors on activity of 3OH-PAME degrading enzyme

Inhibitor	Conc. (mM)	Residual activity (%)				
		XB7	XB102	XB109	XB115	XB122
Ag ⁺	5	0.16 ± 0.1	0.23 ± 0.1	0.20 ± 0.1	0.20 ± 0.0	0.41 ± 0.1
NH ₄ ⁺	5	72.3 ± 8.6	80.1 ± 8.1	79.0 ± 6.8	100.0 ± 2.9	103.5 ± 8.7
Na ⁺	5	29.1 ± 6.5	44.3 ± 3.8	62.7 ± 7.1	62.9 ± 4.4	46.5 ± 5.2
K ⁺	5	49.2 ± 6.0	70.8 ± 3.9	80.5 ± 7.3	72.4 ± 3.2	67.6 ± 4.0
Cu ⁺²	5	504.2 ± 29.1	205.2 ± 11.4	108.2 ± 5.7	181.0 ± 4.5	162.0 ± 10.8
Ca ⁺²	5	217.3 ± 8.2	149.6 ± 6.5	127.1 ± 7.9	122.2 ± 5.4	246.0 ± 12.7
Fe ⁺²	5	13.0 ± 6.1	5.0 ± 2.3	12.5 ± 1.6	13.6 ± 0.2	18.4 ± 3.6
Mg ⁺²	5	84.2 ± 10.0	79.1 ± 3.6	85.0 ± 4.9	78.2 ± 2.4	68.5 ± 4.6
Mn ⁺²	5	43.2 ± 5.0	45.5 ± 3.1	41.2 ± 3.4	97.6 ± 3.7	87.2 ± 5.2
Zn ⁺²	5	107.1 ± 8.4	66.2 ± 4.1	88.9 ± 7.0	59.4 ± 3.6	90.5 ± 6.6
Sn ⁺²	5	0	0	0	0	0
Hg ⁺²	5	0	0	0	0	0
BME	5	1.8 ± 1.4	0.2 ± 0.1	0.0 ± 0.1	0.2 ± 0.1	0.32 ± 0.2
PMSF	1	48.8 ± 11.9	66.7 ± 3.2	39.4 ± 3.1	48.2 ± 0.8	45.0 ± 4.3
EDTA	1	70.9 ± 1.6	56.9 ± 4.1	73.3 ± 4.3	69.1 ± 1.4	41.7 ± 1.5
IdAA	1	78.8 ± 1.9	67.5 ± 2.2	71.6 ± 1.7	79.0 ± 7.3	79.5 ± 2.3
SDS	1	41.6 ± 2.6	53.5 ± 4.1	47.5 ± 0.9	51.3 ± 2.4	61.1 ± 5.0
None	0	100	100	100	100	100

BME: Beta mercaptoethanol, PMSF: Phenyl methyl sulfonyl fluoride, EDTA: Ethylene diaminetetraacetic acid, IdAA Iodoacetic acid.



CHAPTER VII

Summary and conclusion

SUMMARY AND CONCLUSION

Xylem residing bacteria from eggplant, chilli and *S. torvum* were isolated with a main aim to study their antagonism and quorum quenching activities against the bacterial wilt pathogen *R. solanacearum*. Further, XRB exhibiting inhibitory interactions with *R. solanacearum* were studied to determine their plant growth promoting activities, biocontrol and re-colonization ability in eggplant, in order to determine their possible use as biocontrol agents for management of BW in solanaceous vegetables.

Amongst the 167 viable XRB isolated from solanaceous vegetables from Goa, Gram negative bacteria were predominating (59.28%) and Gram positive bacteria comprised of 40.72%. Diversity analysis by ARDRA and identification by 16S rRNA gene sequencing showed that *Proteobacteria* is the dominant phylum comprised 41.81% isolates, followed by *Firmicutes* (29.09%), *Actinobacteria* (25.45%) and *Bacteroidetes* (3.64%) isolated from xylem of solanaceous vegetables. Analysis by PCR-DGGE revealed presence of *Arthrobacter* sp., *Curtobacterium* sp. and *Acinetobacter* sp. which were not found amongst cultured isolates obtained from same xylem sap sample. Results suggest diverse genera of bacteria belonging to four phyla of Eubacteria reside in xylem of eggplant, chilli and *S. torvum*.

Twenty eight out of 167 XRB exhibited antagonism to *R. solanacearum* which included mainly fluorescent and non-fluorescent *Pseudomonas* sp., *Enterobacter* sp., *Bacillus* sp., *Staphylococcus* sp. and *Agrobacterium* sp. XRB of phyla *Proteobacteria* and *Firmicutes* were highly antagonistic to *R. solanacearum*. Antagonistic XRB produced growth promoting substances namely ACC deaminase and indole acetic acid (upto 645 $\mu\text{g.mL}^{-1}$), solubilized phosphate and were putative diazotrophs. These traits of

XRB may be involved in enhancement of seedling vigour and increased shoot length in eggplant. Antagonistic XRB produced several extracellular hydrolytic enzymes mainly cell wall degrading enzymes (endoglucanase, pectinase and polygalacturonase) which may aid in endophytic colonization of eggplant. In addition production of protease and chitinase may be effective in management of fungal diseases of solanaceous vegetables.

Mechanism of antagonism of XRB can be attributed to but not restricted to the production of volatile and diffusible inhibitory compounds mainly antibiotics, bacteriocins, HCN, ammonia, acetoin and siderophores. These compounds may have contributed directly or indirectly to their biocontrol efficacies ranging from 10-100% against BW. Interestingly antagonistic XRB of genera *Agrobacterium* sp.(XB1, XB86 and XB165), *Enterobacter* sp.(XB99 and XB123), *Pseudomonas aeruginosa* (XB7 and XB122) and *Bacillus cereus* (XB177) which prevented wilt were also able to re-colonize eggplant stem and rhizosphere. Studies using GFP tagged XB177 proved that bacteria isolated from xylem could re-colonize xylem vessels of stem and also rhizoplane of eggplant, tomato and chilli when tested under greenhouse conditions. The results indicate that xylem residents may prevent wilt in solanaceous vegetables by niche exclusion and production of volatile and diffusible antimicrobials effective against *R. solanacearum*.

Quorum sensing mediated by 3OH-PAME (synthesized by a product of *phcB* gene) regulates the ability of *R. solanacearum* to cause wilt. In the present study, mutagenesis of *phcB* gene of *R. solanacearum* caused loss in expression of virulence factors (exopolysaccharides and endoglucanase) by *R. solanacearum* and the mutant was avirulent in eggplant and tomato. However the mutant could still colonize eggplant stem and rhizosphere when tested under greenhouse conditions. Based on the results it is concluded that quorum sensing system is a potential target to limit virulence and wilt

infection by *R. solanacearum*. These results encourage further studies on quorum quenching mechanisms against *R. solanacearum* as alternate biocontrol strategies.

Interestingly, eight strains out of 167 XRB degraded 3OH-PAME a quorum sensing molecule of *R. solanacearum*. This indicates that quorum quenching ability is a rare trait amongst the xylem residing bacteria. Amongst the 3OH-PAME degrading XRB (3PD-XRB), two were also antagonistic (XB7 and XB122) to *R. solanacearum*. Degradation of 3OH-PAME to 3OH-palmitic acid by the hydrolysis of ester bond between the methyl group and the 3OH-palmitic acid chain was confirmed by HPLC-MS. Thus the 3OH-PAME degrading enzyme was considered a putative esterase/lipase enzyme and was extracellular in nature. 3PD-XRB hydrolyzed Tween 20, Tween 80 and tributyrin in plate based bioassays. Crude quorum quenching enzyme (QQE) from only six XRB had high specific activity towards 3OH-PAME degradation. Strains XB7 and XB122 which degraded 3OH-PAME were identified as *Pseudomonas aeruginosa*, XB109 and XB115 as *Rhodococcus corynebacterioides*, XB102 as *Stenotrophomonas maltophilia* and XB174 as *Acinetobacter* sp. Crude QQE from XB102 and XB122 reduced expression of endoglucanase and exo-polysaccharides by *R. solanacearum*, indicating that the QQE can interfere in quorum sensing pathway by degrading 3OH-PAME naturally produced by *R. solanacearum*. Five 3PD-XRB (XB7, XB102, XB109, XB115 and XB122) were able to re-colonize eggplant and prevent wilt in eggplant. Quorum quenching enzyme may be possibly involved in wilt prevention by the 3PD-XRB.

