STUDIES ON CHARACTERIZATION, GENOMIC FINGERPRINTING AND MANAGEMENT OF *Erwinia chrysanthemi* CAUSING STALK ROT OF SORGHUM

Thesis

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By

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on characterization, genomic fingerprinting and management of *Erwinia chrysanthemi* causing stalk rot of sorghum"** submitted in partial fulfilment of the requirements for the degree of **Doctor of Philosophy** with major in **Plant Pathology** and minor in **Entomology** of the College of Post-Graduate Studies, G.B. Pant University of Agriculture and Technology, Pantnagar, is a record of *bona fide* research carried out by **Mr. Bhupendra Singh Kharayat, Id. No. 35474** under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been duly acknowledged.

Pantnagar
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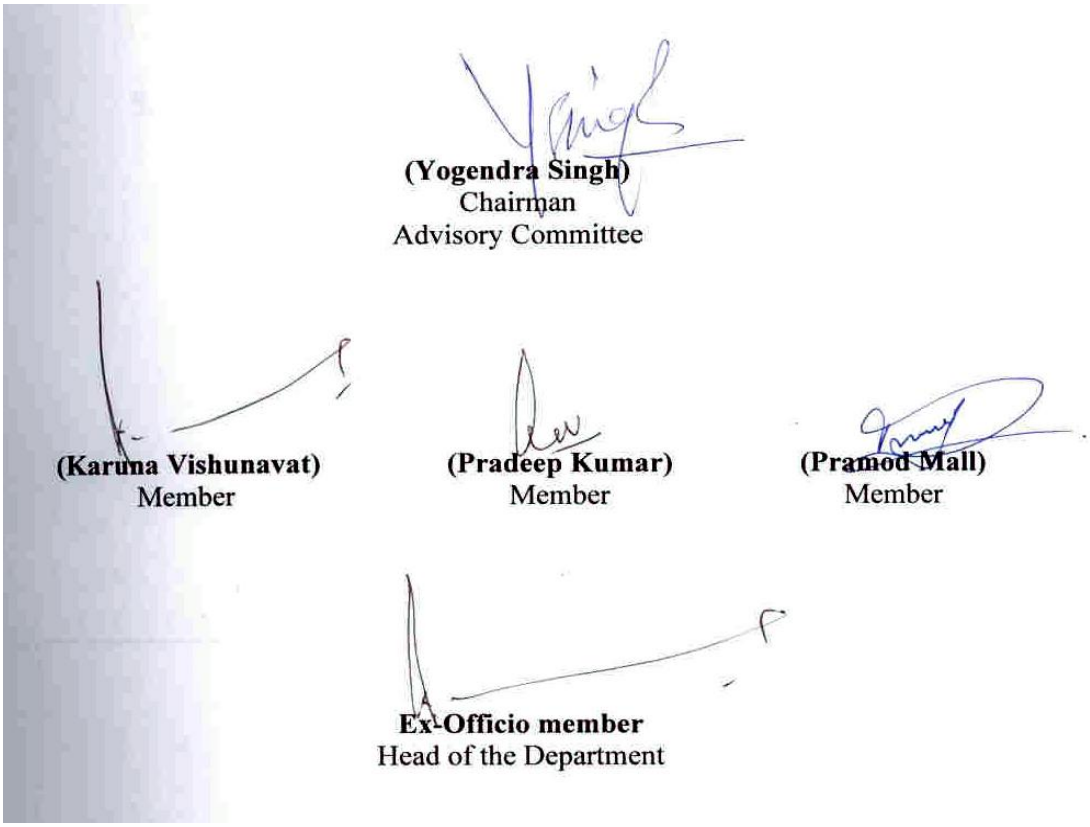
CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Mr. Bhupendra Singh Kharayat, Id. No. 35474,** a candidate for the degree of **Doctor of Philosophy** with major in **Plant Pathology** and minor in **Entomology** agree that the thesis entitled **"Studies on characterization, genomic fingerprinting and management of *Erwinia chrysanthemi* causing stalk rot of sorghum"** may be submitted in partial fulfilment of the requirements for the degree.



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CONTENTS

Abbreviations and Symbols

Abbreviation	Stand for
bp	base pair
BCA	Biocontrol agent
ºC	Celsius
CDS	Coding sequence
CD	critical differences
cfu	colony forming unit
conc.	Concentration
CPV	crystal violet pectate
CRD	complete randomized design
cv	Coefficient of variation
DAS	Day after sowing
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Ech	*Erwinia chrysanthemi*
ERIC	Enterobacterial Repetitive Intergenic Consensus Sequences
EtBr	Ethidium Bromide
Fig.	Figure
g	Gram
gel doc	Gel Documentation
hrs	Hours
LBA	Luria Bertani Agar
LBB	Luria Bertani Broth
$MgCl_2$	Magnesium Chloride
min.	Minutes
ml	Milliliter
mM	Millimolar
NA	Nutrient Agar

NB	Nutrient Broth
ng	Nanogram
NGM	Nutrient Glycerol $MnCl_2$
nm	Nanometer
NYDA	Nutrient Yeast Dextrose Agar
PCR	Polymerase Chain Reaction
PDI	Percent disease incidence
pH	Potential Hydrogen
ppm	Parts per million
Primer F	Primer forward
Primer R	Primer reverse
Psf	*Pseudomonas florescens*
RBD	Radom Block Design
rep	Repetitive Extragenic Palindromic Sequences
RH	Relative humidity
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Second
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
sem	Standard error of mean
TAE	Tris Acetate EDTA
Taq polymerase	*Thermus aquaticus* polymerase
TE	Tris-EDTA
Th	*Trichoderma harzianum*
v/v	Volume/volume
w/v	Weight/ volume
YDCA	Yeast Dextrose Calcium Carbonate agar
µl	Microlitre

Introduction

Chapter 1 INTRODUCTION

The genus sorghum belongs to the tribe Andropogoneae of the family Poaceae and includes both wild species and species cultivated for their grain, fodder, syrup and other commercial purposes. Sorghum is a self pollinating, diploid (2n=20) with a genome (1C=735 Mbp) about 25% the size of maize, or sugarcane. All the cultivated forms are grouped under a single species *Sorghum bicolor* (L.) Moench. *S. bicolor* is typically an annual, but some cultivars are perennial. It grows in clumps which may attain a height upto 3.5 m bearing very wide (upto 12 cm) and long upto 125 cm leaves. It is the fifth most important cereal crop in the world after maize (*Zea mays* L.), wheat (*Triticum vulgare* L.), rice (*Oryza sativa* L.), and barley (*Hordeum vulgare* L.). Worldwide, the area under sorghum is estimated to about 44 million hectares in 99 countries in Africa, Asia, Oceania, and the Americas. Sorghum main producers (in million metric tons) are: USA (11.5), Nigeria (8.0), India (7.5), Mexico (6.3), China (3.1) and Sudan (2.6) **(FAO, 2006).** Sorghum production in India in 2012 occurred 6000 metric tons **(USDA, 2012).**

Sorghum is cultivated widely throughout the arid, semi arid tropics and temperate regions within the latitude of 45^{o} N to 45^{o}S. Its peculiar quality of withstanding drought makes it a potential alternative in dryland and rainfed conditions in semi-arid tropics (SAT) **(Ross and Webster, 1970)**. India ranks first in acreage among the sorghum growing countries and second in production with an area of about 9.49 mha, production of 7.78 mt and productivity of about 981 kg/ha **(Rao *et al.*, 2008)**. In India, it is grown successfully in areas having average rainfall between 500 and 1000 mm with the temperature requirement of 25-30^{o}C. It is grown as *Kharif* crop in North India while in western and southern part of the country it is also grown as *Rabi* crop. More than 60% of the total sorghum is raised as rainfed crop in *Kharif* season (June to Oct.) in *Rabi* (Oct. to Feb.) and summer season, depending upon the weather conditions.

Kharif sorghum occupied an area of 3.46 mha producing 5.01 mt with an average productivity of 885 kg/ha in 2007-2008 and *rabi* sorghum cultivated on 4.57

mha produced about 2.77 mt **(Rao *et al.*, 2008).** The major states in the country where this cereal grain is produced are Maharashtra, Karnataka, Gujarat, Madhya Pradesh, Andhra Pradesh, Rajasthan and Uttar Pradesh. Maharashtra produces the maximum sorghum in India. The nutritional value of sorghum is same as of that of corn and that is why it is gaining importance as livestock feed. Sorghum is also used for production of ethanol, starch, adhesives and paper other than being used as food and feed. In developed countries sorghum is mainly used for animal feed. Recent work has shown that sorghum and millet (*Pennisetum glaucum* (L.) R. Br) are rich in antioxidants and gluten-free, which make them an attractive alternative for wheat allergy sufferers **(Dahlbert *et al.*, 2004).** Sorghum grain has high levels of iron (>70 ppm) and zinc (>50ppm), and is hence being targeted as a means to reduce micronutrient malnutrition globally.

Recently interest in utilization of sweet sorghum for ethanol production has increased in India due to its four times less growing period (4 months) and water requirement (8000 m^3 over two crops) and three times less cost of cultivation than those of sugarcane **(Soltani and Almodares, 1994; Dayakar Rao *et al.*, 2004)**. Also the sweet sorghum is best suited for ethanol as its juice has higher total reducing sugar content compared to sugarcane juice with 90 per cent fermentation efficiency **(Ratnavathi *et al.*, 2004; Huligol *et al.*, 2004)**. The more important is that the ethanol production from sweet sorghum and its use as fuel is environment friendly, as it is of clean burning nature, rating high octane compared to compressed natural gas **(Arbatti, 2001)**.Sorghum plants are known to contain cyanogenic glucoside dhurrin, a group of nitrogenous secondary compounds, which during enzymatic hydrolysis release hydrocyanic acid (HCN), glucose and p-hydroxy benzaldehyde. Cyanogenic glucoside in young sorghum plants accumulate mainly in leaves and at maturity is fairly evenly distributed between leaves and stem **(Morton, 1981; Kim and Voigtlaender, 1985)**. It was found that HCN content at initial stages of the plant growth may play an important role in the resistant reaction in leaf blight diseases of sorghum **(Mohan and Lakshman, 1987).** Sorghum is attacked by a wide range of pathogen because of the diversity of its use and range of environments in which it is cultivated. Stalk rot caused by *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock is one of the most destructive diseases of Sorghum crop in India. The disease

has assumed in severe proportion in Uttarakhand in recent past. The disease mainly affected sorghum stem showing water-soaked symptoms that later turned reddish dark brown color. The infected stem pith disintegrates and show slimy soft-rot symptoms and eventually the whole plant is wilted.

Identification is necessary for the taxonomy and efficient management of any disease inciting microorganism. When an unknown bacterium is isolated in the laboratory, it is usually identified by a combination of information from microscopic observations, i.e. morphology and arrangement of cells; gram staining; cultural (growth) characteristics on cultural medium; biochemical and physiological characterization. Molecular tools are available to identify the organism, but classical approaches combined with it give most authentic results. The investigation is aimed to study the cultural and morphological characteristics of *Erwinia chrysanthemi*, on differential mediums and molecular detection by PCR technique.

The identification and classification of bacteria are of crucial importance in environmental, industrial, medical and agricultural microbiology and microbial ecology. A number of different phenotypic and genotypic methods are presently being employed for microbial identification and classification **(Louws *et al*., 1996)**. Each of these methods permits a certain level of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level. Moreover, each method has its advantages and disadvantages, with regard to ease of application, reproducibility, requirement for equipment and level of resolution **(Akkermans *et al*., 1995)**. Generally, DNA-based methods are emerging as the more reliable, simple and inexpensive ways to identify and classify microbes. In fact, the assignment of genera/species has traditionally been based on DNA-DNA hybridization methods **(Wayne *et al*., 1987)** and modern phylogeny is increasingly based on 16S rRNA sequence analysis **(Woese, 1987; Stackebrandt and Goebel, 1994)**. Advances in molecular biology have provided highly specific methods for classifying, identifying and typing isolates based on nucleic acid sequence data. Taxonomic systems are becoming increasingly important for characterizing microorganisms since they can be applied in epidemiological investigations of disease outbreaks to identify a potential common source and/or to establish grouping strategies for isolates recovered from

several environments. Typing systems based on phenotypic tests have limitations as key phenotypic traits of microorganisms may be inconsistently expressed or may not provide enough discriminative power to separate closely related microorganisms. Therefore, shortcomings of phenotypically based methods have pushed towards the development and employment of methods based on microbial genotypes or DNA sequences minimizing problems with respect to reproducibility and, in some cases, enabling the establishment of large databases (a biological-genotypic database) of characterized organisms. The various molecular fingerprinting methods have advantages and disadvantages when applied to specific situations and objectives. Fingerprinting methods used to discriminate between strains within target species are easy to perform and data interpretation is relatively straight forward. Prokaryotic and eukaryotic genomes contain dispersed repetitive sequences separating longer single-copy DNA sequences. Interspersed repetitive sequences are characterized as relatively short (usually <500 bp), non-coding, dispersed elements in bacterial genomes. Current data supports that repetitive DNA comprises a substantial portion of the microbial genomes.

For the management of soil and seed born bacterial disease chemical and non-chemical methods including biological control agent are used. Because the pathogen invades the inner parts of the plants, the conventional chemical products such as copper may not provide adequate control for the disease. To solve this problem, many scientists have focused on alternative solutions such as biological control in conventional and ecological (organic) agriculture. Biocontrol can be achieved either through introduction of biocontrol agent directly or by adopting practices which favour build up of biocontrol agents under natural conditions. Several research groups have used plant growth promoting rhizobacteria, fluorescent Pseudomonas strains and *Trichoderma* spp. Several researchers have successfully employed antagonistic bacteria, *Streptomyces* and yeasts to control plant bacterial diseases **(Alivizatos and Pantazis, 1992; Ozaktan *et al*., 1999)**. One of the alternative control methods of *E. chrysanthemi* is the use of biological control agents. Biological disease control is an attractive alternative strategy for the control of plant diseases. Meanwhile, it also provides practices compatible with the goal of a sustainable agricultural system. Understanding the mechanisms of biological control of plant diseases through the

interactions between antagonists and pathogens may allow us to select and construct the more effective biocontrol agents and to manipulate the soil environment to create a conducive condition for successful biocontrol. Breeding for resistance, which continues to be the most practical and feasible method to control plant diseases is not able to keep pace with the development of more virulent pathogens.

The study of genomic variability analysis among *E. chrysanthemi* isolates can be used to characterize native population of soft rot *Erwinia* isolates existing in tarai region of district of Uttarakhand, which may be helpful in devising breeding programme for resistance against the disease.

Keeping above factors in view the present study is aimed at following objectives:

1. Characterization of *Erwinia chrysanthemi* isolates.
2. Evaluation of pathogenicity and inoculation methods for creating artificial epidemic under glass house conditions.
3. Study of pathogenic variability existing among *Erwinia chrysanthemi* isolates under glasshouse conditions.
4. Study of genomic variability existing among *Erwinia chrysanthemi* isolates.
5. Evaluation of antagonistic potential of biocontrol agents against test pathogen under *in vitro* and *in vivo* conditions.
6. Glasshouse and field evaluation of vermicompost colonized with biocontrol agents against test pathogen by pre-plant soil drenching method.
7. Screening of chemicals against the test pathogen under *in vitro* and *in vivo* conditions.

Review of Literature

2.1 Historical background and nomenclature

The genus *Erwinia* is named after one of the founder of phytobacteriology, Erwin Frink Smith, and was established by **Winslow *et al*. (1917)** to include the plant pathogenic entereobacteria. Like other entereobacteria, the *Erwinia* are motile by means of several to many peritrichous flagella, gram-negative, non-sporeforming, straight rod with rounded ends, and occurs singly or in pairs. Its size varies from 0.8-3.2×0.5-0.8 μm (average 1.8×0.6 μm) depending on carbon source present in the medium and growth conditions **(Grula, 1970)**. There are 3-14, but more usually 8-11, peritrichous flagellae **(Burkholder *et al*., 1953; Dickey, 1981).** The bacterial family Enterobacteriaceae includes multiple animal and plant pathogens, with the latter belonging to the genera *Brenneria, Dickeya, Enterobacter, Erwinia, Pantoea* and *Pectobacterium* **(Hauben *et al*., 1998; Samson *et al*., 2005)**. In 1917, the genus *Erwinia* was established to encompass all members of the Enterobactericeae that were pathogenic to plants, including both *Erwinia* have strong pectolytic activity, causing soft rots in plants (the "carotovora" group e.g. *Erwinia carotovora* and *E. chrysanthemi*) and non-pectolytic, causing necrotic or wilt diseases (the "amylovora" group e.g. *E. amylovora*). The species *E. chrysanthemi* was created for the *Chrysanthemum morifolium* hollow stalk agent **(Burkholder *et al*., 1953)**. Later studies revealed that strains of *E. chrysanthemi* (*Ech*) cause disease on a wide variety of plant hosts, including 16 dicotyledonous families of plants in 11 orders and 10 monocotyledonous families in five orders **(Samson *et al*., 2005; Ma *et al*., 2007)**. Because of the wide host range of *E. chrysanthemi*, **Lelliott and Dickey (1984)** subdivided the species into six pathovars, namely pvs *chrysanthemi*, *dianthicola*, *dieffenbachia*, *paradisiaca*, *parthenii* and *zeae*, based on host specificity. Later, **Samson *et al*. (1987)** developed a biovar system based on some key stable biochemical characteristics. Initially **Waldee (1945)** proposed the moving pectolytic *Erwiniae* into a new genus *Pectobacterium* on the basis of the unique pectolytic activity of the bacteria. Previous suggestions to separate the pectolytic enterobacteria into the genus *Pectobacterium* had not found favor among phytobacteriologists. It was

not until 1998 that new insights from16S rDNA analysis led to regained impetus for such a move and to this name being largely accepted by the scientific community **(Waldee, 1945; Hauben *et al*., 1998)**. **Hauben *et al*., (1998)** revived the suggestion and added evidence from sequence analysis of the 16S ribosomal DNA of various plant-associated members of the *Enterobacteriaceae* to support the proposal. Although phenotypic characterization and analysis of a single DNA fragment might have been considered insufficient for subdivision at the generic level, the DNA: DNA hybridization study conducted by **Gardan *et al*., (2003)** provides further stimulation to change in favor of the new nomenclature. Whilst the potato pathogens *Pectobacterium carotovorum* subsp. *carotovorum* (syn. *Erwinia carotovora* subsp. *carotovora*) and *Pectobacterium atrosepticum* (syn. *Erwinia carotovora* subsp. *atroseptica*) remain within this genus, further analysis of *Pectobacterium chrysanthemi* using 16S rDNA, DNA–DNA hybridization and biochemical characterization showed that it forms a distinct clade from the pectobacteria, and a new genus, *Dickeya*, was proposed (named after the eminent microbiologist **Robert S. Dickey**).