QQE from five 3PD-XRB was characterized using esterase/lipase substrates namely p-Nitrophenyl acetate and p-Nitrophenyl palmitate. The results of enzyme kinetics indicate that the QQE had higher affinity towards short chain p-Nitrophenyl

acetate. It can be concluded that the QQE are putative esterases but have lower affinity towards long chain fatty esters. Using p-Nitrophenyl acetate as substrate, the QQE from five 3PD-XRB were characterized. Optimum temperature for the activity of QQE ranged between 28°C to 37°C whereas pH optimum was between pH 6.8 to pH 8.0. Effect of 14 enzyme inhibitors was tested, Cu²⁺ and Ca²⁺ enhanced activity whereas Fe²⁺, Sn²⁺, Hg²⁺, Ag⁺ and beta-mercaptoethanol inhibited the activity of all the QQE from 3PD-XRB. Activity was reduced by other inhibitors including the serine esterase inhibitors *viz.* phenyl methyl sulphonyl fluoride and iodoacetic acid.

The results demonstrate the diverse activities of XRB mainly antagonism to *R. solanacearum*, positive effect on growth parameters in eggplant, reduction of wilt incidence in eggplant under greenhouse conditions and quorum quenching by degradation of 3OH-PAME. These traits make XRB from solanaceous vegetables potential candidates as biocontrol agents for management of bacterial wilt.

Future prospects of my Ph. D research which can be evaluated include:

- Determining the nucleotide sequence of gene encoding quorum quenching enzymes in xylem residing bacteria and determining the amino acid sequence of the quorum quenching protein.
- Transgenic eggplant to express bacterial 3OH-PAME degrading quorum quenching enzymes.
- Elucidating whether xylem residing bacteria can induce induced systemic resistance (ISR) in eggplant.
- Studies to determine biosafety of xylem residing bacteria for deploying as biocontrol agents.

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Appendices

Appendix A

Media composition

Tryptic Soy Broth and Agar

Tryptone	17.0 g
Peptone	3.0 g
NaCl	5.0 g
Glucose	2.5 g
K ₂ HPO ₄	2.5 g
Distilled water	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH. For Tryptic Soy agar 15.0 g agar was added per litre of broth, digested and sterilized.

Medium 523 (Viss *et al.*, 1991)

Sucrose	10.0 g
Casein hydrolysate	8.0 g
Yeast extract	4.0 g
KH ₂ PO ₄	2.0 g
MgSO ₄ .7H ₂ O	0.15 g
Distilled water	1000.0 mL
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH.

King's B broth

Peptone	20.0 g
K ₂ HPO ₄	1.5 g
MgSO ₄ .7H ₂ O	1.5 g
Glycerol	10.0 mL
DW	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH. For King's B agar 15.0 g agar was added per litre of broth, digested and sterilized.

King's B broth and agar with rifampin 100 µg. mL⁻¹

KB broth/agar was prepared as given above. 100 µL of 100 mg. mL⁻¹

rifampicin was added every 100 mL sterile molten cooled medium before pouring plates.

Nutrient broth

Peptone	10.0 g
Yeast extract	3.0 g
NaCl	5.0 g
Tryptophan (100mg. mL ⁻¹)	1.0 mL
DW	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH. For nutrient agar, 15.0 g agar was added to one liter nutrient broth. One mL **tryptophan** (100 mg. mL⁻¹) to one liter Nutrient broth for quantify indole acetic acid production.

Salkowsky's reagent for estimation of indole acetic acid production

0.5 M FeCl ₃	1.0 mL
35.0% HClO ₄	50.0 mL

Luria Bertani (LB) broth

Tryptone	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
DW	1000.0 mL
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH. For LB agar 15.0 g agar was added per litre of broth, digested and sterilized. LB agar supplemented with **10mM MgSO₄**: 2.4 g MgSO₄ was added per liter LB agar

Casein hydrolysate Peptone Glucose (CPG) broth

Peptone	10.0 g
Casein hydrolysate	1.0 g
Glucose	5.0 g

pH was adjusted to 7.0 with 0.1 N NaOH. For CPG agar 15.0 g agar was added per litre of broth, digested and sterilized.

TZC agar: One mL of 1% tetrazolium chloride was added per liter CPG agar to make one litre TZC agar. 1% stock of TZC was prepared by dissolving 1.0 g TZC in 100 mL DW and filter sterilized.

Bacto-Peptone Glucose (BG) agar: One g casein hydrolysate was added per liter CPG agar to make one litre of BG agar

Semi selective Medium South Africa (SMSA) (French *et al.*, 1995)

Basal medium

Peptone	10.0 g
Casein hydrolysate	1.0 g
Glycerol (mL)	5.0 mL
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH and sterilized.

For every 250.0 mL molten cooled medium following volumes of stock solutions were added in molten cooled SMSA.

Antibiotic/Compound	Final concentration	Volume /250 mL basal medium
1% polymyxin B sulphate	100.0 ppm	2.5 mL
1% crystal violet	5.0 ppm	125.0 µL
1% TZC	50.0 ppm	1.25 mL
100 mg. mL ⁻¹ Bacitracin	25.0 ppm	6.25 µL
1% Penicillin G	0.5 ppm	12.5 µL
20 mg. mL ⁻¹ Chloramphenicol	5.0 ppm	62.5 µL

Note: kanamycin (50 µg. mL⁻¹) was added in above medium for isolation of *phcB* mutant GB3-2 from rhizosphere soil and eggplant grown in greenhouse conditions

Tween 20 and Tween 80medium

Meat Extract	2.0 g
Peptone	5.0 g
CaCl ₂ .2H ₂ O	0.4 g
DW	1000.0 mL
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH. Tween 20 (Polyoxyethylene-Sorbitan monolaurate) and Tween 80 (Polyoxyethylene-Sorbitan monooleate) are heat sensitive and viscous. Therefore a

10% stock was made in DW, filter sterilized and stored at room temperature. 50 mL of 10% stock was added separately every 200 mL basal medium to make Tween 20 and Tween 80 agar.

Tributylin agar

Peptone	5.0 g
Yeast extract	3.0 g
DW	990.0 mL
Agar	15.0

pH was adjusted to 7.0 with 0.1 N NaOH. Ten mL of sterile tributyrin (Hi-media) was added in 990 mL of above agar and plates were poured

Pectin agar

Citrus pectin	5.0 g
Yeast extract	5.0 g
Agar	15.0 g
pH	7.0
Distilled water	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH.

For visualization of pectinase activity: 2% CTAB solution (2 g CTAB dissolved in 100 mL distilled water) and 1M NaCl (58.44 g NaCl dissolved in 1000 mL distilled water) was used

Skimmed milk agar

Skim milk powder	28.0 g
Casein hydrolysate	5.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Agar	15.0
DW	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH.

Starch agar

Peptone	10.0 g
Yeast extract	3.0 g
NaCl	5.0 g
Starch	10.0 g
Distilled water	1000.0 mL
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH. After incubation plates were flooded with lugols iodine solution for 5 min. Amylase production was indicated by appearance of light brown haloes on dark brown background.

Lugol's Iodine

Potassium iodide (KI)	20.0 g
Iodine (I ₂)	10.0 g
Distilled water	1000.0 mL

Mineral Salts Medium (MSM)

MgSO ₄ .7H ₂ O	0.02
K ₂ HPO ₄	0.09
KCl	0.02
FeSO ₄ .7H ₂ O	0.0002
ZnSO ₄	0.0002
MnSO ₄	0.0002

NH₄NO₃ 0.1
 Colloidal chitin (2.7%) 7.4 g
 Agar 1.5
 Distilled water 100.0 mL
 pH was adjusted to 6.3 with 0.1 N NaOH.

For preparation of colloidal chitin, method described by Hsu and Lockwood (1975) was followed. Briefly, 60 mL conc. HCl was added to 5 g crabs shell chitin powder (Hi-Media Laboratories, Mumbai) and incubated overnight at 4°C. Equal volume of 50% ethanol was added to this mixture and kept overnight at RT. Solution was centrifuged at 10000 rpm for 12 min to remove HCl and alcohol. Pellet was washed with water seven times. Pellet was adjusted to neutral with 1N NaOH. Pellet was washed twice with sterile distilled water. Supernatant was discarded to obtain colloidal chitin. Moisture content was determined using moisture balance (Sartorius, Germany). Based on moisture content amount corresponding to 0.2 % dry weight of colloidal chitin was amended in MSM medium.

Carboxy Methyl Cellulose (CMC) agar

CMC 10.0 g
 NaNO₃ 1.0 g
 K₂HPO₄ 1.0 g
 KCl 1.0 g
 MgSO₄·7H₂O 0.5 g
 Yeast extract 0.5 g
 Glucose 1.0 g
 Agar 15.0 g
 Distilled water 1000.0 mL
 pH was adjusted to 7.0 with 0.1 N NaOH.

For staining: 0.1 % Congo red solution [0.1 g Congo red dissolved in 100 mL distilled water], 1M NaCl [58.44 g NaCl dissolved in 1000 mL distilled water]

Polygalacturonate agar

Polygalacturonic acid 10.0g
 NaNO₃ 1.0 g
 K₂HPO₄ 1.0 g
 KCl 1.0 g
 MgSO₄·7H₂O 0.5 g
 Yeast extract 0.5 g
 Glucose 1.0 g
 Agar 15.0 g
 Distilled water 1000.0 mL
 pH was adjusted to 7.0 with 0.1 N NaOH.

For visualization of polygalacturonase activity: 0.1 % Reuthenium red solution (0.1 g reuthenium red dissolved in 100 mL distilled water) and 1M NaCl (58.44 g NaCl dissolved in 1000 mL distilled water) was used.

Dworkin and Foster's salt medium for ACC deaminase activity

Component	Qty/l	Stock solution	Qty. per 100 mL medium
KH ₂ PO ₄	4.0 g	-	0.4 g
Na ₂ HPO ₄	6.0 g	-	0.6 g
MgSO ₄ ·7H ₂ O	0.2 g	-	0.02 g
FeSO ₄ ·7H ₂ O	1.0 mg	10 mg. mL ⁻¹	2.0 mL
H ₃ BO ₃	10.0 µg	10 mg. mL ⁻¹	20.0 µL

MnSO ₄	10.0 µg	10 mg. mL ⁻¹	20.0 µL
ZnSO ₄	70.0 µg	10 mg. mL ⁻¹	140.0 µL
CuSO ₄	50.0 µg	10mg. mL ⁻¹	100.0 µL
Na ₂ MoO ₄	10.0 µg	10mg. mL ⁻¹	20.0 µL
ACC (0.5M)	3.0 mM	0.5 M	600.0 µL
Distilled water	1000.0 mL		100.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH.

[0.5M ACC was prepared by dissolving 101.1 mg ACC (Sigma Aldrich, USA) in 2.0 mL distilled water and filter sterilized]

CAS Medium for Siderophore detection

Molten cooled King's B agar 900.0 mL

CAS Solution 100.0 mL

CAS (Chrome Azurol Sulfonate) solution was prepared by dissolving 60.5 mg CAS in 50.0 mL DW and to it 10 mL of 1 mM FeCl₃·6H₂O (prepared in 10mM HCl) was added. In the resulting solution, 72.9 mg of HDTMA was dissolved and finally diluted to 100 mL.

Dobereiner's Nitrogen free semisolid medium (Nfb) medium (Eckert *et al.*, 2001)

Malate/malic acid	5.0 g
K ₂ HPO ₄	0.5 g
MgSO ₄ ·7H ₂ O	0.2 g
NaCl	0.1 g
CaCl ₂ ·2H ₂ O	0.02 g
Bromothymol blue (0.5% in 0.2M KOH)	2.0 mL
Vitamin solution	1.0 mL
Micronutrient Solution	2.0 mL
Distilled water	1000.0 mL
Agar	1.8 mL

pH was adjusted to 6.5 with 0.1 N NaOH.

Vitamin and nutrient solutions were prepared as follows

Vitamin solution

Component	Volume for 10 mL
Biotin (10mg. mL ⁻¹)	500.0 µL
Pyridoxine Hydrochloride (10mg. mL ⁻¹)	1000.0 µL
Distilled water	8500.0 µL

Micronutrient Solution

Component	Volume for 2 mL
H ₃ BO ₃ (10 mg. mL ⁻¹)	560.0 µL
MnSO ₄ ·H ₂ O (10 mg. mL ⁻¹)	700.0 µL
ZnSO ₄ ·7H ₂ O (10 mg. mL ⁻¹)	48.0 µL
CuSO ₄ ·5H ₂ O (10 mg. mL ⁻¹)	16.0 µL
Na ₂ MoO ₄ ·2H ₂ O (10 mg. mL ⁻¹)	400.0 µL
Distilled water	276.0 µL

10 X Phosphate Buffered saline (PBS)

NaCl	80.0 g
KCl	2.0 g
Na ₂ HPO ₄ ·2H ₂ O	14.4 g
KH ₂ PO ₄	2.4 g
Distilled water	1000.0 mL

Peptone water (Ammonia production, Gayathri *et al.*, 2010)

Peptone	20.0 g
NaCl	5.0 g
DW	1000.0 mL

Nessler's reagent

Dissolve 50g of KI in 50 mL of cold water. Add a saturated solution of mercuric chloride (22g in 350 mL of water). After mixing a clear solution with whitish orange precipitate is formed that after a few seconds turns bright red-orange. Add 200 mL of 5N NaOH (4.0 g NaOH pellets dissolved in 100 mL distilled water) and dilute to 1 liter. Let the precipitate settle, and draw off the clear liquid and use for testing ammonia in samples

Glycine broth for HCN production

Peptone	10.0 g
Yeast extract	3.0 g
NaCl	5.0 g
Glycine	4.4 g
DW	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH.

Picric acid solution

Picric acid	2.5 g
Na ₂ CO ₃	12.5 g
Distilled water	1000 mL

Sterile filter paper strips (10.5 x 0.5 cm) were dipped in above solution, dried and used for detection of HCN production

Appendix B

Biochemical characterization (media, reagents and procedures)

Gram's Crystal Violet Reagent

Solution A		Solution B	
Crystal violet	2.0 g	Ammonium oxalate	0.8 g
Ethyl alcohol	20.0 mL	Distilled water	80.0 mL

Solution A and Solution B were mixed to obtain crystal violet reagent. Solution was stored for 24 h and filter through Whatman filter paper No. 1 prior to use.

Gram's Iodine (Mordant)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

Stored in amber colored bottled at room temperature

Decolorizer, 95% ethanol in water (v/v)

Gram's Safranin (Counter stain)

Safranin	2.5 g
95% ethanol	100.0 mL

Ten mL of above solution was diluted to 100.0 mL with distilled water prior to use as a counter staining

Gram staining

Single colony of Gram positive XRB freshly grown on nutrient agar was suspended in 200 μ L of normal saline (0.85 % NaCl w/v). Thin smear was made on a grease free slide and heat fixed. Smear was flooded with Gram's crystal violet solution for one min. Smear was rinsed gently under indirect stream of water and flooded with Gram's iodine solution for one min. Slide was rinsed gently under indirect stream of water and decolorizer was flooded until the decolorizer running from the slide came clear of crystal violet. Slide was rinsed gently under indirect stream of water and counter stained with Gram's safranin for 2 min. Slide was air dried and observed under 100 X lens using Olympus CX41 microscope.

Spore staining

For spore staining, smear was prepared as described above. 0.5 % malachite green solution (w/v in water) was flooded over the smear and the slide was steamed for 10 min keeping the smear moist with fresh dye when it evaporated. The smear was washed under running tap water and counter stained with Gram's safranin. Safranin was washed from the smear, air dried and observed under 100 X lens using Olympus CX41 microscope.