The potential to parasitize mammalian host as well as plants by *Ech* have been observed **(Duarte *et al*., 2000)**. The bacterium can adhere to cause a oxidative stress response I and kill cultured human adeocarcioma cells. These bacteria express a surface protein that ears immunological identity to intimin, a protein required for full virulence of eterohemorrhagic ad eteropathogeic *Escherichia coli.* A type III secretion system mutant of *Ech* was observed to have a significantly lower capability of causing death than the wild-type strain in parallel cultures of human colon adenocarcioma.

2.2 Economic importance and geographical distribution

Soft rot erwinias are very important primary pathogens of both growing plants and the harvested crop **(Pérombelon and Kelman, 1980; Kotoujansky, 1987; Agrios, 2005; Toth *et al*., 2003)**. *E. chrysanthemi* is pathogenic to a wide range of tropical and sub tropical crops of diverse botanical families including ornamentals, as well as on greenhouse-grown crops in temperate regions **(Schaad and Brenner, 1977; Dickey, 1979; Pérombelon and Kelman, 1980)**. However, strains from different host plants differ in their specific host range as well as in the pathogenic and

phenotypic properties **(Dickey, 1981; Kotoujansky, 1987; Janse and Ruissen, 1988)**. Due to economic importance, the whole genome of soft rot pathogen has been sequenced in 2010 {*Dickeya dadantii* (strain 3937) (*Erwinia chrysanthemi* (strain 3937)} complete proteome. **http://asap.ahabs.wisc.edu/research-projects/plant-pathogen-genome-projects/dickeya-dadantii-erwinia-chrysanthemi-3937-genome-project.html**}.

2.3 Morphology

E. chrysanthemi (*Ech*) is a motile, gram-negative, non-sporing, straight rod with rounded ends, and occurs singly or in pairs. Its size varies from 0.8-3.2×0.5-0.8 μm (average 1.8×0.6 μm) depending on carbon source present in the medium and growth conditions **(Grula, 1970).** There are 3-14, but more usually 8-11, peritrichous flagellae **(Burkholder, *et al.*, 1953; Dickey, 1981)**. *Erwinia* are the only plant pathogenic bacteria that are facultative anaerobes **(Agrios, 2005)**. On PDA, young colonies are circular, convex, smooth and entire, or sculptured with irregular margins, depending on the moisture content of the growth medium. After 4-5 days colonies become, round, raised centre and lobed periphery, which later becomes feathery or almost coralloid **(EPPO, 1992)**.

2.4 Biology and ecology

E. chrysanthemi is a soft rot pathogen degrading succulent fleshy plant organs such as roots, tubers, stem cuttings and thick leaves. It is also a vascular wilt pathogen, colonizing the xylem and becoming systemic within the plant. This latter aspect is the most alarming when vegetative propagation is involved. The pathogen can remain latent in stock plants (ornamentals, bananas) and can thus be spread in cuttings from them. Tubers are an important source of the disease for potatoes. The bacterium is able to survive in the soil (on plant debris), so that infestation remains between two crops. High humidity and free water favour spread and penetration of the bacteria. Disease development is dependent on high temperatures, generally 25-30°C. Host specialization has not definitely been proved in *E. chrysanthemi*, except in pv. *paradisiaca* **(Dickey and Victoria, 1980; Dickey, 1981)**. The pathogen is ubiquitous and isolates from maize and potato seem to be rather polyphagous, while

Philodendron and *Kalanchoe* do appear to be differential hosts for temperate isolates **(Janse and Ruissen, 1988)**.

The pathogen lives in plant debris and in the roots as well as on soils surrounding the roots of sweet potato and its other hosts. Dissemination is through infected planting material, irrigation water, tools, animal grazing, shoes of laborers, etc. Optimum disease development occurs at 30°C but the bacterium survives in a wide range of temperature below 27°C. No other factors, aside from temperature, have been reported to affect the development of the disease. Pathotypes of *E. chrysanthemi* have been reported to affect other plant species, but no reference was made on sweetpotato.

2.5 Host range

The soft rot, *E. chrysanthemi* attacks the parenchymatous tissues (which comprised primarily of living cells without secondary wall thickening) of wide range of plants species **(Bradbury 1977; Bradbury 1986; Clark and Moyer, 1988)**. The bacteria are distributed worldwide, with *E. chrysanthemi*, which has a higher optimum temperature for growth, being found primarily in the tropics or in temperate region greenhouse **(Pérombelon and Kelman, 1980)**. The soft rot Erwinia are widespread in surface water **(Cother *et al*., 1992; Cother and Gilbert 1990; Harrison *et al*., 1987; McCarter-Zorner *et al*., 1984)**, they are competitive saprophytes in the rhizosphere **(Staghellii 1982)**, and aggressively utilize pectate as carbon source **(Burr and Schroth 1977; Meneley and Stanghellini 1976)**. Pectic enzyme production and sensitivity to desiccation appear to be major factors in the biology of this bacterium. Several taxonomic subgroups have been identified within *E. chrysanthemi*, and some strains display a degree of host specificity **(Boccara *et al*., 1991; Dicky 1979, 1981; Janse and Russen, 1988).** However, the current taxonomic subgroups do not consistently correspond to host origin, and a given strain of *Ech* is generally capale of attacking many plants. The primary hosts are*: Zea mays* (corn*), Sorghum bicolor* (sorghum*), Sorghum sudanensis* (Sudan grass). The secondary hosts include: *Aechmea fasciata* (urn-plant), *Aglaonema pictum* (Chinese evergreen), *Allium fistulosum* (Welsh onion), *Ananas comosus* (pineapple), *Begonia bertini* (begonia), *Brachiara mutica* (tall panicum*), Chrysanthemum* spp. (Chrysanthemum), *Colocasia*

esculenta (taro), *Dahlia* spp. (dahlia), *Daucus carota* (carrot), *Dianthus caryiophyllus* (carnation), *Dieffenbachia* spp. (dieffenbachia), *Dracaena marginata* (Madagascar dragon tree), *Euphorbia pulcherrima (*poinsettia), *Imperata cylindrica* (cogon grass), *Ipomoea batatas* (sweetpotato), *Musa* spp.(plantain), *Oriza sativa* (rice), *Panicum* maximum (Guinea grass), *Parthenium argeratum* (guayule), *Paspalum* sp.(paspalum), *Pennisetum purpureum* (elephant grass), *Petunia hybrida* (Petunia), *Phalaenopsis* sp. (phalaenopsis), *Philodendron* spp. (phylodendron), *Saccharum officinarum* (sugar cane), *Saintpaulia ionantha (*African violet), *Solanum tuberosum* (potato) and *Syngonium podophylum* (nephthytis).

2.6 Phytosanitary risk

E. chrysanthemi is listed as an A2 quarantine pest by EPPO **(OEPP/EPPO, 1982)**. This arose historically from the earlier listing of the carnation pathogen and it does not appear that plant quarantine authorities had other hosts in mind. When bacterial nomenclature was revised in **1980**, **EPPO** specified that the quarantine pests concerned were pathovars *dianthicola* and *chrysanthemi* **(OEPP/EPPO, 1988)**. It was accepted that the major means of infection of these crops, at least in some countries, was the use of infected planting material which according to current trade practice was mostly imported. Thus the local presence of *E. chrysanthemi* was irrelevant (since it could be excluded by very simple precautions), and protection against this pest was perceived as a plant quarantine problem.

Since then, argument has continued on the infraspecific forms of *E. chrysanthemi* which are supposed to be covered by the quarantine pest classification. This debate has been further complicated in two ways: (1) many authorities consider that *E. chrysanthemi* is so widely distributed in the EPPO region that it cannot classify as a quarantine pest; (2) the existence of host-specific pathovars is queried **(Samson *et al.*, 1987)**, at least with respect to carnation and chrysanthemum, which increases yet further the possibility that these crops might be infected from sources other than imported planting material. It is now accepted within EPPO that the risk from *E. chrysanthemi* can be adequately covered by national nuclear-stock certification schemes for the crops concerned and that *E. chrysanthemi* will be deleted from the EPPO A2 list as soon as such schemes have been agreed for carnations **(OEPP/EPPO, 1991)** and

chrysanthemums. No other regional plant protection organization considers *E. chrysanthemi* to be a quarantine pest.

2.7 Symptoms

Plant infection by *E. chrysanthemi* and other soft rot erwinias causes various symptoms, both local and systemic, ranging from tissue maceration to wilts and blights **(Pérombelon and Kelman, 1980; Kotoujansky, 1987; Expert, 1999; Yang *et al*., 2004a)**. Infection generally results in maceration and rotting of parenchymatous tissue of the affected organ but wilting and leaf chlorosis are common early symptoms in vegetatively reproduced crops due to systemic infection of the young plant from inoculum in infected seed material **(Pérombelon and Kelman, 1980)**.

2.8 Penetration

The bacteria may enter the plant host through wounds or normal plant openings on the outside of the stem or leaves, such as hydathodes and stomata, and end up in intercellular spaces in the tissue of the plant stem (**Kelman *et al.*, 1957**, **Mildenhall, 1974**) and multiply profusely in the intercellular spaces where they produce several kinds of enzymes that dissolve the middle lamella and separate the cells from each other, leading to maceration **(Agrios, 2005, Murata *et al*., 1991)**. When inoculated directly into the stem, the soft rot bacteria can colonise the vascular system and their movement up the stem is followed by progressive decay of stem tissue **(Pérombelon and Kelman, 1980)**. Upon plant tissue infection, *E. chrysanthemi* undergoes up- or down-regulation of several genes that encode various functions including virulence factors, adaptation to the apoplast environment, metabolism and protection against the host plant defence machinery **(Aguilar *et al*., 2002; Okinaka *et al*., 2002; Toth *et al*., 2003; Yang *et al*., 2004a)**. Like other many enteric pathogens, such as *Escherichia coli* O157:H7 and *Yersinia pestis*, *Erwinia chrysanthemi* cells do not appear to invade host cells internally in the pathogenic phase. They remain in the intercellular spaces of infected plant tissue and use several secretion systems to inject virulence factors into host cells. In addition to causing local disease, the bacteria may enter vascular elements of infected plants, thereby moving rapidly through the host **(Chatterjee *et al.*, 2000; Collmer and Keen 1986;**

Expert 1999; Hugouvieux-Cotte-Pattat *et al*., 1996; Perombelon and Kelman 1980). Many virulence determinants have been discovered in *E.chrysanthemi*, including the well-studied extracellular enzymes such as pectate lyase, pectinase, and cellulase; siderophore-dependent iron uptake systems, *sap*, and *msrA* gene **(Expert 1999; Franza and Expert 1991; Franza *et al*., 1999; Hassouni *et al*., 1999; Lopez-Solanilla *et al*., 2001; Tardy *et al*., 1997)**. Virulence gene expression in *E. chrysanthemi* is a highly regulated phenomenon affected by a variety of parameters, including temperature, pH, iron levels, growth phase, and population density. Plant tissue breakdown is ensured by in-planta secretion of extra cellular cell wall-degrading enzymes, most important of which are pectinases **(Collmer and Keen, 1986; Kotoujansky, 1987; Barras *et al*., 1994; Shevchik *et al*., 1998; Toth *et al*., 2003)** although cellulases, hemicellulases, proteases, phospholipases, and xylanases may also contribute to the pathogenicity of *E. chrysanthemi* **(Kotoujansky, 1987; Barras *et al*., 1994)**. Pectate lyases attack the α-1,4-glycosidic linkages in pectate by cleaving the glycosidic bonds through β-elimination giving rise to unsaturated products which are then cleaved by polygalacturonase to yield saturated oligomers that can serve as carbon source for the bacteria **(Collmer and Keen, 1986; Kotoujansky, 1987; Barras *et al*., 1994)**. The specific importance of the various pectinolytic enzymes in plant tissue maceration varies from one host-pathogen interaction to another **(Kotoujansky, 1987; Barras *et al*., 1994)** and bacterial pectinases may adapt to the host plant pectins resulting in host specificities among strains **(Kotoujansky, 1987)**. The recent proposal of a new genus name, *Pectobacterium*, is probably related to the importance of pectinolytic enzymes in their pathogenicity. Besides cell wall degrading enzymes, other soft rot bacterial virulence factors include toxins, siderophores, hormones and signalling molecules as well as survival structures such as pili, flagella, lipopolysaccharides, exopolysaccharide slime layers, osmoregulated periplasmic glucans, and outer membrane proteins **(Salmond, 1994, Toth *et al*., 2003)**. *E. chrysanthemi* produces antioxidant enzymes in-planta to repair oxidative damage from plant-generated active oxygen species **(Hassouni *et al*., 1999; Toth *et al*., 2003).**

E. chrysanthemi also produces two important siderophores (chrysobactin and achromobactin) that play important roles in its pathogenicity. Chrysobactin functions

in sequestering free Fe necessary for survival and multiplication of the bacteria in the apoplastic fluid of plants after infection and by so doing, weakens the host plant through Fe deprivation, thus rendering it more vulnerable **(Barras *et al*., 1994; Expert, 1999)**. Chrysobactin is also essential for systemic infection by the bacteria **(Barras *et al*., 1994; Expert, 1999)** while achromobactin is necessary for the onset of local infection **(Expert, 1999)**. *E. chrysanthemi* also produces enterobactin ferrichrome receptors that interact with other bacterial Fe carrier molecules than chrysobactin and function in monitoring the composition of the habitat and to weaken potential competitors through Fe deprivation **(Barras *et al*., 1994)**. Fe chelation is further believed to serve in antioxidant defence of *E. chrysanthemi* against oxidative stress from plant-generated oxidative burst in response to attack **(Toth *et al*., 2003)**.

Despite the importance of pectinases in the virulence of *E. chrysanthemi* the nature of their degradation products determines the outcome of the host-pathogen interaction. Pectic oligomers with a low degree of polymerisation would be used for production of inducers of pectinases while those with a high degree of polymerisation would activate plant defence systems like synthesis of phytoalexins, PR proteins (chitinase, β-glucanase) and lignin **(Collmer and Keen, 1986; Barras *et al*., 1994)**. The most important virulence factor of *E. chrysanthemi* lies on its ability to secrete plant cell wall degrading enzymes, key among which are pectinases **(Collmer and Keen, 1986; Kotoujansky, 1987; Barras *et al*., 1994).** *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock was designated as a new species based on strains that had been isolated from *Chrysanthemum morifblium.* Similarities and differences in phenotypic characteristics have been noted for strains of *E. chrysanthemi* from different hosts. Preliminary studies indicated that strains isolated from the same host usually were similar even though the host plants had been collected from different locations. The latter observation suggested that the hosts of *E. chrysanthemi* may be attacked by specific types of the pathogen which might be recognizable by phenotypic characteristics.

Soft-rot *Erwinia* including *Ech*, *Ecc*, and *Eca* are characterized by their ability to produce an array of pectolytic enzymes including pectin methylesterase (PME), polygalacturonase (PG), pectin lyase (PNL), and pectate lyase (PL). The PLs produced by *Ech*, *Ecc*, and *Eca* in cultures are usually present in multiple (3-5)

isozymic forms, which can be readily identified by isoelectric focusing (IEF) gel electrophoresis and overlay enzyme staining techniques **(Ried and Collmer, 1986)**. In *Ech*, a second set of PL isozymes that appear to be inducible only in the presence of plant constituents has been detected by using these techniques **(Kelemu and Collmer, 1993)**. The biological and pathological function of each pectic enzyme produced by soft-rot *Erwinia* has not been fully determined. It also remains unclear if production of certain pectic enzymes is restricted to specific tissues or organs or limited to specific developmental stages in plant. With the aid of molecular genetic technologies, results from previous studies **(Kotoujansky, 1987)** have shown that no single pectic enzyme produced by soft rot *Erwinia* is absolutely required for the pathogen to initiate the disease development. However, the PL isozymes, especially the alkaline PLe, have been repeatedly shown to display the highest degree of tissue-macerating ability *in vitro* and are assumed to be the principal enzyme responsible for the development of soft-rot *in vivo*. Production of PME, *PG* and PNL by soft-rotting bacteria does not appear to play an important role in initiating the disease development but is expected to enhance the survival and proliferation of the bacteria in plants and in natural environments. Inactivation of PME, PG, or PNL genes by marker exchange mutagenesis did not significantly affect the tissue-macerating ability of the mutants **(Collmer and Keen, 1986).** Purified PG by itself is sufficient to induce soft rot of potato tuber slices but not purified PME or PNL. Production of PNL by soft rot *Erwinia* **(McEvoy *et al*., 1990)** and *Pseudomonas* spp**. (Sone *et al*., 1988)** is inducible only by DNA-damaging agents such as UV irradiation and Mitomycin-C treatment. The ecological and pathological importance of PNL production by *Erwinia* and *Pseudomonas* remain obscure and may act as a part of host defence mechanisms against the adverse environmental conditions. The role of PME produced by soft rotting bacteria in soft-rot pathogenesis is also minimal and non-essential. However, it may be possible that a concerted action of PME and PL is required for more efficient degradation of native pectins in plant cell walls.