Carbohydrate utilization

Basal broth

Peptone	10.0 g
NaCl	5.0 g
Bromothymol blue	0.02 g
Distilled water	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH

Bromothymol blue pH breakpoint- Yellow (pH 6) to Blue (pH 7.6)

Ten % sugar solutions were prepared (arabinose, fructose, glucose, maltose, raffinose, sucrose, trehalose, lactose, mannitol, galactose, xylose, sorbitol, lactose) and autoclaved separately for 10

min. Sugar solutions were added separately to basal medium for each test with the final concentration of 1%. Twenty μL of 24 hour old culture was inoculated in 5 mL sugar broth and incubated at $28\pm 2^\circ\text{C}$ for 24 h to 4 days. Color change from blue to yellow indicated utilization of sugars and acid production by XRB.

Growth at 4°C and 41°C

Single colony of cultures was streaked on nutrient agar (Appendix A). Plates were incubated at $4\pm 2^\circ\text{C}$ and $41\pm 2^\circ\text{C}$ for 48 h. Plates incubated at $28\pm 2^\circ\text{C}$ served as control. Growth at different temperatures was compared visually.

Growth in 7% NaCl

Single colony of cultures was streaked on nutrient agar (Appendix A) amended with 7% NaCl (w/v) and incubated at $28\pm 2^\circ\text{C}$ for 48 h. Cultures was streaked on nutrient agar and incubated at 28°C served as control. Growth in presence or absence of NaCl was compared visually.

Catalase test

Used for detecting the ability of organism to produce the enzyme catalase. Single colony of XRB grown on nutrient agar was picked and placed in 50.0 μL of H_2O_2 solution on a clean glass slide. Appearance of bubbles indicated liberation of O_2 from H_2O_2 . During the metabolism of sugars, H_2O_2 is formed as a byproduct and is toxic to bacteria. Enzyme catalase breaks down the toxic H_2O_2 and releases O_2 .

Oxidase test

Used to determine whether an organism possesses enzyme oxidase. Single colony of XRB grown in nutrient agar was spotted on the Oxidase reagent discs (1% tetra methyl para phenylene diamine di-hydrochloride (Hi-Media Laboratories, Mumbai) and allowed to dry. Color change of the inoculum to dark blue indicated the presence of enzyme cytochrome c oxidase and the obligate aerobic nature of the bacteria tested. In this test bacteria utilize the oxidase reagent as terminal electron acceptor and reduce it to a blue colored compound indophenol blue.

Indole test

Single colony of XRB was grown in 5.0 mL tryptone water (Hi-Media Laboratories, Mumbai) for 24 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. Indole produced was detected adding 10-12 drops of Kovac's reagent. Positive indicated by production of red ring in the culture broth. This test is used to determine the ability of an organism to split indole from the amino acid tryptophan using the enzyme tryptophanase (tryptophan indole lyase). Tryptophan is converted to indole and pyruvic acid. Indole reacts with aldehyde to produce a quinoidal red compound. Appearance of red color layer indicates indole production. Negative reaction is indicated by appearance of yellow layer.

Kovac's reagent

p-Dimethyl aminobenzaldehyde	2.5 g
HCl (37%)	12.5 mL
Isoamyl alcohol	37.5 mL

MR-VP broth

Peptone	7.0 g
K_2HPO_4	5.0 g
Dextrose	5.0 g
DW	1000.0 g

pH was adjusted to 7.0 with 0.1 N NaOH

Methyl red test

Single colony of XRB was inoculated in five mL MR-VP broth and incubated at for 24 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. After incubation 3-4 drops of methyl red solution was added. Appearance of red color indicated acid production. Negative test was indicative of appearance of yellow color. Methyl red test is used to determine the ability of an organism to produce mixed acid (lactic, acetic, succinic, formic) plus H_2 and CO_2 from glucose fermentation that lowers the pH to lower than 5.0. These organisms also produce great amounts of gas due to the presence of the enzyme formic hydrogen lyase.

Voges-Proskauer test

Single colony of XRB was inoculated in five mL MR-VP broth and incubated at for 24 h at $28 \pm 2^\circ\text{C}$ with constant shaking at 140 rpm. One mL of alpha naphthol (barrits reagent A) and one mL 40% KOH (Barrits reagent B) were added and mixed. Tubes were allowed to stand for 1 h and the results were observed. Appearance of wine red color indicated production of acetoin. Solution turned brown in absence of acetoin production. Bacteria produce acetyl methyl carbinol (acetoin) as the end product of sugar fermentation. Acetoin is converted to diacetyl in presence of oxygen and alkaline conditions produced by adding KOH and alpha naphthol in the barrits reagent. Acetoin/ 2,3 butanediol and ethanol producing organisms cause less lowering of the pH than the methyl red positive organisms.

Voges proskauer reagent (Barrits reagent)

Reagent A: 5% 1-Naphthol (alpha naphthol): 5.0 g in 100.0 mL of absolute ethanol

Reagent B: 40% KOH solution: 40g KOH in 100.0 mL Distilled water

Add 0.5 mL of reagent A and then 0.5 mL of reagent B and mix well. Appearance of red color indicated production of acetoin from glucose.

Simmon citrate medium

Na. Citrate	2.0 g
NaCl	5.0 g
$(\text{NH}_4)_2\text{SO}_4$	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
K_2HPO_4	1.0 g
Bromothymol blue	0.08 g
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH

Citrate utilization

Single colony of XRB was streaked on Simmon's Citrate slant and incubated at $28 \pm 2^\circ\text{C}$ for 24 - 48 h. Color change of the medium from green to blue is indicative of the ability of the an organism to utilize citrate as the sole source of carbon with production of the enzyme citratase. The media contains sodium citrate as the carbon source, and ammonium salts as the nitrogen source, with bromothymol blue as the pH indicator. An organism that uses citrate breaks down the ammonium salts to ammonia, which creates an alkaline pH.

Moeller's Lysine decarboxylase broth

Peptone	5.0 g
L-lysine	10.0 g
Yeast extract	5.0 g
Glucose	0.5 g
Bromocresol purple	0.005 g

pH was adjusted to 6.8 with 0.1 N NaOH

Lysine decarboxylase test

Single colony of XRB was inoculated in Moeller's lysine decarboxylase broth, overlaid with mineral oil and incubated at $28 \pm 2^\circ\text{C}$ for 24 -48 h. Bacteria able to ferment glucose produce acid and lower the pH of the medium, changing the color from purple to yellow indicated by bromocresol purple indicator dye. In the acidic conditions, bacteria able to decarboxylate amino acid lysine release amine cadaverine and increase the pH causing a color of the medium to revert back to purplish. A yellow color after 24 hours indicates inability of bacteria to decarboxylate lysine.

Christensen's Urea agar

Peptone	1.0 g
NaCl	5.0 g
Na ₂ HPO ₄	1.2 g
KH ₂ PO ₄	0.8 g
Phenol red	0.012 g
Agar	15.0 g

pH was adjusted to 7.0 with 0.1 N NaOH

Fifty mL of 40% filter sterilized urea solution was added every 950 mL molten cooled basal medium

Urease test

Single colony of XRB was streaked onto Christensen's urea agar slant and incubated at $28 \pm 2^\circ\text{C}$ for 24 -48 h. Bacteria able to produce enzyme urease hydrolyse urea to ammonia and turn the color of medium to pink indicated by phenol red indicator dye.