2.9 Isolation, purification and preservation

Methods of isolation for plant pathogenic bacteria have been recommended by several workers **(Starr, 1947; Elliot, 1951; Society of American Bacteriologist, 1957; Schaad, 1980)**.

2.10 Detection and inspection

Since soft rots and particularly wilts are not symptoms specific to *E. chrysanthemi*, the identity of the causal bacterium must be checked. Latent infections can be detected in cuttings or tubers. Selective pectate media have been devised for specific isolation of pectolytic erwinias **(Van Vuurde and Roozen, 1990)**. Further characterization is needed to reach the species level. Tolerance to temperature and erythromycin may be used for direct differential isolation **(Pérombelon and Hyman, 1986)** but with precautions **(Janse and Spit, 1989)**. Antisera and ELISA kits are commercially available to detect *E. chrysanthemi*. The antibodies are generally directed against O-serogroup 1, recognizing only 68% of the strains **(Samson *et al.*, 1990)**. Inoculated artificially into aubergines, *E. chrysanthemi* from potatoes can cause symptoms resembling those caused by *Clavibacter michiganensis* subsp. *sepedonicus* **(Persson and Janse, 1988)**.

2.11 Taxonomy

The soft rot erwiniae are members of the Enterobacteriaceae, along with other plant pathogens such as *Erwinia amylovora* and human pathogens such as *Escherichia coli*, *Salmonella* spp. and *Yersinia* spp. Although the genus name *Erwinia* is most often used to describe the group, an alternative genus name *Pectobacterium* was recently proposed for the soft rot species. The genus *Erwinia* was first described in 1917 to encapsulate all members of the Enterobacteriaceae that cause disease on plants, irrespective of their relatedness to other members of the family **(Pérombelon, 1990)**. Identification of pectolytic erwinias is traditionally based on biochemical and phenotypic characteristics **(De Boer and Kelman, 2000)**, and more recently molecular techniques have also been applied. As a result, over the years this has caused many nomenclatural difficulties and has led to the relocation of various species into other genera, notably *E. stewartii* to *Pantoea stewartii* **(Mergaert *et al.*, 1993)**, *E. herbicola* to *Pantoea agglomerans* **(Gavini *et al.*, 1989)**, *E. dissolvens* to *Enterobacter dissolvens* **(Brenner *et al.*, 1986)** and *E. salicis* to *Brenneria salicis* **(Hauben *et al.*, 1998)**. It has also been suggested by **Hauben *et al.*, (1998)**, on the basis of 16S rDNA sequence analysis, that the soft rot erwinias be renamed *Pectobacterium carotovorum* sp. *atrosepticum* (for *Eca*), *Pectobacterium carotovorum* sp. carotovorum (for *Ecc*) and

Pectobacterium chrysanthemi (for *Ech*), supporting an earlier proposal by **Waldee (1945)** to rename the group similarly. However, at present '*Pectobacterium*' has not been widely adopted by the '*Erwinia*' research community. The name *Erwinia chrysanthemi* is given precedence here solely on the basis of greater usage within the scientific literature. **Gardan *et al.* (2003)** elevated three subspecies of *Pectobacterium carotovorum* to species level (*P. atrosepticum*, *P. betavascularum* and *P. wasabi*). Presently, the taxonomy of *Erwinia* is in a state of flux **(Yap *et al.*, 2004)**, and the proposed name *Pectobacterium* by **Hauben *et al.* (1998)** has not yet been accepted by many researchers working with this group of bacteria.

2.11.1 Taxonomic position

Kingdom	Bacteria
Phylum	Protobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Erwinia*
Species	*chrysanthemi* **Burkholder *et al.*, 1953**

Synonyms: *Pectobacterium parthenii* var. *dianthicola*, *Pectobacterium parthenii* var. *chrysanthemi*, *Pectobacterium parthenii*, *Pectobacterium chrysanthemi* = *Dickeya chrysanthemi*, *Pectobacterium chrysanthemi* **(Burkholder *et al.*, 1953) Brenner *et al.*, 1973** (Approved Lists 1980) emend. **Hauben *et al.*, 1998.,** *Pectobacterium chrysanthemi*, *Pectobacterium cartovorm* f. sp. *chrysanthemi*, *Pectobacterium carotovorum* var. *chrysanthemi*, *Erwinia chrysanthemi* **Burkholder *et al.* 1953** (Approved Lists 1980), *Erwinia carotovora var. chrysanthemi*, *Dickeya chrysanthemi* **(Burkholder *et al.*, 1953) Samson *et al.*, 2005**, *Dickeya chrysanthemi*, "*Pectobacterium parthenii*" **Hellmers 1958**, "*Pectobacterium parthenii* var. *chrysanthemi*" **(Burkholder *et al.*, 1953) Hellmers 1958**, "*Pectobacterium cartovorum* f. sp. *chrysanthemi*" **Dowson 1957**, "*Pectobacterium carotovorum* var. *chrysanthemi*" **(Burkholder *et al.*, 1953) Graham and Dowson 1960**, "*Erwinia carotovora* var. *chrysanthemi*" **(Burkholder *et al.*, 1953) Dye 1969**.

2.12 Characterization of test bacterium

2.12.1 Biochemical and physiological characterization

The isolation of soft rot bacterium becomes difficult as large number of saprophytes invade the rotten tissue. In order to get rid of these saprophytes, various investigators have tried certain specific media and techniques **(Friedman, 1964, Berhares, 1968 and Cother, *et al*., 1980)**. A medium containing tetrazolium salts, used for differentiating mutants/ isolates have been modified and used to differentiate pathogenic isolates of *Pseudomonas solanacerum* **(Kelman, 1954)** on this medium. *E. chrysanthemi* pv *zeae* had been shown to be differentiated from virulent ones depending on the colony with deep red centre and narrow colourless border **(Thind and Payak, 1979)**.

The selectivity and differentiating abilities of most media are based on preventing or retarding the growth of cells by using inhibitors of metabolic pathways and antibiotics that suppress the proteins and nucleic acid synthesis. **Kodo and Heskett (1970)** designated five selective media to isolate the bacterium from different plant pathogenic genera with accuracy and most efficiently from soil and infected plant parts. Pectolytic soft rot *Erwinia* species are capable of producing cell wall-degrading pectolytic (pectinase) enzymes which enable the bacteria to macerate the parenchymatous tissues of their plant hosts. Thus, the virulence of the bacteria depends largely on the ability of the species or strains to secrete enough of the pectic enzymes **(Boccara *et al.*, 1988)**. The breaking down of pectin or polypectate by pectic enzymes produced by the bacteria have been utilized as the basic principle in the development of most selective media for detection of the bacteria from samples **(Graham 1958; Cuppels and Kelman 1974; Stewart 1962; Perombelon and Burnett 1991)**. The crystal violet-sodium polypectate medium (CVP) developed by **Cuppels and Kelman (1974)** based selective medium (CVP) for isolation of erwinias dependent on the quality of the polypectate used seemed to be the best selective medium developed for detection and enumeration of the soft rot erwinias **(Pierce and McCain, 1992)**. The superiority of the medium was based on high selectivity, good recovery and the formation of characteristic deep cavities on the medium by the soft rot erwinias. Unfortunately, the lack of sodium polypectate from Sunkist (Sunkist

Ltd., California, USA), which was used in the original preparation, led to the use of sodium polypectate from other sources. Results were, however, not satisfactory in many cases due either to poor gelling quality, or the bacteria were unable to break down the polypectate **(Cother, 1980)**. The isolation of *Erwinia spp.* using the selective media is based on the ability of the pathogen to hydrolyze polypectate and to form typical cup-shaped pits (cavities) in the medium. As was shown by **Perombelon (1971)**, other pectolytic bacteria occurring in the soil, especially certain pseudomonads, also form cavities, but these are shallow and quite distinct from those caused by varieties of *E. carotovora,* which are easily recognized even when there is overcrowding by other organisms. All the pectolytic isolates are currently being characterized using physiological and biochemical tests selected according to keys of **Buchanan and Gibbons (1974), Bradbury (1988), Holt *et al*. (1994) and Schaad *et al*. (2001)**.

2.11.2 Molecular characterization

Traditionally, the characterization and identification of pectolytic Erwinias are based on biochemical and phenotypic characteristics **(De Boer and Kelman, 2000)** and recently molecular techniques are also applied **(Baghee-Ravari *et al*., 2011)**. The most commonly used methods are biochemical tests **(Dickey and Kelman, 1988)** and pathogenicity profiling **(Smith and Bartz, 1990)**. Previous attempts to group *E. chrysanthemi* strains on the basis of host range or on the basis of physiological, biochemical, and serological properties, have shown that there is a great deal of variability in this species **(Zaidi *et al.*, 2002).** Phenotypic studies using biochemical and physiological methods on soft rot Erwinias have been and are still undertaken in parallel to other methods to identify and characterize the group **(Dye, 1968, 1969; De Boer *et al*., 1987; Toth *et al*., 1999; Seo *et al*., 2003; Yahiaoui *et al*., 2003, Rahman *et al*., 2012)**. Fatty acid analysis proved to be a reliable and accurate method to identify many organisms, including soft rot bacteria, and could distinguish sub-species **(Persson and Sletten, 1995; Seo *et al*., 2002)**. However, these molecular tools which have been used previously can be used only with purified bacteria. Thus, a tool to specifically identify *E. chrysanthemi* that takes into account the previously reported diversity is still needed. Several studies have shown that the pectinases,

particularly the pectate lyases (PL), are involved in the phytopathogenicity of *E. chrysanthemi* **(Barras *et al*., 1977; Boccara *et al*., 1991; Kotoujansky, 1987)**. Electrofocusing of PL and RFLP analyses of PL-encoding genes (*pel*) both revealed polymorphism that might be used to differentiate *E. chrysanthemi* strains **(Boccara *et al*., 1991; Ried and Collmer, 1986)**. Furthermore, PCR-amplified fragments of *pel* genes were used recently to differentiate *Erwinia carotovora* subspecies in an RFLP analysis of the amplified fragments. **Nassar *et al.* (1996)** successfully developed a procedure for the identification of *E. chrysanthemi* using species specific PCR primers (ADE1 (5'-GATCAGAAAGCCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3') derived from *pel*ADE, which are pectate lyase encoding genes of *E. chrysanthemi.* Molecular methods has been used to identification of the *E. chrysanthemi* strains using the primer pairs (ADE1 and ADE2) directed toward a species-specific region of the pelADE genes by several other workers **(Henz *et al*., 2006; Kaneshiro *et al*., 2008; Czajkowski *et al*., 2009; Diallo *et al*., 2009; Keith and Sewake, 2009; Tsror *et al*., 2009;)** and ERWFOR (5'-ACGCATGAAATCGGCCATGC-3') and CHRREV (5'-AGTGCTGCCGTACAGCACGT-3'), directed toward a species-specific region of the 16S rRNA gene **(Smid *et al*., 1995, Keith and Sewake, 2009).**

2.13 Diversity analysis: Genomic fingerprinting

Genetic diversity at the subspecific level is recognized in *E. carotovora* serotypes based on differences in the lipopolysaccharide O antigen **(De Boer *et al*., 1979)** and in differences in the O and H antigens of *E. chrysanthemi* **(Janse and Ruissen, 1988)**. Additional subspecific diversity is evident in discriminatory phage sensitivity **(Toth *et al*., 1999)**, and banding patterns achieved by PCR–RFLP protocols **(Boccara *et al*., 1991; Nassar *et al*., 1996; Waleron *et al*., 2002)**. There are also significant phenotypic differences among strains within subspecies**.**

In recent years, improved genomic fingerprinting protocols have revealed hitherto unrealized levels of genetic diversity within populations of bacteria. Application of PCR with specific primers is a notable example. Among the techniques frequently applied to type bacteria, the repetitive element polymorphism (rep)-PCR (**Versalovic *et al*., 1991; Martin *et al*., 1994; Versalovic *et al*., 1994**)

can be used to identify species-specific markers. Characteristic prokaryotic repeats such as the enterobacterial repetitive intergenic consensus (ERIC) sequences and the repetitive extragenic palindrome (REP) sequence motif have been found in numerous different enterobacterial species as well (**Versalovic *et al*., 1991)**. The REP sequence element was discovered in 1982 **(Higgins *et al*., 1982)**, whereas the ERIC motifs, initially named intergenic repeat units, were described in detail in 1990 **(Gilson *et al*., 1990)**. In the early 1990s, another dispersed-repeat motif was identified in *Streptococcus pneumoniae*. This is so-called BOX Repeat **(Martin *et al*., 1992)**. All of these prokaryotic moieties represent repetitive DNA that is not organized in tandem repeats but is scattered throughout the entire genome of microorganisms. Repetitive Extragenic Palindromic Polymerase Chain Reactions (rep-PCR) fingerprints bacterial genomes based on strain-specific patterns derived from PCR amplification of repetitive DNA elements present within bacterial genomes. The palindromic nature of repetitive elements (ie. BOX, ERIC and REP primers) across the microbial genome and their ability to form stem-loop structures leads to the generation of unique fingerprint patterns. Microbial fingerprinting is extensively used in diagnostic bacteriology (**Oyarzabal *et al*., 1997**), in ecological and evolutionary genetical studies **(Van Belkum *et al*., 2001)** and in search and discovery programmes designed to detect new microbial products **(Goodfellow and O'Donnell, 1989; Bull *et al*., 1992, 2000)**.

rep-PCR genomic fingerprinting, a DNA amplification based technique, which has been found to be extremely reliable, reproducible, rapid and highly discriminatory **(Versalovic *et al*., 1994; Louws *et al*., 1996)**. The rep-PCR primer targets to conserved motifs in bacterial repetitive elements scattered in the whole genome, referred to as enterobacterial repetitive intergenic consensus (ERIC) (**Hulton, 1991**), repetitive extragenic palindromic (REP) (**Gilson, 1984**), and BOX (**Martin, *et al*., 1992**), producing a PCR product profile which is generally specific to a given strain. rep-PCR is a fingerprinting technique that uses repetitive sequences (mostly of unknown function, that are interspersed throughout the DNA) present in the genomic DNA of bacteria. For example, repetitive palindromic units are present at about 500-1000 copies in the DNA of *Escherichia coli* and *Salmonella typhimurium*. REP-PCR uses 35-40 bp repetitive extragenic palindromic sequences,

BOX-PCR uses the 154 bp so-called BOX element and ERIC-PCR uses 124-127 bp enterobacterial repetitive intergenic consensus sequences. More primer sets against different repetitive elements have been developed. Which method yields the most discriminative patterns has yet to be determined empirically. Several families of repetitive sequences are interspersed throughout the genome of diverse bacterial species **(Lupski and Weinstock, 1992)**. Three families of repetitive sequences have been studied in most detail, including the 35-40 bp repetitive extragenic palindromic (REP) sequence, the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and the 154 bp BOX element **(Versalovic *et al.*, 1994)**. These sequences appear to be located in distinct, intergenic positions all around the chromosome. The repetitive elements may be present in both orientations on the chromosome, and PCR primers have been designed to "read outward" from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX **(Versalovic *et al.*, 1994)**. The use of the above primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX sequences. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR, respectively, and rep-PCR collectively **(Versalovic *et al.*, 1991; 1994)**. rep-PCR is discriminative at very low taxonomic level, usually strain level, and is very useful in epidemiological and environmental studies tracking of strains. It is plasmid-DNA independent. The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint **(Versalovic *et al.*, 1994)**. These fingerprints resemble "bar code" patterns analogous to UPC codes used in grocery stores **(Lupski 1993)**. rep-PCR genomic fingerprinting makes use of DNA primers complementary to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes of most Gram-negative and several Gram-positive bacteria **(Lupski and Weinstock, 1992)**. The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies and strain level. Rep-PCR genomic fingerprinting protocols have been applied successfully in many medical, agricultural, industrial and environmental studies of microbial diversity **(Versalovic *et al.*, 1994)**. In addition to studying diversity, rep-PCR genomic fingerprinting has become a valuable tool for the identification and classification of bacteria, and for

molecular epidemiological studies of human and plant pathogens **(Van Belkum *et al.*, 1994; Louws *et al.*, 1996; Versalovic *et al.*, 1997).** rep-PCR primers complement these repetitive sequences and allow for specific binding providing reproducible, unique rep-PCR DNA fingerprint patterns. The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of *Escherichia coli* and *Salmonella*. The family of REP elements is generally between 33 and 40 bp in length, has 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* or *Salmonella*. The amplified DNA fragments, when separated by electrophoresis, constitute a genomic fingerprint that can be employed for subspecies discrimination and strain delineation of bacteria and fungi. The application of rep-PCR to microbes has proven a discriminatory and reproducible tool for microbial subtype analyses and for microbial ecology investigations.