Hugh-Leifson Oxidative fermentative test medium

Component	g.L ⁻¹
Peptone	2
NaCl	5
K ₂ HPO ₄	3
1% Aq. Bromothymol blue	3 mL
Glucose	10
D/W	1000 mL
Agar	15

pH was adjusted to 7.0 with 0.1 N NaOH

Bromothymol blue pH breakpoint- Yellow (pH 6) to Blue (pH 7.6)

Hugh Leifson's Oxidative-fermentative test

This test is used to determine the ability of gram negative bacteria to utilize sugars oxidatively or by fermentation or their inability to utilize a particular sugar. Acids produced by fermentation can be detected easily but those produced oxidatively are weak acids and can be detected by this test. The medium contains more amounts of carbohydrates (1%) and less amounts of protein sources (0.2%). Lower N/C ratio causes production of more amounts of acids which diffuse easily in semisolid medium. XRB were stab inoculated half way in of hugh-leifson medium. One tube inoculated with each organism was overlaid with sterile mineral oil to maintain anaerobic conditions. Tubes were incubated at $28 \pm 2^\circ\text{C}$ for 48 h. Fermentative organisms produce acid in the aerobic and anaerobic tubes as indicated by appearance of yellow color. Oxidative organisms produce acid in the aerobic tube while non saccharolytic organisms cannot utilize sugars. Non saccharolytic organisms may produce amines by utilization of peptone and cause the medium to turn blue.

Nitrate broth

Component	g.L ⁻¹
Peptone	5.0 g
KNO ₃	0.2 g

pH was adjusted to 7.0 with 0.1 N NaOH

Reagents for nitrate reduction

Sulphanilic acid solution

Component	g.L ⁻¹
Sulphanilic acid	0.5 g
Glacial acetic acid	30 mL
D/W	100 mL

Alpha naphthylamine solution

Component	g.L ⁻¹
Alpha naphthylamine	0.2 g
Glacial acetic acid	30 mL
D/W	120 mL

Nitrate reduction test

Single colony of XRB was inoculated in five mL nitrate broth and incubated at for 24 h at 28 ± 2°C with constant shaking at 140 rpm. This test detects the ability of an organism to use nitrate as terminal electron acceptor. Nitrate is reduced to nitrite by nitrate reductase. Nitrite is detected by adding one mL each of alpha-naphthyl amine and sulphanilic acid to culture tube. Appearance of red color indicated that nitrate was reduced to nitrite. No color change was observed in negative test.

Gelatin liquefaction test (Balan *et al.*, 2012)

Peptone	10.0 g
Yeast extract	3.0 g
NaCl	5.0 g
K ₂ HPO ₄	0.5 g
KH ₂ PO ₄	0.5 g
Gelatin	0.05 g
Agar	15.0 g
Distilled water	1000.0 mL

pH was adjusted to 7.0 with 0.1 N NaOH

Plates were incubated at 30°C for 48 h and after spot inoculation of fresh culture and flooded with Mercuric chloride solution for detection of gelatin liquefaction.

HgCl ₂	15.0 g
Conc. HCl	20.0 g
Distilled water	To 100.0 mL

Gelatin liquefaction test

This test detects the ability of the organism to degrade gelatin by producing proteases. Single colony of XRB was spot inoculated on gelatin agar. Plates were incubated at 28 ± 2°C for 48 h and flooded with Mercuric chloride solution for detection of gelatin liquefaction. Clear haloes around the inoculum indicated gelatin liquefaction.

Motility

Motility was checked by hanging drop method. 5 µL suspension made from a single colony was spotted on a coverslip. The coverslip was quickly inverted and placed over the cavity in a cavity slide and observed under 40x magnification using phase contrast microscopy. Presence of motile cells was observed at the periphery of the drop (Czaban *et al.*, 2007).

Appendix C

Buffers and Reagents for DNA work

0.5M EDTA pH 8.0: 146.12 g EDTA was dissolved in 600 mL DW, pH adjusted to 8.0 with NaOH and diluted to 1000 mL

1M Tris-HCl pH 8.0: 121.1 g of Tris base Dissolve above quantity in 600 mL DW adjust pH to 8.0 with HCl and dilute to 1000 mL.

TE buffer

Reagent	Working concentration	Volume for 100 mL solution
1 M TrisCl pH 8.0	10 mM	1.0 mL
0.5 M EDTA pH 8.0	1 mM	0.2 mL
Distilled water	-	to 100.0 mL

5M NaCl : 14.6 g of sodium chloride was dissolved in distilled water and final volume was made up to 50 mL.

CTAB/NaCl solution: 2.05 g of NaCl was dissolved in 40 mL distilled water and 5.0 g CTAB was added while heating and stirring. After CTAB dissolved final volume was adjusted to 50 mL with distilled water.

RNase (20 μ g. mL⁻¹)

One mg. mL⁻¹ stock of RNase (Chromous Biotech, Bangalore) was prepared in sterile DW. From the stock aliquots of 1 X TE buffer were made containing RNase at the concentration of 20 μ g. mL⁻¹.

50 X Tris acetate EDTA (TAE) Buffer

Tris Base	242.0 g
Glacial Acetic acid	57.1 mL
0.5 M EDTA, pH 8.0	100 mL
Total volume	1000 mL

For 1X TAE, 20.0 mL of the above stock was diluted to 1000.0 mL using sterile distilled water and stored at RT

Reagents for PCR-DGGE

45% Polyacrylamide solution

Acrylamide: Bis-acrylamide = 29:1

Dissolve 43.5 g of acrylamide and 1.5 g of bisacrylamide in prewarm sterile water and make up the volume to 100 mL in a premarked glass bottle.

High Denaturant Solution (80% denaturant)

Component	Concentration	Quantity
45% Polyacrylamide	8%	17.7 mL
50X TAE Buffer	1 x	2.0 mL
Formamide	32%	32.0 mL
Urea	5.6 M (33.6%)	33.60 g

Dilute to 100 mL with distilled water

Low Denaturant Solution (20% denaturant)

Component	Concentration	Quantity
45% Polyacrylamide	8%	17.7 mL
50X TAE Buffer	1 x	2.0 mL
Formamide	8%	8.0 mL
Urea	1.4 M (8.4%)	8.40 g
Dilute to 100 mL with distilled water		

Stacking gel (5.0% polyacrylamide without denaturant)

Component	Concentration	Volume in μL
45% Polyacrylamide	5%	550.0
50X TAE Buffer	1 x	100.0
10% Ammonium per sulphate (APS)	0.16%	40.0
TEMED	1.4%	7.0
Water		4303.0

Buffers for silver staining of DGGE gels

Fixer solution (10% ethanol in 0.5% acetic acid)

Reagent	Volume
Glacial acetic acid	5.0 mL
Ethanol	100.0 mL
Total volume	1000.0 mL

Impregnation solution

Reagent	Volume
AgNO_3	1.5 g
Formaldehyde (37%)	1.5 mL
Total volume	1000.0 mL

Prepare fresh and store in dark until use

Developer solution

Reagent	Volume
NaOH	15 g
Formaldehyde (37%)	2.0 mL
Total volume	1000.0 mL

Prepare fresh and store in dark until use

Stop solution (10% ethanol in 0.5% acetic acid)

Reagent	Volume
Glacial acetic acid	5.0 mL
Ethanol	100.0 mL
Total volume	1000.0 mL

3M Sodium acetate (Molecular weight: $82.3 \text{ g}\cdot\text{mol}^{-1}$)

24.69 g sodium acetate was dissolved in 100 mL distilled water .