Versalovic *et al.* (1994) elegantly discusses both the theoretical and technical aspects of repetitive sequence-based PCR in which REP, ERIC, or BOX motifs are used to delineate strain relatedness. It was demonstrated that due to the specificity of the repeat PCRs, crude cell lysates can be used as amplification templates. Moreover, even material directly derived from infections can be used immediately without a bacterial cultivation step. Theoretically, this enables bacterial detection and typing in a single PCR test. Amplicons derived from repeat PCRs show a very high degree of variability, but some of the fragments have been successfully used as species-specific DNA probes **(Giesendorf *et al.*, 1993).** Linkage between rep-PCR results and the geographic origin of bacterial strains has been recognized in various studies **(Scortichini *et al.*, 2001; Mkandawire *et al.*, 2004). Louws *et al.* (1994)** believe that one of the important reasons for this phenomenon is that the selection for one geographically suitable area can have influence on the genetic map of bacterium and also dispersion of these repetitive units in the genome of bacterium.

2.14 Management

2.14.1 Biological control

Pesticides present the only group of chemicals that are purposely applied to the environment with aim to suppress plant and animal pests and to protect

agricultural and industrial products. However, the majority of pesticides is not specifically targeting the pest only and during their application they also affect non-target plants and animals. Repeated application leads to loss of biodiversity. Many pesticides are not easily degradable, they persist in soil, leach to groundwater, surface water and contaminate wide environment. Depending on their chemical properties they can enter the organism, bioaccumulate in food chains and consequently influence also human health.The use of chemical pesticide and other agro chemicals are getting reduced/being banned globally because of their toxic effects on human beings and his live stock, residual toxicity, environmental problems, pest out-breaks and drastic effects on beneficial insects. Therefore, now it is imperative to develop a holistic system of tackling pests to make it more eco-friendly, economically viable and socially acceptable for the farmers. In the WTO regime, it is absolutely necessary to limit the usage of chemicals, to remain in the world market and sustain the competition. In this regard to tackle the major pests and diseases of major crops the biological control approaches are gaining momentum.

Overall, intensive pesticide application results in several negative effects in the environment that cannot be ignored. Increasing concern regarding food safety and environmental pollution, as well as legislative pressures to reduce the number of approved active pesticide ingredients, has generated an interest in compost and other biological control agents to prevent and control plant diseases. Several fungal and bacterial biocontrol agents have been used for achieving disease control of various plant species. Among fungi *Trichoderma* is most researched group and has been found to be useful against aerial, roots and soil pathogens **(Weller, 1988; Van Loon *et al*., 1998; Herman *et al*., 2004)**. Protection of root from infection of the fungus with microbial inoculants is targeted for eco-safe management of this disease. Biological suppression has proved to be a reliable component of integrated disease management of phytopathogenic fungi following greenhouse **(Paulitz and Belanger, 2001)** and field demonstration of several biocontrol agents. Among the bacterial biocontrol agents, fluorescent *Pseudomonas* spp. have emerged as the largest and potentially promising group of rhizobacteria involved in the biocontrol of plant diseases **(O'Sullivan and O'Gara, 1992)**. These bacteria are ideally suited as soil inoculants because of their rapid and aggressive colonization of the root. This

feature alone is suggested as a disease control mechanism by preventing the invasion of detrimental soil microorganisms onto the root surface **(Altman, 1970)**. *Pseudomonas florescens* species produce antibacterial and antifungal substances **(Burr *et al.*, 1978; Lindberg 1981; Thomashow and Weller 1988; Daniel *et al.*, 1992; Verges and Richard 1994; Rosales *et al.*, 1995)**. It inhibits microorganisms by several mechanisms, for example siderophore production **(Loper and Buy *et al.*, 1991)**, antibiotic production **(Howell and Stipanovic 1979, 1980; Dahiya *et al.*, 1988; Howie and Suslow 1991)** or by the production of the secondary metabolite 2, 4-diacetylpholroglucinol **(Dunne *et al.*, 1996)**. It has been reported to play a role in controlling plant diseases and increasing the growth yields of dome for example, **Burr *et al.*, (1978)** reported that *P. florescens* exhibited antibiosis *in vitro* against *Erwinia carotovora* and rapidly colonized roots of potato seed pieces and increased crop yield in field trials. Secondary metabolites like antibiotics, cyanide and siderophores have also been reported to be associated with the antagonism exerted by fluorescent *Pseudomonas* spp. It should be recognized, however, that there are problems of variable results obtained in different soil types **(Burr and Caesar, 1984; Hase *et al.*, 2001)** and the inadequate survival of strains **(Hase *et al.*, 2000)** on seeds prior to planting, preventing the commercial use of many of these biological control agents. Various scientists from across the world have reported the non-pathogenic fluorescent pseudomonads as effective biological agents against species of *Erwinia* and other pathogens **(Singh and Sinha, 2005)**. **Shanmugam *et al.* (2003)** identified that the mixture of *Pseudomonas fluorescence* and *P. putida* was more effective than either *Pseudomonad* alone in inhibiting the growth of *Erwinia* spp. Using composts in agriculture to minimize organic wastes and to reduce the addition of fertilizers and fungicides in crop production is highly effective. The use of compost as a peat substitute to control root pathogens was first suggested by **Hoitink *et al.* (1975)**. Since then, several soil-borne plant pathogens have been reduced by using composts made of different raw materials **(Borrero *et al.*, 2004; Cotxarrera *et al.*, 2002; Hoitink and Boehm, 1999; Hoitink and Fahy, 1986; Litterick *et al.*, 2004)**. However, the capacity of composts to suppress *Rhizoctonia solani*, a pathogen that affects both seedlings and adult plants of many species, remains limited **(Hoitink and Boehm, 1999; Scheuerell *et al.*, 2005)**. The capacity of certain

composts to repress *R. solani* may be due to the presence and activity of specific antagonists **(Kuter *et al*., 1983; Scheuerell *et al*., 2005; Tuitert *et al*., 1998)** and depends on the degree of compost decomposition **(Hoitink and Boehm, 1999)**. Moreover, matured composts can sustain biological control agents, whereas immature composts do not support them, negatively affecting the growth of crop plants and possibly containing pathogen populations **(De Ceuster and Hoitink, 1999; Litterick *et al*., 2004)**.

2.14.2 Vermicompost

Chemical fertilizers and pesticides decrease soil fertility and cause health problems to the consumers. An excessive reduction in soil biodiversity, especially the loss of species with key functions, may result in severe effects including the long-term degradation of soil and the loss of agricultural productive capacity. Soil health and soil quality are fundamental to the sustained productivity and viability of agricultural systems worldwide. Efforts to increase soil quality remain the most attractive approach for reducing damage from root diseases, and there is agreement that the use of cover cropping and green manuring will, in time, provide suppression of soil pathogens **(Stone *et al*., 2004; Van Bruggen and Termorshuizen, 2003, Zaidi, 2004.**). However, the time required is dependent on several factors including choice of cover crop, rate of residue decomposition, soil type, and pathogen pressure. This lag period can vary considerably and may be costly for both small and large growers. Several studies have demonstrated strong correlations between earthworm densities and parameters that define the physical and biological health of soil **(Edwards, 1998)**. The utilization of organic wastes through earth-worms is called vermicomposting. Vermicompost is produced by biodegradation of organic material through interactions between earthworms and micro-organisms. It is a technical method of producing compost in an ecologically sound, economically viable and sociologically acceptable manner. Although **Darwin (1881)** first drew attention to the great importance of earthworms in the decomposition of dead plants and the release of nutrients from them, it was necessary to wait more than a century until this was taken seriously as a field of scientific knowledge or even a real technology. Vermicomposting is a mesophilic bio-oxidative process in which

detritivorous earthworms interact intensively with microorganisms and soil invertebrates within the decomposer community, strongly affecting decomposition processes, accelerating the stabilization of organic matter, and greatly modifying its physical and biochemical properties **(Edwards and Bohlen, 1996; Dominguez, 2004; Edwards *et al*., 2004)**. Physical assessments of soil quality including bulk density, pore size, water infiltration rate, soil water content, and water-holding capacity are improved by earthworm activity **(Edwards and Arancon, 2004)**. Many consider earthworm numbers to be a reflection of soil quality **(Buckerfield, 1997; Muys and Granval, 1997)**. Additionally, studies have determined that earthworms serve crucial roles in other areas essential to healthy agroecosystems **(Edwards and Arancon, 2004; Edwards and Bohlen, 1996)**. High earthworm populations increase nutrient availability **(Devliegher and Verstraete, 1997; Subler, *et al.*, 1997)** and produce plant growth **hormones (Atiyeh, *et al*., 2002; Canellas *et al*., 2002)**. **Scheu (2003)** reviewed 67 earthworm studies and found that 79% reported increased plant biomass in the presence of earthworms. The deep burrows made by the earthworms, *Lumbricus terrestris* (Canadian night crawler) and *L. rubellus* (Red earthworm), break up hardpans in poorly drained soils, promote aggregate structure, and facilitate percolation **(Ehlers, 1975; Joschko *et al*., 1982)**. Earthworms also detoxify soil by aiding in pesticide degradation, soil remediation, and land restoration **(Edwards and Arancon, 2004)**. Microorganisms produce the enzymes that cause the biochemical decomposition of organic matter, but earthworms are the crucial drivers of the process as they are involved in the indirect stimulation of microbial populations through fragmentation and digestion of fresh organic matter, which results in a greater surface area available for colonization, thus dramatically increasing microbial activity. Porosity, drainage, water holding capacity and microbial activity are high in vermicompost. Earthworms also modify microbial biomass and activity through stimulation, digeston, and dispersion in the casts and interact closely with other biological components of the vermicomposting system, thereby affecting the structure of the microflora and microfuna communities **(Dominguez *et al*., 2003; Lores *et al*., 2006)**. Vermicompost can supply the full requirement of major nutrients for plant growth *i.e.* N, P, K; secondary nutrients *i.e.* Ca, Mg, S and also micro nutrients *i.e.* Cu, Zn, Mn and Fe. The average nutrient content of vermicompost is

much higher than in the other types of compost **(Singh, 1996).** On the other hand vermicompost is a rich source of vitamins and growth hormones like gibberellins which regulate plant growth. **Fragoso *et al*. (1996)** reported that single earthworm has to digest organic matter up to 5-30 times of its body weight per day in order to enhance the growth of beneficial soil bacteria which are the most divers and speediest agents for decomposing organic matter. At present information on the role of local earthworm fauna in soil fertility improvement is scanty and inconclusive. Earthworms vermicompost is proving to be highly nutritive 'organic fertilizer' and more powerful 'growth promoter' over the conventional composts and a 'protective' farm input (increasing the physical, chemical & biological properties of soil, restoring and improving its natural fertility) against the 'destructive' chemical fertilizers which has destroyed the soil properties and decreased its natural fertility over the years. Vermicompost retains nutrients for long time and while the conventional compost fails to deliver the required amount of macro and micronutrients including the vital NPK to plants in shorter time, the vermicompost does.

Vermicompost is a nutritive 'organic fertilizer' rich in NPK (nitrogen 2-3%, phosphorus 1.55-2.25% and potassium 1.85-2.25%), micronutrients, beneficial soil microbes like 'nitrogen-fixing bacteria' and 'mycorrhizal fungi' and are scientifically proving as 'miracle growth promoters and protectors' **(Sinha *et al*., 2009)**. **Kale and Bano (1986)** reported as high as 7.37% nitrogen (N) and 19.58% phosphorus as P_2O_5 in worm's vermicast. **Suhane (2007)** showed that exchangeable potassium (K) was over 95% higher in vermicompost. There are also good amount of calcium (Ca), magnesium (Mg), zinc (Zn) and manganese (Mn). Additionally, vermicompost contains enzymes like amylase, lipase, cellulase and chitinase, which continue to break down organic matter in the soil (to release the nutrients and make it available to the plant roots) even after they have been excreted. **(Chaoui *et al*., 2003; Lunt and Jacobson, 1994; and Tiwari *et al*., 1989)**. Annual application of adequate amount of vermicompost also lead to significant increase in soil enzyme activities such as 'urease', 'phosphomonoesterase', 'phosphodiesterase' and 'arylsulphatase'. The soil treated with vermicompost has significantly more electrical conductivity (EC) and near neutral pH **(Tiwari *et al*., 1989)**.

Vermicompost has very 'high porosity', 'aeration', 'drainage' and 'water holding capacity'. They have a vast surface area, providing strong absorbability and retention of nutrients. They appear to retain more nutrients for longer period of time. Study showed that soil amended with vermicompost had significantly greater 'soil bulk density' and hence porous and lighter and never compacted. Increase in porosity has been attributed to increased number of pores in the 30-50 μm and 50-500 size ranges and decrease in number of pores greater than 500 μm **(Lunt and Jacobson, 1994; Nighawan and Kanwar, 1952)**. It has been found to influence on all yield parameters such as-improved seed germination, enhanced rate of seedling growth, flowering and fruiting of major crops like wheat, paddy, corn, sugarcane, tomato, potato, brinjal, okra, spinach, grape and strawberry as well as of flowering plants like petunias, marigolds, sunflowers, chrysanthemums and poinsettias. In all growth trials the best growth responses were exhibited when the vermicompost constituted a relatively small proportion (10%-20%) of the total volume of the container medium. **Suhane *et al*., (2008)** asserts that vermicompost is at least 4 times more nutritive than cattle dung compost. Application of vermicompost @ 25 quintal/ha in farm wheat crops supported yield better than chemical fertilizers. It was 40 quintal/ha on vermicompost and 34.2 Q/ha on chemicals. And when same amount of agrochemicals were supplemented with vermicompost the yield increased to about 44 Q/ha which is over 28% and nearly 3 times over control. On cattle dung compost applied @ 100 Q/ha (4 times of vermicompost) the yield was just over 33 Q/ha. Application of vermicompost had other agronomic benefits. It significantly reduced the demand for irrigation by nearly 30-40%.

2.14.2.1 Suppression of soil borne plant diseases by vermicompost

Suppression of soil borne diseases has been reported for several kinds of composts. It has been demonstrated to reduce bacterial disease and anthracnose on fruit and increased yield in organically-produced tomatoes produced in soil amended with compost. Vermicomposts has been widely used in agriculture not only for its beneficial effects on soil structure and biota but also for its ability to inhibit plant pathogens, for example, **Asciutto *et al*. (2006)** reported that vermicompost (25%) controlled *Rhizoctonia solani* that causes damping off in Patience-plant (*Impatiens*

walleriana). The suppression of disease caused by soil borne pathogens on application of vermicompost has been reported **(Somshekara *et al,* 2001)**. Several other studies have been reported the inhibition of plant pathogens by earthworm secretions. **Reiten and Salter (2002)** have reported strong inhibition for *X. campestris pv. carotovora* using compost tea under *in-vitro* conditions. Their studies indicate that compost tea can be used to control *X. campestris pv. carotovora* both in the laboratory studies as well as in the field. **Shobha and Kale (2008)** have reported that gut and body wall extracts had both antibacterial and antifungal activities by forming total inhibition zones, whereas coelomic fluid was found to be only antibacterial at the used concentrations under *in-vitro* conditions. Body wall and gut extracts were found to be inhibitory to *Xanthomonas campestris, Ralstonia solanacearum, Erwinia carotovora, Fusarium oxysporum* and *Botryodiplodia theobromae*. Colemic fluid was inhibitory to *X. campestris* and *E. carotovora.* There was no inhibitory effect recorded by any of the extracts at the used concentrations during their study on *Rhizactonia solani, Alternaria solani* and *Sclerotium rolfsii.* Only delayed sporulation was observed in all the cases, but this was only a visual observation. The mixed extract when tested on *F. oxysporum* has shown a strong clear inhibition zone. **Hudson and Berman (1994)** have reported strong suppression of *Rhizoctonia,* on application of compost to the soil. **Khalifa (1965)** reported suppression and control of *Fusarium* using compost. Body wall and gut extracts have showed inhibitory effect on *F. oxysporum* in studies. Apart from the soil borne plant pathogens, animal fungal pathogens *Candida albicans, Cryptococcus neoformans* and *Trycophytan metagrophyte* **(Subhashini, 2005)**, were also found to be inhibited by the body wall, gut and coelomic extracts. **Reddy *et al*. (2012)** have found that soil application of vermicompost coupled with seed treatment with 10% aqueous extract of vermicomposted neem for one hr can significantly reduce the incidence of bacterial spot caused by *X. campestris* in tomato. **Hameeda *et al*. (2007)** shown significant improvement in shoot length (1-12%), leaf area (20-34%), plant biomass (9-27%) and root volume when vermicompost was applied in sorghum field. Further, vermicompost application also suppresses the growth of many fungi, like *Pythium, Rhizoctonia* and *Verticillium*, as a result, many plant diseases are suppressed when vermicompost is applied in ample quantity in the field **(Hoitink and Fahy, 1986)**.