Composition for 5X Gitschier's buffer used for BOX PCR (Kogan *et al.* 1987)

Component	Standard stock	Working for 5X	Required volume in μL for 1 mL 5X Gitschier buffer
(NH ₄) ₂ SO ₄	1M	83 mM	83
Tris-HCl pH 8.0	1M	335 mM	335
MgCl ₂	1M	33.5 mM	33.5
EDTA	10mM	0.0325 mM (32.5 μM)	6.5
Beta mercapto ethanol	14.3M	148.72 mM	10.4
Distilled water	-	-	531.6
Total volume			1000.0

Reagents for plasmid isolation by Alkaline lysis method (Birnboim and Doly, 1979)

Alkaline lysis solution I

Reagent	Stock solution	Working concentration	Volume per 100 mL
Glucose	1M	50mM	500 μL
EDTA pH 8.0	0.5 M	10mM	200 μL
Tris-HCl pH 8.0	1 M	25 mM	250 μL
RNase	10 mg. mL ⁻¹	20 $\mu\text{g. mL}^{-1}$	2 mL
Distilled water			To 100 mL

Autoclave at 121°C, 15 lbs pressure for 15 min and store at 4°C

Alkaline lysis solution II (0.2N NaOH, 1% SDS) Prepare fresh every time before use

Reagent	Stock solution	Working concentration	Volume per 100 mL
NaOH	2 N	0.2 N	10 mL
SDS	10%	1%	10 mL
Distilled water			80 mL

Alkaline lysis solution III

Reagent	Volume per 100 mL
Potassium acetate (5M)	60 mL
Glacial acetic acid	11.5 mL
Distilled water	28.5 mL

Buffers for preparing chemically competent *E. coli* cells, based on buffers given by Hanahan (1983)

Transformation Buffer I (TFB I)

Component	Molecular weight	For 500 mL	For 100 mL
30 mM KOAc	98.14	1.47g	0.294
50 mM MnCl ₂	197.9	4.97g	0.994
100 mM KCl	74.56	3.73g	0.746
10 mM CaCl ₂	147.02	0.73g	0.146
15% glycerol (w/v)		75.00g	25.00 g
Distilled water		To 500 mL	To 100 mL

Transformation Buffer II (TFB II)

Component	Molecular weight	For 100 mL
75 mM CaCl ₂	147.02	1.10g
10 mM KCl	74.56	0.07g
15% glycerol (w/v)		15.00g
Distilled water		To 100 mL

TFB-I and TFB-II were sterilized using 0.45 µm filter and stored at 4°C.

EP buffer for making electrocompetent XB177 (Dunn et al., 2003)

(0.5 mM MgCl₂, 272 mM sucrose, 0.2 mM K₂HPO₄, 50 µM KH₂PO₄)

Reagent	Stock solution	Working concentration	quantity per 200 mL
MgCl ₂	1 M	0.5 mM	0.1 mL
Sucrose (342.3 g.mol ⁻¹)	-	272 mM	18.62 g
K ₂ HPO ₄	10mM	0.2 mM	4.0 mL
KH ₂ PO ₄	10mM	50 µM	1.0 mL
Distilled water	-	-	To 100.0 mL

EP buffer was sterilized using 0.45 µm filter and stored at 4°C.

Sodium borate buffer (SB buffer, 20X stock, 200mM sodium borate)

20 X Sodium Borate (SB) Buffer

NaOH	8.0 g
Boric acid	47.0 g

Above ingredients were dissolve in 900 mL distilled water and diluted to 1000 mL, pH 8.2. For 1X SB buffer, 20.0 mL of the above stock was diluted to 400.0 mL using sterile distilled water and stored at RT

Appendix D

(Material and reagents for enzyme characterization)

Activation of dialysis tubing

Dialysis tubing was cut into 15-20 cm length pieces and immersed into 500 mL of 2% NaHCO₃/1mM EDTA solution in a glass beaker. Tubing was boiled for 10 min and rinsed thoroughly with sterile distilled water. Tubing was boiled in distilled water for 10 min. Distilled water was decanted and the tubing was immersed in 50% Ethanol/1mM EDTA and stored at 4°C until use. The tubing was rinsed with distilled water from inside and outside before use.

2% NaHCO₃/ 1mM EDTA solution

10 g NaHCO₃ was dissolved in 400 mL distilled water, one mL 0.5 M EDTA (pH 8.0) was added and the volume was brought to 500 mL with distilled water.

50% Ethanol/1mM EDTA solution

250 mL ethanol was added to 150 mL distilled water, one mL 0.5 M EDTA (pH 8.0) (Appendix C) was added and the volume was brought to 500 mL with distilled water.

Saturated Ammonium sulphate solution

383 g of (NH₄)₂SO₄ was dissolved in 500 mL distilled water.

Table to determine volume of saturated ammonium sulfate solution required for 100 mL solution

Initial conc.	Final conc.																	
	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
	Milliliters saturated ammonium sulfate solution to add to 100 mL of solution																	
0	11	18	25	33	43	54	67	82	100	122	150	186	233	300	400	567	900	1900
5	6	12	19	26	36	46	58	73	90	111	138	171	217	280	375	533	850	1800
10	0	6	13	20	29	38	50	64	80	100	125	157	200	260	350	500	800	1700
15		0	6	13	21	31	42	55	70	89	113	143	183	240	325	466	750	1600
20			0	6	14	23	33	45	60	78	100	129	167	220	300	433	700	1500
25				0	7	15	25	36	50	67	88	114	150	200	275	400	650	1400
30					0	8	17	27	40	56	75	100	133	180	250	367	600	1300
35						0	8	18	30	44	63	86	117	160	225	333	550	1200
40							0	9	20	33	50	71	100	140	200	300	500	1100
45								0	10	22	38	57	83	120	175	267	450	1000
50									0	11	25	43	67	100	150	233	400	900
55										0	13	29	50	80	125	200	350	800

60											0	14	33	60	100	167	300	700
65												0	17	40	75	133	250	600
70													0	20	50	100	200	500
75														0	25	67	150	400
80															0	33	100	300
85																0	50	200
90																	0	100

Adapted from Scopes (1987), Initial conc.: Initial concentration of ammonium sulfate(% v/v), Final conc.: Final concentration of ammonium sulfate(% v/v), Values given are the amount of saturated $(\text{NH}_4)_2\text{SO}_4$ solution required to bring a solution of known initial $(\text{NH}_4)_2\text{SO}_4$ concentration to a desired final $(\text{NH}_4)_2\text{SO}_4$ concentration. The values in this table are based on the following equation: Volume of saturated $(\text{NH}_4)_2\text{SO}_4$ to add to 100 mL = $100(C_2 - C_1)/(100 - C_2)$, where C_1 is the initial concentration of ammonium sulfate (% v/v) and C_2 is the final concentration of ammonium sulfate (% v/v).

5X Bradford's reagent for protein estimation

Reagent	Quantity required
Coomassie Brilliant Blue G250	0.05 g
Ethanol	25 mL
Phosphoric acid	42.5 mL
Total volume	To 100 mL

The solution was filtered through Whatman filter paper number 1 and stored in amber colored bottles at 4°C. Aliquot was diluted to 1 X prior to assay.

Preparation of 2 mM p-nitrophenyl acetate (Molecular weight 181 g.mol⁻¹)

2mM stock of substrate was made by dissolving 36.2 mg p-nitrophenyl acetate in 1 mL DMSO, which was then mixed in 99 mL 20 mM phosphate buffer pH 6.8 with simultaneous vigorous swirling to ensure complete dispersion of p-nitrophenyl acetate in buffer (Vercoe et al., 2010). Stock was stored at 4°C in amber colored bottle until use.

Preparation of 2 mM p-nitrophenyl palmitate (Molecular weight 377.52 g.mol⁻¹)

2mM stock of substrate was made by dissolving 75.5 mg p-nitrophenyl palmitate in 1 mL of 1:4 acetonitrile: 2-propanol solution, which was later suspended in 99 mL of 1% acetonitrile, 4% 2-propanol solution (Rhee et al., 2005) with simultaneous vigorous swirling to ensure complete dispersion of p-nitrophenyl palmitate (Vercoe et al., 2010). Stock was stored at 4°C in amber colored bottle until use.

Preparation of p-Nitrophenol for standard curve of p-Nitrophenol

(Molecular weight 139.1 g.mol⁻¹)

1mM stock was prepared by dissolving 13.9 mg p-Nitrophenol in 20mM phosphate buffer, pH 6.8 and stored at 4°C in amber colored bottle until use.

Preparation of 250 mM Na₂CO₃ (for terminating esterase reaction)

(Molecular weight 105.98 g.mol⁻¹) 2.64 g was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water.