Sometimes, vermicompost also controls the population of plant parasitic nematodes **(Johnston *et al*., 1995; Arancon *et al*., 2006)**. More importantly, research conducted in Australia has shown that earthworms were associated with decreased incidence of field diseases of clover, grains, and grapes incited by *Rhizoctonia* spp. **(Stephens and Davoren, 1997)** and *Gaeumannomyces* spp. **(Clapperton *et al*., 2001; Davoren, 1994)**. **Edwards and Arancon (2004)** have shown that vermicomposts, an end-product of the breakdown of organic matter by earthworms, are disease suppressive. Earthworm castings are rich in nutrients and support a diverse microbial community **(Lunt and Jacobson, 1944; Parle, 1963)**. Castings are also rich in calcium humate, a binding agent **(Edwards and Arancon, 2004)** that reduces desiccation of individual castings and favors the incubation and proliferation of beneficial organisms, such as *Trichoderma* spp. **(Tiunov and Scheu, 2000)**, *Pseudomonas* spp. **(Schmidt *et al*., 1997)**, and mycorrhizal spores **(Doube *et al*., 1995, Gange, 1993)**. **Clapperton *et al*. (2001)** showed that earthworms increase communities of gram negative bacteria and concluded that any disease suppression was mediated by enhancing beneficial microbes. Although other microbial communities have been associated with disease suppression, such as filamentous actinomycetes **(Postma *et al*., 2005)** and Mn-reducing microbes **(Elmer, 1995**; **2003)**, the effect of earthworms on these communities has not been studied. Several reports that vermicompost extracts are effective antimicrobial agents against soil-borne pathogens **(Szczech *et al*., 1993; Orlikowski, 1999; Rodriguez *et al*., 2000; Szczech and Smolinska, 2001; Edwards and Arancon, 2004; Zaller, 2006)** and do not produce any residual effects.

2.14.3 Chemical control

In vitro screening for the efficacy of different agrochemicals (copper bactericides, antibiotics and oxolinic acid) has been found effective to inhibit the growth of the bacterium *Erwinia* sp. Among them, oxylinic acid was the most effective **(Hseu, *et al*., 2008)**. Seed, soil or foliage treatment with chemicals has not been found effective in controlling Erwinia soft rot diseases **(Christensen and Wilcoxan, 1966)**. In India, a large number of chemicals have been evaluated against the soft rot bacteria both *in vitro* and *in vivo*. A good number of antibiotics,

dithiocarbamates, sulphur fungicides and potassium permanganate have shown inhibitory effects in vitro **(Sabet, 1956; Thompson, 1965; Chakravarti and Rangrajan, 1966; Rangrajan and Chakravarti, 1969; Rangarajan and Chakravarti, 1970; Thind and Payak, 1972; Alberghina, 1974; Shina and Prasad, 1977, Randhawa *et al*., 1979)**. **Singh *et al.* (1980)** tested, antibiotics, fungicides and stable bleaching power against *Erwinia carotovora* subsp. *carotovora* in vitro, and found complete inhibition of bacterium by Streptomycin. **Sani and Prashar (1981)** found that 1000 ppm concentration of stable Bleaching Power was inhibitory against *Erwinia carotovora* subsp. *carotovora*. This pathogen was also found sensitive to Streptomycin, Tetracyclin, Ampicllin and Chloramphenical but not Penicillin **(Mahmoud, *et al*., 1981)**. Streptocyclin alone and with Blitox-50 WP was found to be most effective against stalk rot of maize in glasshouse. Soil drenching with stable bleaching power was recommended in standing crop **(Lal and Saxena, 1979; Thind and Payak, 1984). Hepperly and Davila (1987)** tested 22 antibiotics against Erwinia isolate of sorghum *in vitro* and *in vivo*, and reported that Tetracyclin, Gentamycin and Chloramphanicol showed sensitivity, but antibiotics of penicillin group appeared insensitive. Sensitivity of *Erwinia chrysanthemi* to Erythromycin also has been recommended as the diagnostic criteria to distinguish it from other *Erwinia* species **(Sradbury, 1977)**.

Materials and Methods

3.1.1 Sample collection

On the basis of visual observation, infected plants with typical soft rot symptoms, samples were collected from 37 different locations of Tarai region of district U.S. Nagar (Uttarakhand) in the growing season 2010-11. From each location, at least 5 rotted plants were collected randomly in "W" shaped pattern from the field. Samples were kept in polythene bags labeled for specific location and then these samples were kept in refrigerator at 4 °C and isolation of suspected bacterium was made for confirmation.

3.1.2 Isolation of pathogen

Isolation of bacterium was done as per the method described by **Janse (2005)**. Pieces of tissues taken from the margin of healthy and diseased tissue were briefly disinfected with 70% alcohol and placed in a tube with sterile water. Tissue were left for 30 min in suspension so that the bacteria could diffuse out of the tissue. Subsequently 100 µl of the suspension was plated onto Nutrient Agar medium.

3.1.3 Purification and preservation of bacterium

Purification of bacterium was done on yeast dextrose calcium carbonate medium (YDC) by streaking freshly growing single colony from CPV medium and incubated at 28°C for five days. *Ech* isolates were stored in NA slants at 4ºC.

3.2 *In vitro* evaluation

3.2.1 Biochemical and physiological characterization of *Ech* isolates

3.2.1.1 Morphological

3.2.1.1.1 Colony characters on culture medium

3.2.1.1.1.1 Logan's medium

The bacterial isolates were transferred onto Logan's medium by streaking bacterial suspension of 10^4 cfu/ml, and incubated at 27 °C for 24 h. After incubation *Ech* shows dark red colonies with a red centre. The experiment was replicated thricely.

3.2.1.1.1.2 YDC

The slanted surface of the test medium YDC was streak-inoculated with a loopful of the inoculum and incubate at 27 ºC. Observation on growth and the development of a blue coloration (alkaline reaction) for 7, 14 and 21 days were recorded. A light blue colour is considered to be a doubtful or negative reaction. The experiment was replicated thricely.

3.2.1.1.1.3 NYDA

Streak-inoculated the slanted surface of the test medium NYDA with a loopful of the inoculum and incubate at 27 ºC. Observe for growth and the development of colony for 7 days. The experiment was replicated thricely.

3.2.1.1.1.4 Cell shape and size by SEM study

SEM protocol

1. Centrifugation of bacterial broth at 8000rpm
2. Collect and wash the pellet with phosphate buffer saline for 3 times
3. Added 0.25% gluteraldehyde (in Na-phosphate, pH 7.4) for 1 h
4. Incubate at room temperature for 30 minutes
5. Then overnight incubation
6. Washed with Na- phosphate buffer for 3 times
7. Collection of the pellet by centrifugation
8. Dehydrolysis the sample by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100% and for each ethanol volume incubate for 15 minutes
9. Incubation in 100% ethanol for 1 hour
10. Preparation of SEM stub by applying the adhesive tape and then adding the bacterial sample on the tape.

3.2.2 Biochemical and physiological testing

3.2.2.1 Citrate utilization

The citrate test performed by inoculating 100 µl of a 10^4 cfu/ml of *Ech* into plate containing the Simmon's Citrate Agar medium, where sodium citrate is the only

source of carbon and energy. Then all plates were incubated at 37 °C for 48 hrs. Bromothymol blue is used as an indicator from sodium carbonate an alkaline product, which changes the colour of indicator from green to blue and this constitutes a positive test.

3.2.2.2 3% KOH test (Suslow *et al*., 1982)

Placed approximately 50 µl of 3% (w/v) KOH on a clean glass slide. Aseptically transfered bacterial cells from an agar plate to the drop of KOH with a sterile toothpick. Agitated the cells with the toothpick. The viscosity of the drop increased and a string of "goo" easily picked up with the tip of the toothpick, the cells are gram-negative. If the viscosity does not greatly increase, the cells are gram-positive. The experiment was replicated thricely.

3.2.2.3 Sensitivity to erythromycin at 15µl/ml (Dickey and Kelman, 1988)

Sensitivity of erythromycin at 15µl/ml conc. was determined on Nutrient Agar medium. Sterile paper disk was dipped in erythromycin at 15µl/ml conc. solution and placed aseptically in NA medium. Blank disk which was dipped in sterile water, placed on same plate served as control and Incubated at 27 °C for 48 hrs. The experiment was replicated thricely.

3.2.2.4 Differentiation of *Erwinia* spp. at different temperatures

NA plates were lightly touch spot-inoculated with a 48 h culture using a straight inoculating needle. Plates were also, spread-inoculate with 100 µl of a 10^4 cfu/ml culture using a glass rod spreader. Then plates were incubated at 27, 37 and 39 °C for 48 h and check plates for growth relative to 27 °C. Triplicate plates were evaluated for each temperature.

3.2.2.5 Blue pigment production on NGM medium (Lee and Yu, 2006)

Medium was allowed to cool upto 45 ^{0}C, and poured into Petri dishes under aseptic conditions. A bacterial streak showing a dark brownish to blue color was considered a positive reaction. For observation of colony morphology, 100 µl of bacterial suspension (10^3 CFU/ml) was pipetted onto the surface of the NGM agar plate, and spread evenly with an L-shaped rod. The plate was incubated for 2 to 3 days at 28.8°C with three replications prior to observation. A bacterial streak showing

a dark brownish to blue color was considered a positive reaction. *E. chrysanthemi* is the only species in the genus Erwinia that is able to produce a water-insoluble blue pigment, known as indigoidine **(Starr *et al.*, 1966).** Accordingly, the formation of blue pigment, which imparts a blue color to bacterial colonies, can be used as a chemotaxonomic trait for rapid identification of *E. chrysanthemi*.

3.2.2.6 Dip pit formation on CPV medium

Tests for the development of liquefied pits were observed on CVP (crystal violet pectate) medium as described by **Cuppels and Kelman (1974)**. The water-bacterial suspension of each isolate was uniformly distributed with a glass rod on the surface of medium. Observations of pits created around colonies were done within five days of incubation at 27°C. The isolation of *Erwinia* spp**.** using the selective media is based on the ability of the pathogen to hydrolyze polypectate and to form typical cup-shaped pits (cavities) in the medium. As was shown by **Perombelon (1971)**, other pectolytic bacteria occurring in the soil, especially certain pseudomonads, also form cavities, but these are shallow and quite distinct from those caused by varieties of *E. carotovora,* which are easily recognized even when there is overcrowding by other organisms. The experiment was replicated thricely.

3.3 Biocontrol agents

Twenty one isolates of *Trichoderma harzianum* and twelve isolates of *Pseudomonas fluorescens* used in investigation were obtained from Biocontrol Lab. of Department of Plant Pathology, G. B. Pant University of Agriculture and Technology, Pantnagar.

3.3.1 Evaluation of biocontrol agents

3.3.1.1 Efficacy of *T. harzianum* isolates against *Ech* by using dual culture method

Twenty one *T. harzianum* isolates (Th-2, 4, 5, 9, 10, 14,18, 19, 21, 28, 31, 32, 36, 38, 37, 39, 43, 56, 60,75 and Th-R) taken from Biological control laboratory, Pantnagar, were screened for their antagonistic potential against the pathogen following dual culture technique **(Morton and Stroube, 1955)**. Twenty ml of sterilized and melted Nutrient Agar (NA) was aseptically poured in a sterilized 90 mm

diameter petri plates and allowed to solidify. Five mm disk dipped in suspension (conc.10^4 cell/ml) of 24h old culture of test bacterium *Ech* and biocontrol agents cut with the help of sterilized cork borer from the edge of 4 days old culture, were placed on solidified NA in such a manner that they lie just opposite to each other. Inoculated petri plates were incubated at 28 ± 1^0C. NA amended petri plate and inoculated centrally with 5mm disk dipped in same suspension of pathogen served as control. The process was replicated 3 times for four days. Experiments were conducted in completely randomized design (CRD) with three replications.

3.3.1.2 Efficacy of *P. florescens* isolates against *Ech* by using dual culture method

Twelve *P. fluorescens* isolates (Psf-2-7, 11, 12, 18, 24, 25, 28 and 31) taken from Biological control laboratory, Pantnagar, were screened for their antagonistic potential against the pathogen following dual culture technique **(Morton and Stroube, 1955)**. Twenty ml of sterilized and melted Nutrient Agar (NA) was aseptically poured in a sterilized 90 mm diameter petri plates and allowed to solidify. Five mm disk dipped in suspension (conc.10^4 cell/ml) of test bacterium *Ech* as well as biocontrol agents from the 24h old culture and were placed on solidified NA in such a manner that they lie just opposite to each other. Inoculated petri plates were incubated at 28 ± 1^0C. NA amended petri plate and inoculated centrally with 5mm disk dipped in same suspension of pathogen served as control. The process was replicated 3 times for four days. Experiments were conducted in completely randomized design (CRD) with three replications.

3.4 Evaluation of chemicals

3.4.1 Efficacy of chemicals against *Ech* at different conc. by using disc diffusion method

Kirby–Bauer and Stokes' method (Hedges, 1999) was used for antibiotic susceptibility test. Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven at 180^0C for 20min. 100 µl of the test bacterium was flooded over the inoculated Nutrient Agar plate under aseptic condition. Then, the discs of different antibiotics of 50, 100 and 200ppm conc. were dispensed onto the surface of the inoculated Nutrient Agar plate under aseptic condition. Each disc was pressed down to ensure complete

contact with the agar surface. Only 4 discs (three conc. of chemical and one control) were placed on each 90 mm plate. Control disc used was dipped in sterile water only. After 48 hours of incubation, at 28 ± 1^0C each plate is examined for the resulting zones of inhibition. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc.

3.5.1 Genomic fingerprinting of different *Ech* isolates

3.5.1.1 DNA extraction (Murray and Thompson, 1980)

For DNA extraction of the isolates nutrient broth was prepared with 3.0 g of beef extract and 5.0 g of peptone dissolved in one litre of distilled water. The pH of the medium was adjusted to 6.8-7.2 before sterilization. Thirty ml of nutrient broth in 150 ml Erlenmeyer flasks were sterilized by autoclaving. A single 24h old colony was inoculated in nutrient broth and grown overnight at 28^0 C in an incubator shaker. Bacterial cells were harvested by centrifugation at 12,000 rpm for 10 minutes. Resulting supernatant was diacarded and cell pellet was suspended in 2 ml TE buffer and vortexed. 250µl of 10% SDS and 50µl proteinase K (10mg/ml) was then added and the tubes were incubated by gentle rocking at 37^0C for 1 h. in a water bath. After 1h of incubation 0.45 ml of 5M NaCl was added and mixed thoroughly by inverting the tubes several times. 0.4ml CTAB (10% CTAB in 0.7M NaCl) was then mixed thoroughly and the tubes were incubated at 65^0C for 20 minutes. After incubation equal volume of Chloroform: Isoamyl Alcohol (24:1) was added and shaken vigorously for at least 30 minutes and then centrifuged at 15,000 rpm for 30 minutes at room temperature to separate the phases. Aqueous phase was then transferred in a fresh tube leaving the interface behind. DNA was precipitated by adding equal volume of cold isopropanol and mixed until a stringy white DNA pellet precipitated and condensed into a tight mass. After centrifugation the resulting pellet was washed with 1 ml of 70% ethanol and then air dried. Finally the pellet was dissolved in 200-400 µl of TE buffer. DNA minipreparation for large number of samples was done by scaling down the entire procedure 10 times and DNA isolation was done in eppendorf tubes instead of Oakridge tubes. Isolated DNA was treated with 100µg/ml RNAse by incubating it at 37^0C for 1 hour. Stocks prepared and source of chemicals during DNA isolation are given in Appendix III and Appendix IV, respectively

3.5.1.2 DNA purification

For DNA purification following steps was performed

1. 2 µl RNAse A was added to the ependorf tube containing 200 µl of extracted DNA and then incubated for 3 h at 37^0C in a water bath.
2. The DNA was further extracted with equal volume of Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1, v/v) and centrifuged at 10,000 rpm for 12 min at 10^0C.
3. Supernatant was taken into a fresh eppendorf tube, 0.6 volume ice cold Isopropanol and 0.1 volume of ice cold Sodium acetate (3 M) were added and the mixture was kept at – 20^0C for at least 2 h.
4. The mixture was then centrifuged at 10,000 rpm at 10^oC for 12 min.
5. Supernatant was removed using a micropipette and pellet was washed with 70% ethanol and dried completely.
6. The DNA pellet was redissolved in minimum amount of TE buffer.

3.6 Evaluation of genetic diversity

Genetic diversity analysis of isolates of *Ech* was evaluated by repetitive sequence based PCR (rep-PCR). rep-PCR protocols has been given in Appendix II.

3.6.1 Oligonucleotide primers for rep-PCR

Polymerase chain reaction (PCR) targeting repetitive DNA sequences (rep-PCR) such as repetitive extragenic palindromic sequences (REP) **(Versalovic *et al*., 1991)**, enterobacterial repetitive intergenic consensus sequences (ERIC) **(Hulton *et al*., 1991)** and BOX-PCR based on primers targeting the highly conserved repetitive DNA sequences of the Box A subunit of the BOX element **(Versalovic *et al.*, 1991)** were used for PCR amplification. The primers used for PCR amplification were BOX (5' CTACGGCAAGGCGACGCTGACG 3'), REP (REP1 5' IIIICGICGICATCIGGC 3' and REP2 5' ICGICTTATCIGGCCTAC 3') and ERIC (ERIC1 5'ATGTAAGCTCCTGGGGATTCAC 3' and ERIC2 5' AAGTAAGTGACTGGGGTGAGCG 3'). Collectively, the PCR protocols with these primers are referred as rep-PCR.

3.6.1.1 Agarose gel eletrophoresis

After completion of PCR, samples were stored at 4^0C until gel electrophoresis. A 20 µl portion of each amplified PCR product was separated on 2 % agarose gel in 0.5 X TBE, stained with ethidium bromide (EtBr) and photographed on an UV transilluminator (Biored). Amplified products were resolved for 6 hrs at 125V.

3.6.1.2 Cluster analysis

rep-PCR generated banding pattern of each isolate were compared visually and grouped according to unique banding patterns. Combined rep-PCR data were used to detect haplotypic diversity from all the locations sampled. Each unique fingerprint generated by the PCR techniques was regarded as a haplotype. For the technique, representative isolates of a haplotype and isolates with unique banding pattern were electrophoresed on the same gel to confirm band identities and differences. Patterns were converted into binary data, i.e., the presence and absence of bands at different positions were recorded as 1 and 0, respectively, at each position along the lane.

A pairwise comparision of strains was done by NTSYS-pc **(version 2.11s; Rohlf, 2003)** with Jaccard's coefficient of similarity. Similarity coefficients were calculated by using the un-weighted pair-group method arithmetic mean (UPGMA) **(Sneath and Sokal, 1973)**. Dendograms were generated with the SAHN (sequential, agglomerative, hierarchical, and nested clustering methods) using the UPGMA method. Bootstrap analysis using the computer program Winboot **(Yap and Nelson, 1996)** was used to assess the robustness of the groupings produced by cluster analysis. The phenograms were reconstructed by repeated sampling with replacement, and the frequency with which a particular grouping formed was used as a measure of the strength of the grouping **(Felsenstein, 1985; Hedges, 1992 and Yap and Nelson, 1996)**.

3.7 Glasshouse experiment

3.7.1 Pathogenicity test and artificial inoculation technique

3.7.1.2 Inoculation techniques and disease assessment

For inoculation, *Ech* Pantnagar isolate was used. Experiments were conducted in glasshouse using healthy seeds of susceptible sweet sorghum cultivar SPSSV 6.

Ten seeds were sown in 30 cm plastic pots filled with sterilized soil. As to obtain 21 days old seedlings for inoculation, these pots were kept in glasshouse and irrigated with water regularly to maintain high moisture conditions. Before inoculation only 5 seedlings per pot were maintained, rest were uprooted. Bacterial cell suspension was prepared from 24hrs old culture of *Ech* and adjusted to 2×10^8cells/ml (cfu) by adding sterilized distilled water and 0.7% (v/v) of Tween-40 (surfactant). Twenty one days old plants were inoculated with this bacterial suspension by six different methods viz. stem injection, midrib injection, cotton wool, tooth pick, leaf whorl inoculation and root tip cut and dip. For control only sterilized water was used. Immediately after inoculation plants were placed in moist chamber for 48-72 hours and then transferred in glasshouse having a temperature of about 30 ± 1^0C and relative humidity >90%. The symptoms expressed were studied and re-isolation of the pathogen was made. Experiment was conducted using completely randomized design (CRD) with three replications. The inoculation test, as above was repeated once more to confirm the result. Disease assessment was done based on percentage of plants showing stalk rot symptoms in relation to total inoculated plants after one week of inoculation **(Hartman and Kelman, 1973)**. Three parameters *viz.*, percent lodging, number of internodes crossed and length of spread lesion (observed visually after splitting-opening the infected stalks) by the *Ech* were used.

3.7.1.2.1 Inoculation technique

3.7.1.2.1.1 Midrib injection method

Bacterial cells suspension was inject-inoculated (Plate 1A) with a 21G hypodermic needle into the midrib of leaf of 21 days old susceptible plants while in control plants midrib was inject-inoculated with sterilized water as per the method described by **Goszczynska*, et al*., (2007)** and **Hseu *et al*., (2008)**.

3.7.1.2.1.2 Cotton wool method

Non-absorbent cotton wool was dipped in bacterial cell suspension and rubbed gently on the both surfaces of leaves to expose the maximum stomatal opening (Plate 1B). Cotton wool dipped in sterilized water was used to rub the leaves of control plants.

3.7.1.2.1.3 Stem injection method

Bacterial suspension was inject-inoculated (Plate 1C) with a 21G hypodermic needle into the vicinity of a growing point of 21 days old susceptible plants as described by earlier investigators **(Thind and Payak, 1978; Aysan *et al.*, 2005; Ruz *et al.*, 2008; Kutama *et al.*, 2011)**. Control plants were inject-inoculated with sterilized water only.

3.7.1.2.1.4 Tooth-pick method

Sorghum stalks were inoculated with *Ech* using the wooden toothpick method (Plate 1D) of inoculation as described by **Young (1943); Crall (1952); Hildebrand (1953) and Clements *et al.*, (2003)**. Tooth picks were boiled thoroughly in water for two hours to remove resin, gum or any other toxic substances that might inhibit the growth of *Ech*. After boiling, they were washed thoroughly in fresh tap water, and then tooth picks were dried in sun. About 10 tooth picks were placed in 100 ml flasks, in such a way that the pointed end of tooth picks faced away from the base and was autoclaved at 15 pounds psi (temperature 121^0C) for 20 minutes. *Ech* was inoculated to 100 ml flasks containing sterilized LB, under aseptic condition, incubated at 28^0C and a rich suspension of bacterial cell was made within 7days. This suspension was further poured into tooth pick containing flasks to cover lower $1/3^{rd}$ of the tooth picks under aseptic condition and flasks were incubated for seven days at 28^0C, by the time, tooth picks were covered with the bacterial growth and were ready for inoculation. To confirm that toothpicks were colonized by *Ech*, infested toothpicks were streaked onto petri plates amended with NGM and growth with blue pigment was observed. A sterile pointed iron needle (1-2 mm diameter) with a wooden handle was used to make a hole in the stem, to facilitate tooth pick insertion. Tooth picks were introduced obliquely into the stalk in 21 days old plants. The control plants were inoculated with a non-infested and sterilized toothpick. Care was taken not to insert the toothpick too deeply in order to avoid splitting of the stalk. Cares were taken to ensure that drought stress conditions prevailed at the time of toothpick insertion.

3.7.1.2.1.5 Leaf-Whorl inoculation method

Leaf-Whorl inoculation method was adapted from **Hartman and Kelman (1973)** used in corn for inoculation of *Erwinia* spp. without causing injury. Bacterial

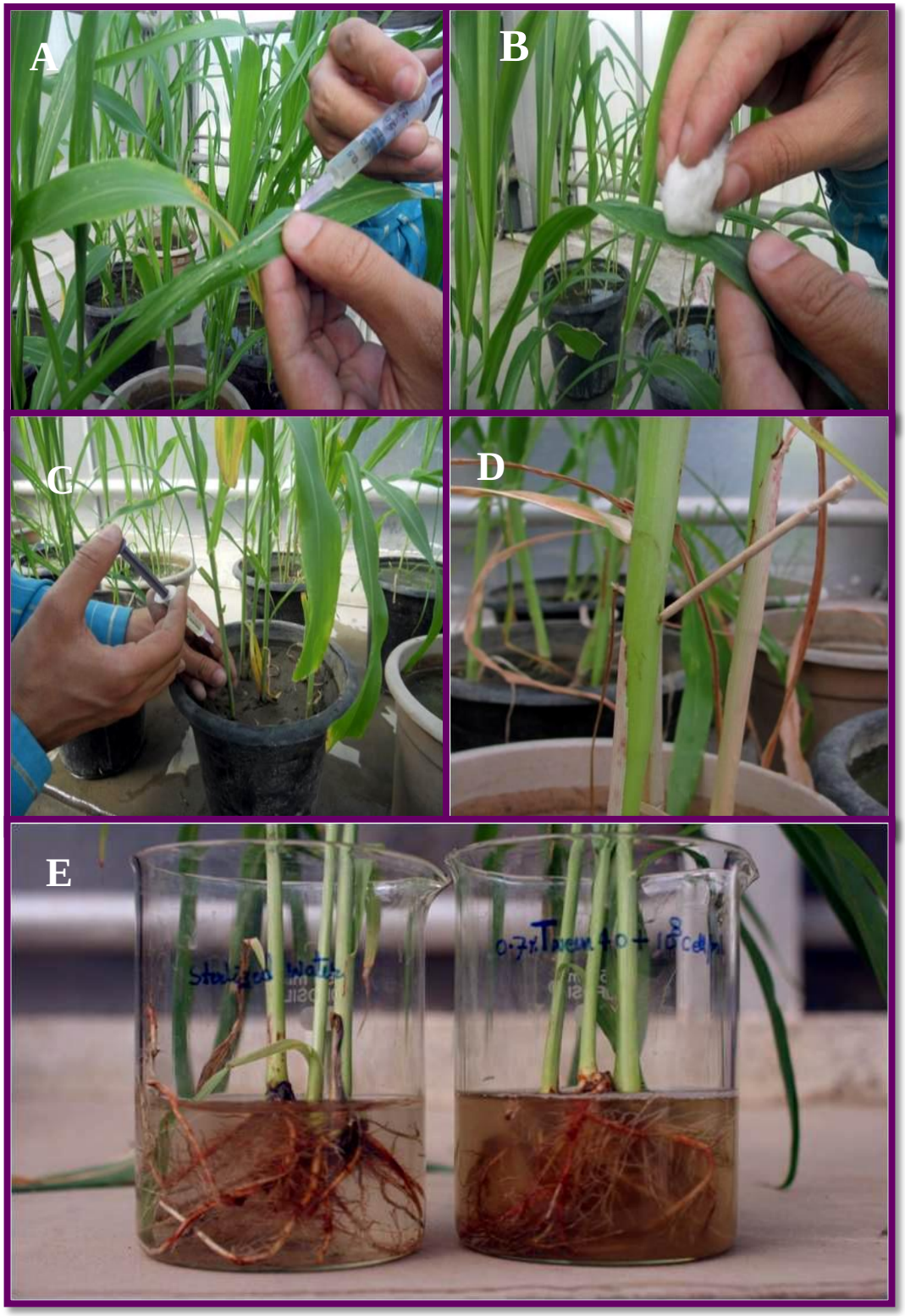


Plate 1

Artificial inoculation methods under glasshouse conditions

A. Midrib injection inoculation, B. Cotton wool inoculation, C. Stem injection inoculation, D. Tooth-pick inoculation, F. Root tip cut & dip inoculation

suspension was sprayed in leaf whorls (2ml/whorl) with the help of atomizer without causing any injury. Care was taken not to disturb the plants after inoculation so that maximum inoculum was retained in the leaf-whorls. Plants sprayed with sterilized water served as control.

3.7.1.2.1.6 Root tip cut and dip method

Root tip cut and dip method (Plate 1E) was adapted from **Bolick (1960)** used to prove pathogenicity of *Erwinia chrysanthemi* in chrysanthemum cause bacterial bud blight. Twenty-one days old plants grown in nursery were watered well 1hr before lifting the plants. Care was taken, not to damage the root and crown portion while uprooting the plants. The adhered soils to the roots were washed gently in tap water. Then again plants roots were washed in sterilized water to avoid the undesirable soil-born microbes or pathogens. The roots cut off about 3cm from the tip portion of main or primary root, and immediately dipped in 250ml beaker containing 100ml of bacterial suspension for 3minutes only. After dipping root in a bacterial suspension, the plants were transplanted immediately into pots filled with sterilized, well watered soil in the glasshouse. The roots of plants used for control were dipped in sterilized water.

3.8 Virulence/Aggressiveness variability analysis under glasshouse conditions

Twenty one taxonomically characterized isolates of *Ech*, collected from Tarai region of district U.S. Nagar (Uttarakhand), were evaluated for their variability in virulence/aggressiveness on SSPV6 cultivar. Differences in virulence/aggressiveness were measured by key parameter: variability in terms of onset of infection, rate of stem lesion (host tissue colonization) expansion, percent disease severity (PDS) and percent toppling (top rot). Based on the aforesaid parameter isolates were designated into 3 main categories: most virulent/aggressive, moderately virulent/aggressive, and least virulent/aggressive. Each isolate was inoculated by two methods: leaf-whorl and stem inoculation as described in evaluation of inoculation methods. Second inoculation was also done after 3 days of first inoculation. Inoculated plants were immediately placed in a humid chamber (RH >90%, 30 ± 2°C) for 24 h, then transferred to the glasshouse where symptom progression was observed at 5, 10 and 15 days after inoculation. Pots were randomized (CRD) prior to inoculation.

3.9 Biological and chemical control

Glasshouse experiments were conducted with five potential *T. harzianum* and *P. fluorescens* isolates and five antibiotics to evaluate their efficacy on seed germination, stem diameter, root length, plant height and disease severity reduction (Table 3.9). BCA isolates and antibiotics found effective in reduce disease severity under glasshouse experiments were further evaluated for efficacy under field conditions.

Table 3.9: Methods of application of bioagents and antibiotics in glasshouse experiments

Treatment	**Methods of application**
Bioagents	**Set-A**
Vermicompost colonized isolates of bioagents	1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray
Isolates of bioagents only	**Set-B** 1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-C** 1. One foliar spray 2. Two foliar spray
Chemicals	**Set-A** 1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-B** 1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-C** 1. One foliar spray 2. Two foliar spray

For all the treatment, twenty one days old sorghum seedlings (SSPV6 cultivar) were artificially inoculated by using suspension containing 0.7 % of Tween-40 (v/v) + 2×10^8 cells/ml (cfu) of *Erwinia chrysanthemi* (*Ech*) Pantnagar isolate by leaf- whorl spray and stem inoculation technique. Control plants were inoculated with sterilized water only.

Following formula was used for all treatment to calculate:

A. Germination percent after 15 days of sowing:

$$\text{Germination \%} = \frac{\text{seed germinated}}{\text{Total seeds sown}} \times 100$$

B. Percent disease severity after 45 and 60 days of sowing:

$$\text{Percent disease severity (PDS)} = \frac{\text{Sum of numerical rating}}{\text{Total no. of samples} \times \text{Maximum rating grade}} \times 100$$

3.9.1 Biological control

3.9.1 Mass multiplication of bioagents

3.9.1.1 *Trichoderma harzianum* isolates

Mass multiplication of five isolates of *Trichoderma harzianum* found best *in vitro* was done on barnyard millet (*Echinocloa frumentacae*, vernacular: Jhangora) grains (Plate.2C, D). Grains were soaked in water for 12hrs and filled in 500ml Erlenmeyer flask (@ 100g/ flask). These flasks were incubated at 121.1 ^{0}C, 15 lbs psi for 20min. after cooling to room temperature, the flasks were inoculated with 4-5 mycelial disc (7mm) cut from freshly growing (3 days old) culture of *T. harzianum* isolates and incubated at 28^{0}C for 15 days. Fully colonized Jhangora grain with mycelial growth were air dried in open shade and ground with the help of Willy Mill to get fine powder. This powder was passed through 50 to 80 mesh sieves, simultaneously to obtain a pure powder.

3.9.1.2 *P. fluorescens* isolates

Five isolate of *P. fluorescens* found best under *in vitro* condition against *Ech* were mass multiplied on King's B broth (Plate.2 A, B). The isolates were inoculated in the 250 ml flask containing 100ml KB broth and incubated for 48 h at 28$\pm$1^{0}C.

3.9.2 Colonization of vermicompost by *T. harzianum* and *P. fluorescens* isolates under shade conditions for glasshouse and field experiments

Vermicompost used for colonization of *T. harzianum* and *P. fluorescens* isolates was taken from LRC, Pantnagar, which was prepared by using manure worm, *Eisenia foetida* and African Night crawlers *Eudrilus eugeniae*. Polythene bags of 5kg capacity were used for colonization of bioagents in which 3kg of freshly prepared vermicompost was filled and was mixed with 5gm (1×10^8 cfu) of talc based formulated powder of each *T. harzianum* and *P. fluorescens* isolates (Plate 3).

3.9.3 Pre-plant soil drenching experiment

3.9.3.1 Pre-plant soil drenching with vermicompost colonized bioagents

Pre-plant soil drenching was done with bioagents colonized vermicompost @ 1kg/pot and 2.5 kg/4 m^2 plots in glasshouse and field conditions respectively. Three replications were maintained for each treatment in glasshouse and field conditions.

3.9.3.2 Pre-plant soil drenching with vermicompost colonized bioagents and foliar spray experiment

Pre-plant soil drenching was done in pots as in aforementioned manner. Twenty one days old seedlings were artificially inoculated using suspension containing 0.7 % of Tween-40 (v/v) + 2×10^8 cells/ml (cfu) of *Erwinia chrysanthemi* (*Ech*) Pantnagar isolate by leaf- whorl spray and stem inoculation. Control plants were inoculated with sterilized water only. Two sets of experiment were conducted viz. First set; Pre-plant soil drenching + one foliar spray and second set: Pre-plant soil drenching + two foliar sprays; In both the sets first spray of *T. harzianum* and *P. fluorescens* isolates @ 5g spore (or cell, talcum powder based)/lit. of water, was given after one week of inoculation. In second set of experiment second spray was given after 15 days of first spray. The observations on disease severity were recorded in 0-5 scale 60 DAS for each treatment taking random samples.