0.1 M Citrate-Phosphate buffer

0.1M Citric acid -1-hydrate (Molecular Weight 210.14 g.mol⁻¹)

2.101 g was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

0.2 M Na₂HPO₄.2H₂O (Molecular weight 177.99 g.mol⁻¹)

3.559 g was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

pH	Volume of 0.1M Citric acid -1-hydrate (mL)	Volume of 0.2 M Na ₂ HPO ₄ .2H ₂ O (mL)	Volume of distilled water
4.0	30.7	19.3	50.0
6.0	17.9	32.1	50.0

0.1 M phosphate buffer (1L)

1M K₂HPO₄ (Molecular Weight 174.18 g.mol⁻¹)

17.41 g of K₂HPO₄ was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

1M KH₂PO₄ (Molecular Weight 136.09 g.mol⁻¹)

13.60 g of K₂HPO₄ was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

pH	Volume of 1 M K ₂ HPO ₄ (mL)	Volume of 1M KH ₂ PO ₄ (mL)	Volume of distilled water
6.8	49.7	50.3	900.0
7.0	61.5	38.5	900.0
8.0	94.0	6.0	900.0

0.1 M Glycine-NaOH buffer

0.2 M Glycine (Molecular Weight 75.07 g.mol⁻¹)

1.501 g was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

0.2 M NaOH (Molecular weight 40.0 g.mol⁻¹)

0.8 g was dissolved in 80 mL distilled water and diluted to 100 mL with distilled water

pH	Volume of 0.1M Glycine (mL)	Volume of 0.2 M NaOH (mL)	Volume of distilled water
10.0	50.0	32.0	118.0

1.5 M Tris, pH 8.8

Molecular weight of Tris Base: 121.1 g.mol⁻¹

For 1.5 M Tris pH 8.8, 18.665 g of tris base was dissolved in 60 mL distilled water. pH was adjusted to 8.8 using HCl and diluted to a final volume of 100 mL.

0.5 M Tris, pH 6.8

Molecular weight of Tris Base: $121.1 \text{ g}\cdot\text{mol}^{-1}$

For 0.5 M Tris pH 6.8, 6.055 g of tris base was dissolved in 60 mL distilled water. pH was adjusted to 6.8 using HCl and diluted to a final volume of 100 mL.

Acrylamide solution

Thirty gram acrylamide and 0.8 g bisacrylamide were dissolved in 50 mL distilled water and the solution was diluted to 100 mL and stored in amber colored bottle at 4°C.

10% Ammonium per sulfate

One gram APS was dissolved in 10 mL distilled water. APS was prepared fresh every day before use in polyacrylamide gels.

Resolving gel: 8% polyacrylamide

Standard concentration	Required concentration	Required volume (mL)
Distilled water	-	6.9
30% acrylamide solution	8%	4.0
1.5 M Tris (pH 8.8)	0.38 M	3.8
10% SDS	0.1%	0.15
10% Ammonium per sulfate	0.1%	0.15
TEMED	0.06%	0.009
Total Volume		15.0

Stacking gel: 4% polyacrylamide

Standard concentration	Required concentration	Required volume (mL)
Distilled water	-	2.2
30% acrylamide solution	4%	0.67
0.5 M Tris (pH 6.8)	0.125 M	1.0
10% SDS	0.1%	0.04
10% Ammonium per sulfate	0.1%	0.04
TEMED	0.01%	0.004
Total Volume		4.0

3X Laemmli Dye (Laemmli, 1970)

Standard concentration	Required concentration	Required quantity
Distilled water		To 10 mL
0.5 M Tris pH 6.8	0.15 mM	3.0 mL
Beta mercaptoethanol (14.2 M)	14.2 M	0.209 mL
SDS	6%	0.6 g
Bromophenol blue	0.3%	0.03 g
Glycerol	30%	3.0 mL

Five microliters of 3X laemmli dye was mixed with 10 μL of sample, boiled for 3 min and used for SDS PAGE. For semi native PAGE for activity staining, laemmli dye devoid of beta mercaptoethanol was used and sample was not boiled in the dye.

5X stock of Tris-Glycine buffer

15.1 g Tris base, 94 g glycine and 1 g SDS were dissolved in 800 mL distilled water and diluted to 1000 mL with distilled water. 5X stock was diluted to 1x with distilled water for use as running buffer (25mM Tris, 250mM Glycine, 0.1% SDS)

Coomassie Brilliant Blue (CBB) staining solution

Coomassie Brilliant Blue R-250	0.25 g
Methanol	900 mL
Glacial acetic acid	100 mL

Destaining solution

Methanol	900 mL
Glacial acetic acid	100 mL

Metal ion stocks

Preparation of stock solutions of inhibitors and metals for enzyme assays

Inhibitor	Molecular weight g.mol⁻¹	Quantity (mg)*
Phenyl methyl sulfony fluoride (PMSF)	174.2	87.1
Sodium dodecyl sulphate (SDS)	288.372	144.1
Beta mercaptoethanol (BME) 14.3M	-	34.9 µL
EDTA, 100mM	-	1000.0 µL
Iodo acetic acid (IdAA)	185.95	92.9
CuSO ₄ .5H ₂ O	249.68	124.5
FeSO ₄ .7H ₂ O	278.015	139.0
MgSO ₄ .7H ₂ O	246.47	123.2
HgCl ₂	271.0	135.5
SnCl ₂ .2H ₂ O	225.63	112.5
CaCl ₂ .2H ₂ O	147.02	73.5
(NH ₄) ₂ SO ₄	132.13	66.0
NaCl	58.44	29.2
KCl	74.56	37.2
MnSO ₄ .H ₂ O	169.02	84.5
ZnSO ₄ .7H ₂ O	287.54	143.7
AgNO ₃	169.87	84.9

* quantity in mg or µL for preparing 10 mL of 50 mM stock solution in distilled water.

Appropriate volumes for concentrations of 1 mM and 5 mM were used in enzyme assays to determine effect of inhibitors in activity of QQE.

Appendix E

(Miscellaneous reagent stocks and standard curves)

2% H₂SO₄ in methanol

0.2 mL concentrated H₂SO₄ was mixed in 9.8 mL methanol and used immediately for esterification.

Saturated NaCl solution: 3.615 g of NaCl was dissolved in 12 mL of DW.

Saturated NaHCO₃ solution: 1.035 g of NaHCO₃ was dissolved in 10.5 mL of DW

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) 20 mg. mL⁻¹ 2% stock

100 mg X-gal (Chromous Biotech, India) was dissolved in 5 mL DMSO, sterilized using syringe filter (0.22 μm) and stored at -20°C.

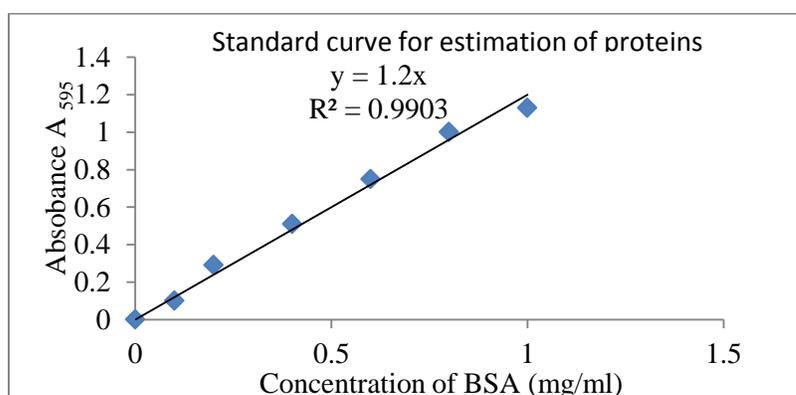


Fig. E1. Absorbance at 595 nm vs different concentrations of BSA (mg. mL⁻¹) for determining amount of protein concentration of QQE by Bradford's assay

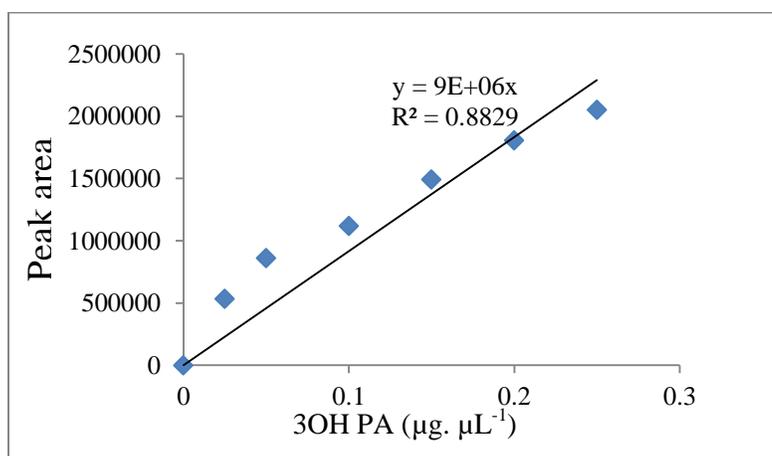


Fig. E2. Peak area vs different concentrations of 3OH-PA (μg.μL⁻¹) for determining amount of 3OH-PA liberated by enzymatic degradation of 3OH-PAME.

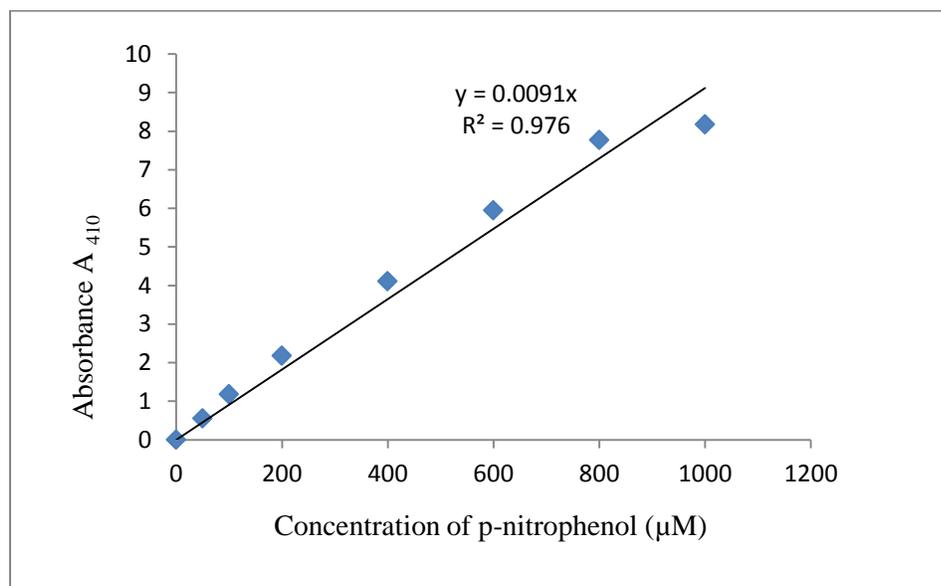


Fig. E3. Absorbance at 410 nm vs different concentrations of p-Nitrophenol (µM) for determining amount of p-Nitrophenol liberated from activity of QQE against p-Nitrophenyl acetate and p-Nitrophenyl palmitate.

Table E1. Number of bacterial strains used in various experiments presented in different chapters.

Experiment	Criteria for selection	No.	Chapter
ARDRA	All XRB in collection	167	III
Antagonism to <i>R. solanacearum</i>	All XRB in collection	167	III
Identification by 16S rRNA gene sequencing	Antagonistic XRB and ARDRA representatives	55	III
Identification by biochemical tests	Antagonistic XRB	28	III
Extracellular hydrolytic enzymes	Antagonistic XRB	28	III
In vitro production of plant growth promoting substances	Antagonistic XRB	28	III
Seedling vigor enhancement	Antagonistic XRB	28	III
Growth promotion in eggplant under greenhouse conditions	Antagonistic XRB which enhanced seedling vigour	23	III
Antibiotic susceptibility testing	Antagonistic XRB	28	III
Diffusible and volatile inhibitory compounds	Antagonistic XRB	28	IV
Determination of BCE	Antagonistic XRB with GPE>10%	16	IV
Niche exclusion/ re-colonization of eggplant	Antagonistic XRB with BCE>20%	11	IV
GFP Tagging	Systemic colonizer of eggplant	1	IV
Strains of <i>R. solanacearum</i> used for mutagenesis	Highly pathogenic and genetically diverse strains	2	V
Screening for quorum quenching	All XRB in collection	167	VI
Characterization (<i>in vitro</i> and <i>in planta</i>)	3OH-PAME degrading XRB	8	VI
HPLC-MS analysis of 3OH-PAME degradation	3OH-PAME degrading XRB	8	VI
Specific activity against 3OH-PAME, p-nitrophenyl acetate and p-nitrophenyl palmitate	3OH-PAME degrading XRB	8	VI
Identification by 16S rRNA gene sequencing	3OH-PAME degraders with high specific activity against 3OH-PAME (two XRB Identified in chapter III)	3 (2)	VI
Purification and characterization of 3OH-PAME degrading enzyme	3OH-PAME degraders with high specific activity against 3OH-PAME	5	VI

List of Publications

Articles

Achari G. A. and Ramesh, R. 2014. Diversity, biocontrol, and plant growth promoting abilities of xylem residing bacteria from solanaceous crops. *International Journal of Microbiology*, <http://dx.doi.org/10.1155/2014/296521>

Achari G. A. and Ramesh, R. 2015. Characterization of bacteria degrading 3-hydroxy palmitic acid methyl ester (3OH-PAME), a quorum sensing molecule of *Ralstonia solanacearum*. *Letters in Applied Microbiology*, **(Accepted)**.

Poster presentations

Achari, G. A. and Ramesh, R. 2013. Xylem bacteria for the plant growth promotion and pathogen inhibition in brinjal. In abstracts of “10th National symposium on biotechnological approaches for plant protection: constraints and opportunities” (pp 112-113 from 27-29, January 2013 held at ICAR Research Complex for Goa, Old Goa, Goa.

Achari, G. A., Ramesh, R and Gaitonde, S. 2012. Diversity, antagonistic and growth promotion ability of xylem residing bacteria isolated from eggplant and chilli. In abstracts of American Society of Microbiology 112th General Meeting, 2012 held during 16-19, 2012 at San Francisco, California USA.

Achari, G. A. and Ramesh, R. 2011. Antagonistic and growth promotion activity of xylem residing bacteria isolated from eggplant and chilli. In: National symposium on “Microbial diversity and its applications in health, agriculture and industry”. March 4-5, 2011, ICAR Research Complex for Goa, Old Goa, Goa, pp 51-52.

Fellowship during PhD

Fulbright Nehru Doctoral and Professional Research Fellowship funded by the U.S. Department of State and United States-India Educational Foundation for carrying out part of the Ph. D. work at the **Department of Plant Pathology, Ohio Agricultural Research Development Centre-The Ohio State University**, Wooster, Ohio 44691, USA, from 4th November, 2011 to 3rd August, 2012.

Other co-authored publications

Ramesh, R., **Achari, G. A.** and Gaitonde, S. 2014. Genetic diversity of *Ralstonia solanacearum* infecting solanaceous vegetables from India reveals the existence of unknown or newer sequevars of Phylotype I strains. *European Journal of Plant Pathology*, **140**: 543-562. DOI: 10.1007/s10658-014-0487-5.

Ramesh, R., Gaitonde, S., **Achari, G.**, Asolkar, T., Singh, N.P., Carrere, S., Genin, S. and Peeters, N. 2014. Genome sequencing of *Ralstonia solanacearum* biovar 3, phylotype I, strains Rs-09-161 and Rs-10-244, isolated from eggplant and chili in India. *Genome Announcement*, **2(3)**:e00323-14. doi:10.1128/genomeA.00323-14.

Ramesh, R., Gaitonde, S. and **Achari, G.** 2013. Genetic diversity of *Ralstonia solanacearum*, a phytopathogenic bacterium infecting solanaceous vegetables using REP-PCR and PFGE. In: (Microbial diversity and its applications. 2013. (Eds.) S.B. Barbuddhe, R. Ramesh and N.P. Singh). New India Publishing Agency, New Delhi pp 119-128. ISBN: 9789381450666.

Ramesh, R., Anthony J., Jaxon, T.C.D., Gaitonde, S. and **Achari, G.** 2011. PCR based sensitive detection of *Ralstonia solanacearum* from soil, eggplant, seeds and weeds. *Archives of Phytopathology and Plant Protection*, **44(19)**:1908-1919.