3.9.3.3 Foliar spray experiment

Ten healthy seeds were sown in pots filled with sterilized soil. These pots kept in glass house were maintained and inoculated with the pathogen, similarly as described above. Two sets of experiment were conducted viz. first set: one foliar

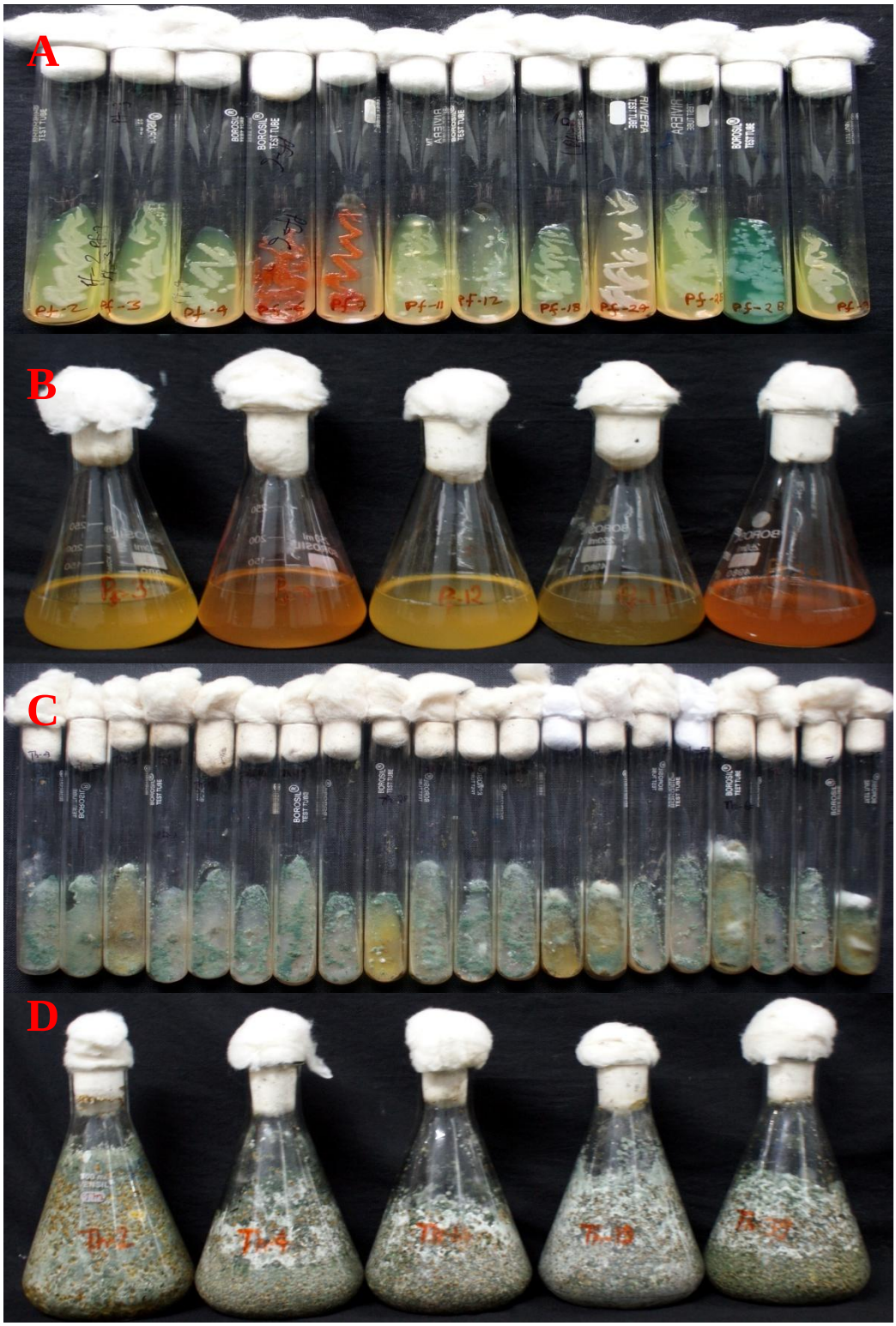


Plate 2

A. *Pseudomonas fluorescens* isolates used in study
B. Mass multiplication of *P. fluorescens* isolates in King's Broth
C. *Trichoderma harzianum* isolates used in study
D. Mass multiplication of *T. harzianum* isolates

Plate 3

A. Pollybag filling with vermicompost at LRC, Pantnagar,
B. Vermicompost inoculation with *T. harzianum* isolates
C. Vermicompost inoculation with *P. fluorescens* isolates
D. Colonization on vermicompost by *T. harzianum* isolates
E. Close-up view of colonized vermicompost by *T. harzianum* isolate
F. Control : No observable fungal mycelial growth on vermicompost

spray and second set: two foliar sprays. Plant in both sets were sprayed with *T. harzianum* isolates and *P. fluorescens* isolates @ 5g spore (or cell, talcum powder based) /lit. of water after one week of inoculation. In second set of experiment second spray was given after 15 days of first spraying. The observations on disease severity were recorded in 0-5 scale 60 DAS for each treatment taking random samples

3.9.4 Chemical control

3.9.4.1 Pre-plant soil drenching experiment

Pre-plant soil drenching was done with six chemicals (viz. Blitox-50, Chloramphanicol, Tetracyclin, Bleaching powder, Streptomycin and Streptocyclin). Suspensions of these chemicals were applied @ 2.5% in pots containing sterilized soil. After proper mixing of chemicals suspensions in soil seed sowing was done. The observations on disease severity were recorded 45 and 60 days and on seed germination, plant height and stem diameter after 60days after sowing, for each treatment random samples were taken.

3.9.4.1.1 Pre-plant soil drenching and foliar spray experiment

Pre-plant soil drenching was done in pots as per aforementioned manner. Two sets of experiment were conducted viz. First set: Pre-plant soil drenching + one foliar spray, second set: Pre-plant soil drenching + two foliar sprays. In both the sets first spray of chemicals @ 2.5g/lit. of water was given after one week of inoculation. In second set treatments were resprayed at 15 days of first spray. The observations on disease severity were recorded 45 and 60 days and on seed germination, plant height and stem diameter after 60days after sowing, for each treatment random samples were taken.

3.9.4.1.2 Foliar spray experiment

Two sets of experiment were conducted viz. first set; one foliar spray and second set; two foliar sprays. In both the sets first spray of chemicals @ 2.5g/lit. of water was given after one week of inoculation. In second set treatments were resprayed at 15 days of first spray. The observations on disease severity were recorded 45 and 60 days and on seed germination, plant height and stem diameter after 60days after sowing, for each treatment random samples were taken.

3.10 Field trials

Field experiments were conducted during the *Kharif* season of 2012 at Livestock Research Centre, G.B. Pant University of Agriculture and Technology, Pantnagar to evaluate the efficacy of selected bioagents in controlling Erwinia stalk rot of sorghum. Trials were laid out in Randomized Block Design (RBD) with three replications. For all the treatment, twenty one days old sorghum seedlings (SSPV6 cultivar) were artificially inoculated using suspension containing 0.7 % of Tween-40 (v/v) + 2×10^8 cells/ml (cfu) of *Erwinia chrysanthemi* (*Ech*) Pantnagar isolate by using leaf-whorl spray and stem inoculation technique. Control plants were inoculated with sterilized water only. Observations on disease severity were recorded 45 and 60 days after sowing. The observations on seed germinations and yield (biomass) parameter were taken 15 and 60 days after sowing respectively.

3.10.1 Biological control

T. harzianum and *P. fluorescens* isolates found effective under glass house experiments were further evaluated in field trials, as per procedure described in glasshouse experiments.

3.10.1.1 Pre-plant soil drenching experiment

3.10.1.1.1 Pre-plant soil drenching with vermicompost colonized bioagents

Pre-plant soil drenching was done with bioagents colonized vermicompost @ 2.5kg/4 m^2 at 10cm depth furrow and then seed sowing was done under field conditions (Plate 3 A, B). Three replications were maintained for each treatment field conditions. The observations on plant height and stem diameter were recorded 45 DAS whereas disease severity was recorded two times 45 and 60 days after sowing. The observation on yield (biomass) parameter was taken 60 days after sowing. Disease severity recorded in 0-5 scale 45 and 60 DAS for each treatment taking random samples.

3.10.1.1.2 Pre-plant Soil drenching and foliar spray experiment

Pre-plant soil drenching was done and foliar spray was done as per procedure described in field and glass house experiment respectively. The observation on disease severity recorded in 0-5 scale 45 and 60 DAS for each treatment taking random samples.

Plate 4

A & B. Pre-plant soil drenching with bioagents colonized vermicompost in field at LRC, Nagla

Table 3.10: Methods of application of bioagents and chemicals in field experiments

Treatment	**Methods of application**
Bioagents	**Set-A**
Vermicompost colonized isolates of bioagents	1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray
Isolates of bioagents only	**Set-B**
	1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-C** 1. One foliar spray 2. Two foliar spray
Chemicals	**Set-A**
	1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-B** 1. Pre-plant soil drenching 2. Pre-plant soil drenching + one foliar spray 3. Pre-plant soil drenching + two foliar spray **Set-C** 1. One foliar spray 2. Two foliar spray

3.10.1.1.3 Foliar spray experiment

Two sets of experiment were conducted viz. first set; one foliar spray and second set; two foliar sprays. In both the sets first spray with *T. harzianum* and *P. fluorescens* @ 5.0g/lit. of water was given after one week of inoculation. In second set treatments were resprayed at 15 days of first spray. The observations on disease severity were recorded 45 and 60 days and on plant height and stem diameter after 60days after sowing, for each treatment random samples were taken. The observations on seed germinations and yield (biomass) parameters were taken 15 and 60 days after sowing respectively.

3.11 Chemical control

Chemicals found effective in glass house experiments were further evaluated in field trials.

3.11.1 Pre-plant Soil drenching experiment

Five chemicals viz. Blitox-50, Chloramphanicol, Tetracyclin, Bleaching powder and Streptocyclin were used for pre-plant soil drenching. The solution of these chemicals were drenched in 10cm depth furrow and then seed sowing was done. The observations on plant height and stem diameter were recorded 45 DAS whereas disease severity was recorded two times 45 and 60 days after sowing.

3.11.2 Pre-plant soil drenching and Foliar spray experiment

Seed treatment and foliar spray was done as per procedure described in glass house experiments. The observations on disease severity were recorded in 0-5 scale 45 and 60 DAS. For each treatment random samples were taken.

3.11.3 Foliar spray experiment

Two sets of experiment were conducted viz. first set; one foliar spray and second set; two foliar sprays. In both the sets first spray of chemicals @ 2.5g/lit. of water was given after one week of inoculation. In second set treatments were resprayed at 15 days of first spray. The observations on disease severity were recorded 45 and 60 days and on plant height and stem diameter after 60 days after sowing, for each treatment random samples were taken.

3.12 Population dynamics studies of earthworms in rhizospheric soil of sorghum

3.12.1 Collection of soil samples

Ten random samples of rhizospheric soil of sorghum plants were collected from the plot with different treatment (Pre-plant soil drenching with bioagents colonized vermicompost, soil drenching with vermicompost only, soil drenching with bioagents only, soil drenching with antibiotic and control plot) of research trial at Pantnagar. Samples were collected in the month of September in growing season 2012. Soil sampled from 10 cm widget diameter from surface and at a depth of 15 cm in rhizospheric soil of sorghum plants. Number of young, adults and heap of castings made by earthworms (in 10cm×15cm area) were taken into considerations.

3.13.1 Artificial inoculation in field experiments

3.13.1.1 Leaf-Whorl inoculation method

Leaf-Whorl inoculation method was adapted from **Hartman and Kelman (1973)** used in corn for inoculation of *Erwinia* spp without causing injury. All the plants in each row were artificially inoculated by spraying the bacterial suspension in leaf whorls (2ml/whorl) with the help of atomizer without causing any injury between 5-7 pm after 21 days of sowing as night temperature and humidity are conducive for infection. Care was taken not to disturb the plants after inoculation so that maximum inoculum was retained in the leaf-whorls. Plants sprayed with sterilized water served as control.

3.13.1.2 Stem inject-inoculation

Bacterial suspension was inject-inoculated with a 21G hypodermic needle into the vicinity of a growing point of 21 days old plants as described by earlier investigators **(Thind and Payak, 1978; Aysan *et al*., 2005; Ruz *et al*., 2008; Kutama *et al*., 2011)**. All the plants in front row were only inoculated. Control plants were inject-inoculated with sterilized water only.

3.14 Preparation of inoculum on Luria broth

The test bacterium *E. chrysanthemi* isolated and purified from fresh diseased stalk of sorghum was used throughout the investigation. For preparation of inoculum

Luria broth medium was used. Single typical colony of *E. chrysanthemi* was inoculated in each flask aseptically and then flasks were incubated at 28 ± 1^0C for 24hrs. During incubation, the flasks were regularly shaken for uniform bacterial growth on broth then used as inoculum for artificial inoculation in field experiments.

3.15 Disease observation

Observation on severity of the disease was recorded in 0 to 5 scales modified and adapted from **Muhammad (1983)** used for evaluation of corn germplasm against Erwinia stalk rot as follows:

0 = No symptoms,

1 = Initial small necrotic areas/ partial rotting at the base of the whorl/stalk,

2 = 25-49% dark brown, water-soaked, soft or slimy at the base of the whorl, disintegration of pith tissues at a single internode, premature of wilting uppermost leaves,

3 = 50-74%, decay spreading rapidly crossing 2-3 internodes in collapsed plant,

4 = 75-100% of tissue rotted with foul smell at the base of whorl/ extensive necrosis/ soft rotting with visible external symptoms,

5 = lodging accompanied by extensive necrosis/ rotting of leaf /stalk tissue usually having a very strong foul smell

3.16.1 Preparation of the field

The field was first got ploughed in the first week of June using soil turning plough. The field was then harrowed twice by using disk harrow for better pulverization and leveled with leveler.

3.16.2 Fertilizer schedule

Recommended dose of P_2O_5 (50 Kg/ha) and K_2O (40 Kg/ha) in the form of single superphosphate and Muriate of Potash were broadcasted and mixed thoroughly before sowing. Nitrogen in the form of Urea @ 120 Kg/ha was applied in two split doses. First half was applied along with phosphorus and potash whereas, the second half was top dressed after 40 days of sowing.

3.16.3 Field layout

The experiment was carried out in randomized block design with three replications. Germplasm was planted in five rows of 6 m length in the plots at the Livestock Research Centre of the University during *Kharif* season of 2012 with RBD. The spacing dimensions were 45 × 15 cm.

3.16.4 Sowing and post sowing operations

The sowing was done on 18th May during *Kharif* season, 2012. The spacing dimensions were 45 × 15 cm. The seed was sown @ 15 Kg/ha at the depth of 3-4 cm. Weeding and irrigation were done from time to time, as and when required. Thinning was done to maintain the distance of 15 cm between plant to plant after 25 DAS. Two sprays of Thiodan 35 EC (0.1 %) were done to protect the crop from insect damage, first at the appearance of the sorghum shootfly (*Antherigona soccata*) and then after 15 days of first spray.

3.17 Statistical analysis

The data was analyzed statistically at the computer centre of G.B. Pant University of Agriculture and Technology, Pantnagar, using Completely Randomized Design (CRD) and Randomized Block Design (RBD). The treatments were compared by the means of critical differences (CD) at 5% level of significance.

Results and Discussion

4.1 Symptomatology

Stalk rot of sorghum caused by *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock is one of the most destructive diseases of sorghum crop. The disease mainly affected sorghum stem showing water-soaked symptoms that later turned reddish dark brown colour. The infected stem pith disintegrated and showed slimy soft-rot symptoms and eventually the whole plant wilted. Losses are due to (1) premature plant death (2) harvest losses associated with stalk breakage or lodging (Plate 1). Problems with ear rots are usually greater where lodging occurs, particularly when the harvest season is wet. This uncommon disease usually occurs about midseason in hot, damp weather as a tan to dark brown, water-soaked, soft or slimy disintegration of pith tissues at a single internode (Plate 5B). Affected stalks suddenly collapse and are usually twisted (Plate 5E). The tip of the uppermost leaves often wilt, followed by a slimy soft rot at the base of the whorl. The decay spreads rapidly downward until the affected plants collapse. Lodged plants usually have a foul odor. Bacterial ooze may be seen sometimes on the affected stalk in natural conditions (Plate 5 F).

Top rot symptoms

The first visible symptoms are wilting and drying up of the tips of the middle leaves of the whorl (Plate 6). At the same time, a soft rot develops in the stalk at the base of the whorl. A rapid decay downwards through the stalk is observed and the tops of affected plants droop. The cluster of leaves can easily be pulled out, showing a soft, rotted condition at the breaking point near the base of the whorl.

Basal or stalk rot symptoms

The infected tissue becomes brown, soft, and water-soaked. Internally, the stalk turns into a soft mass of disintegrated tissue (Plate 7, 8). At this stage, the plants usually topple over. A foul odour and the presence of dipterous larvae on and in decaying tissues are characteristic symptoms of this disease. The rot may involve only one or two internodes, or the entire length of the stalk, which finally dries up and its interior turns into a shredded mass of fibrous tissue.

Wilting of the upper- and inner-most leaves symptom was observed first. When infected leaves were pulled, they separated from the stalk at the point of intense bacterial rot near the stem's apex. The disjointed apex appeared as a mushy foul smelling whip. The following three symptoms were observed: flaccid leaves, greasy leaves and finally withered leaves. The pith is disintegrated and often reduced to a slimy consistency at the juncture with the stem. **Saxena *et al*. (1991)** reported this bacterium causing stalk and top rot of sorghum under natural conditions in India during 1987-88 crop season in sorghum field at Pantnagar, Uttarakhand. The disease was wide spread and affected 60-80% of plants in different sorghum genotypes. The disease mainly affects sorghum stem showing water-soaked symptoms that later turn reddish dark brown colour. The infected stem pith is disintegrated and show slimy soft-rot symptoms with foul-smell and eventually the whole plant wilts **(Zummo, 1969; Hseu *et al*., 2008; Hepperly and Ramos-Davila, 1987)**. The rot may involve only one or two internodes, or the entire length of the stalk, which finally dries up and its interior turns into a shredded mass of fibrous tissue. Lower leaves and leaf sheaths covering the internodes are chlorotic, and the rind is pale-straw instead of green in color. The economic, biomass and grain yield losses due to rapid progress of this bacterial soft rot disease is one of the most destructive feature in natural condition. The disease appears before the onset of flowering. Cloudy weather, relatively high temperature (>30 ^{o}C) and frequent rainfall favor disease epidemic **(Saxena *et al*., 1991)**.

4.2 *In-vitro* experiment

4.2.1 Biochemical and physiological characterization *E. chrysanthemi* isolates

The identification of plant bacteria has traditionally been based on the biochemical behaviour and the cell and colony morphology of these organisms. Biochemical tests are reliable and specific, and have been extensively used. On the basis of a most widely recommended set of biochemical and physiological testing to differentiate presumptive *Erwinia*; test bacterium was screened for characterization upto species level by using set of tests (Table 4.1, Plate 9, 10) and study of cell morphology and size using SEM (Plate 11). On NA, young colonies are circular,

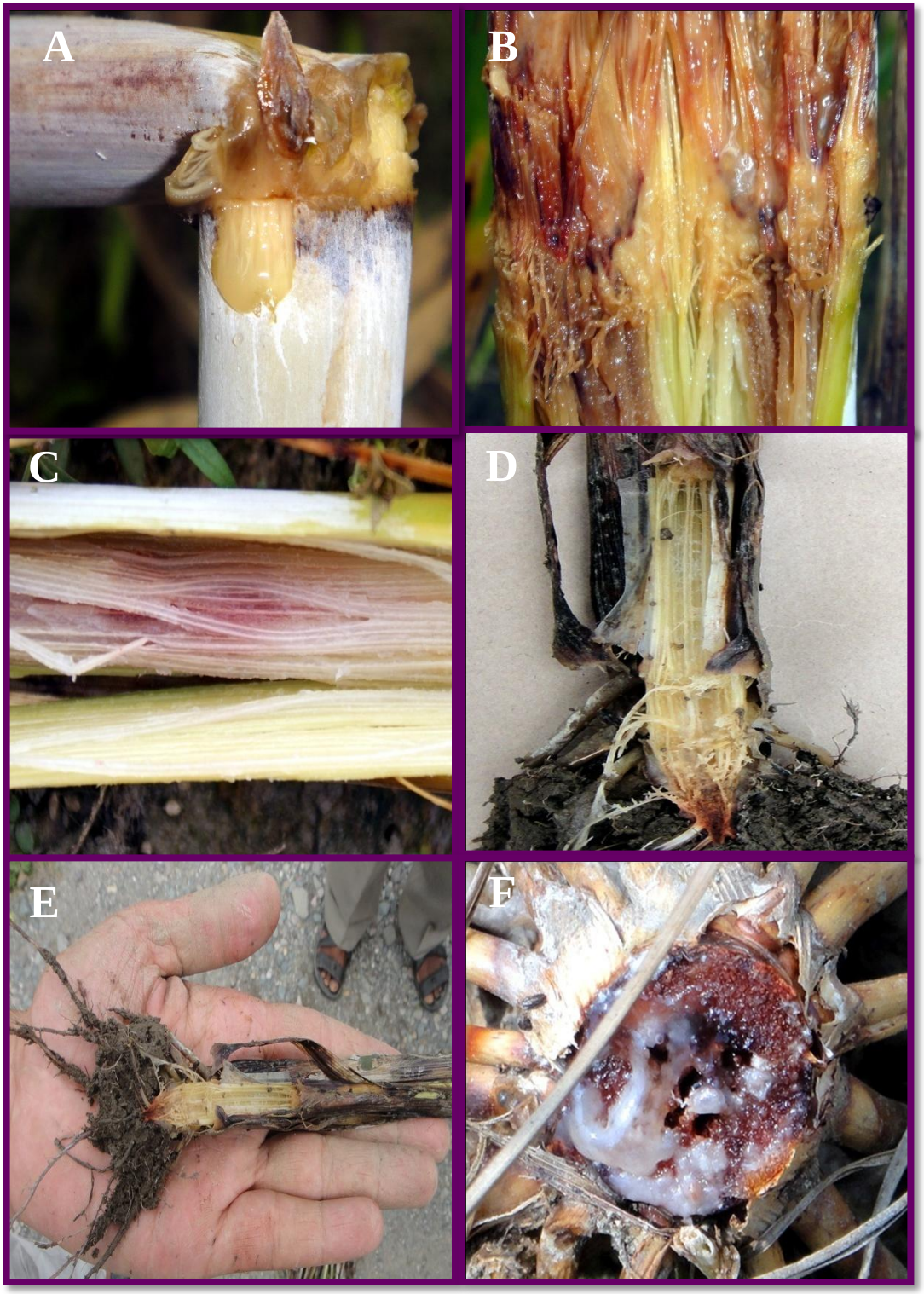


Plate 5

A. Rotted stalk with typical bacterial exudates
B. Typically rotted stalk of sorghum
C. Internal discoloration
D & E. Basal rot with lower internode & bacterial slime
F. Bacterial exudates in natural conditions

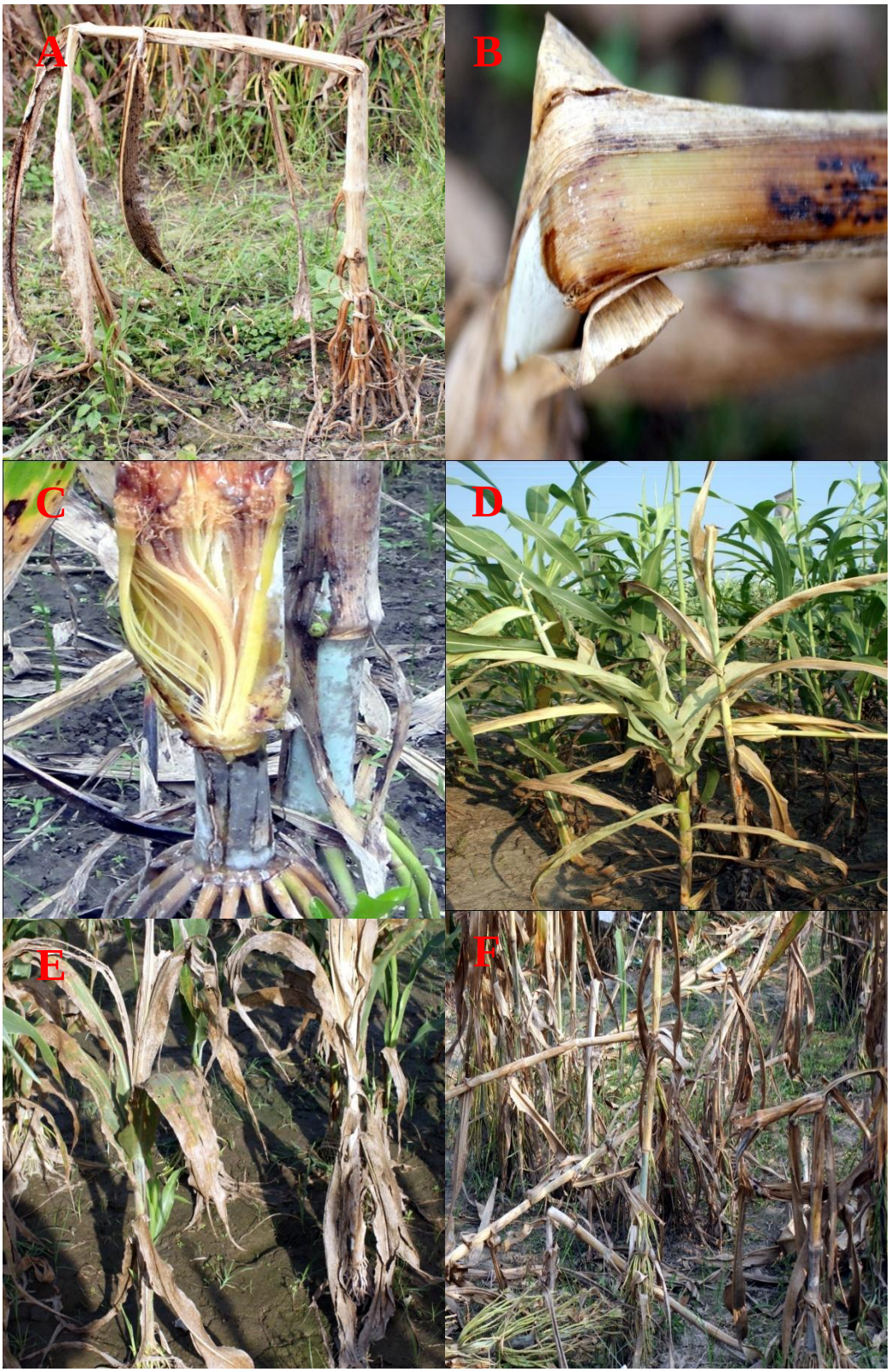


Plate 6

Field view of typical stalk rot symptoms caused by *Erwinia chrysanthemi*

Plate 7

A. Soft mass of disintegrated tissue mostly initiating at nodes
B . Internodes becoming soft and slimy
C. Internal discoloration
D & E. Basal rot with lower internode
F. Root infection of *Erwinia chrysanthemi*

Plate 8

Soil as a source of inoculum

A. Soft rotted stalk at soil line

B. Infection route of secondary roots of sorghum

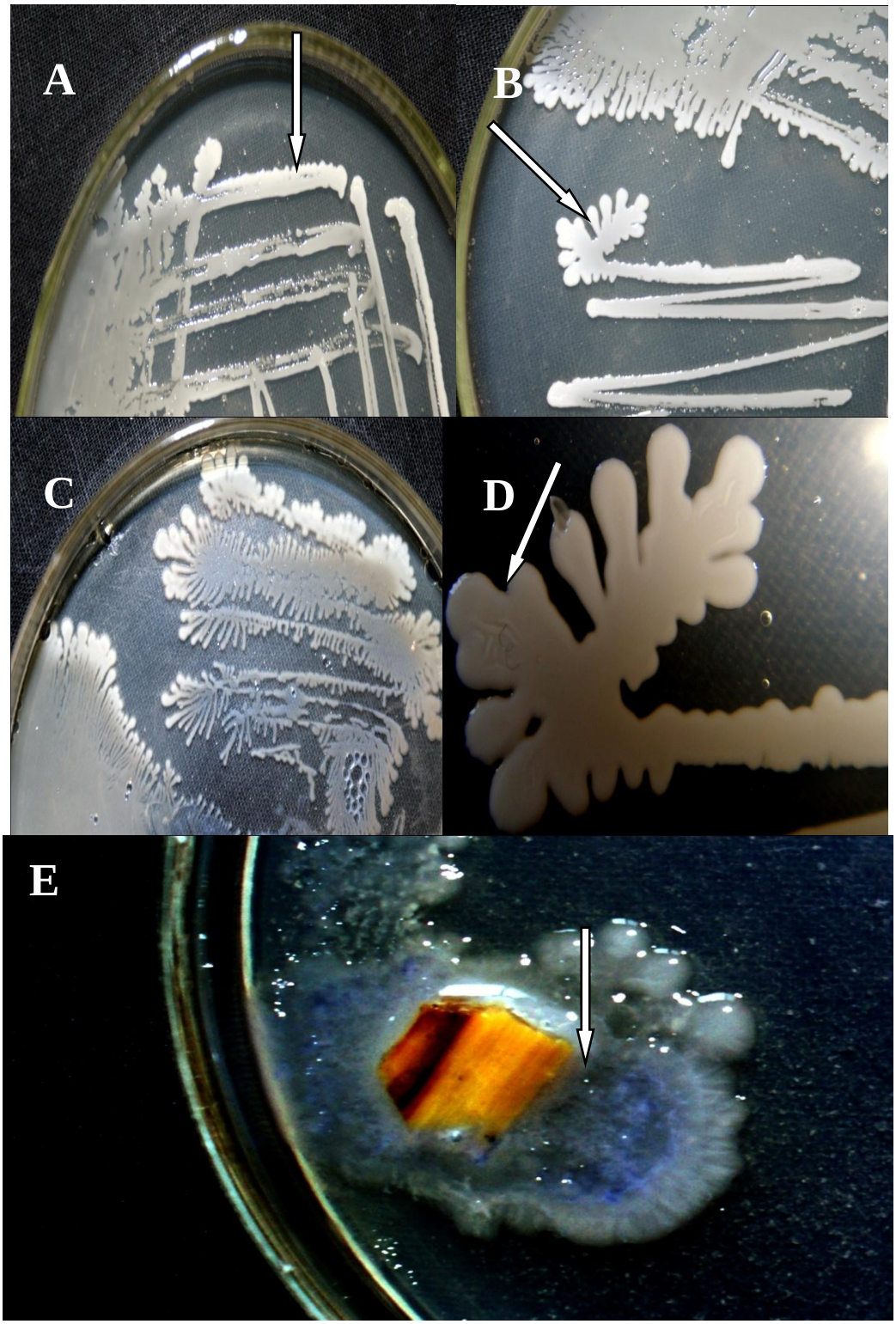


Plate 9

A. 4 days old colony with undulate or lobed margin, which later becomes feathery
B. 6 days old colony with initial feathery growth
C. 8 days old colony with feathery growth
D. Close-up of undulate or lobed margin
E. Colony with water-insoluble blue pigment (Indigoidine) on NGM agar medium

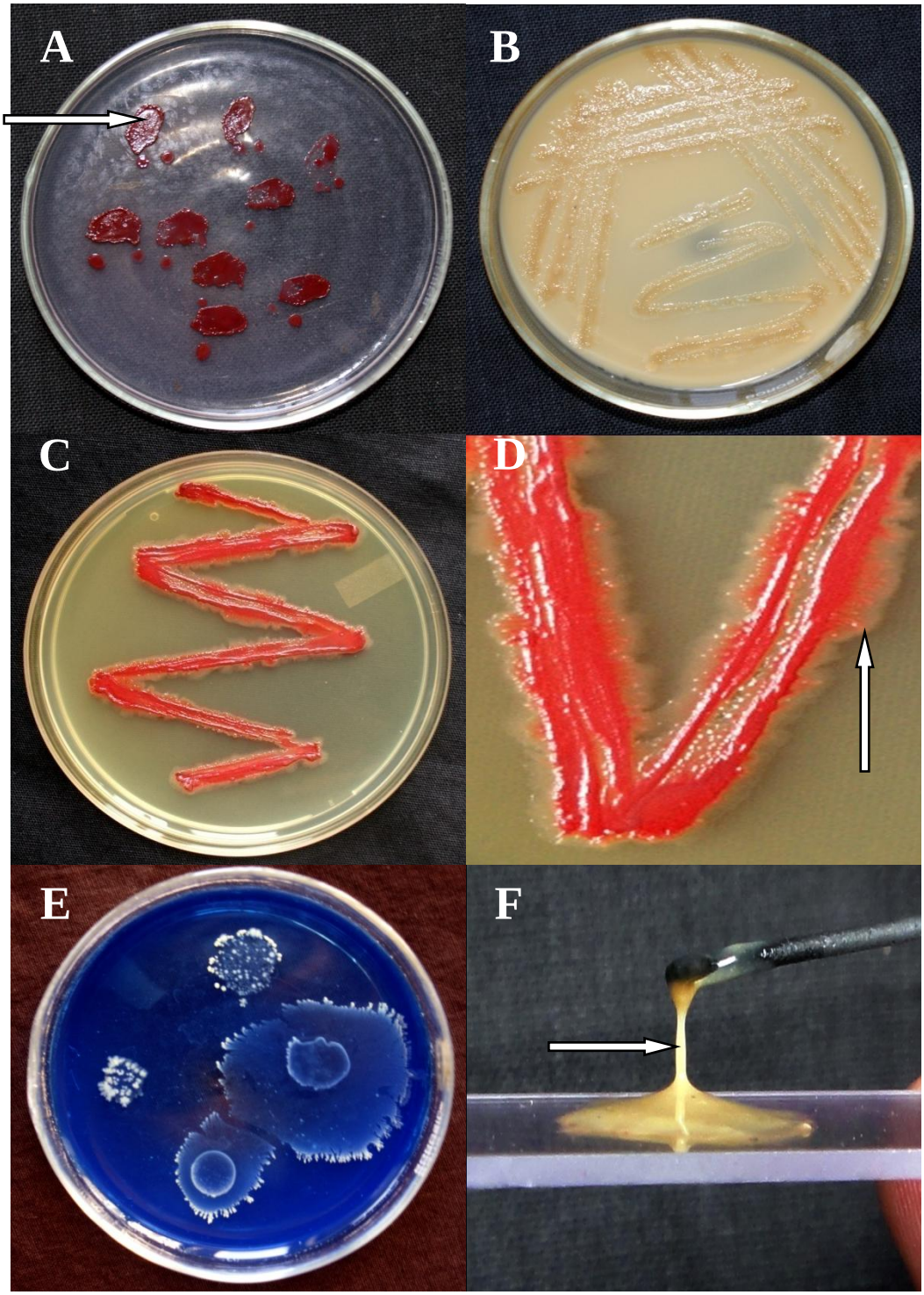


Plate 10

A. Deep pit on CPV medium + 0.5 % TZC solution
B. Brown colony on YDC medium
C. Ceramis colony with red centre on Logan's medium by *E. chrysanthemi*
D. Close-up of colony
E. Citrate utilization by *E. chrysanthemi*
F. String formation in 3% KOH test

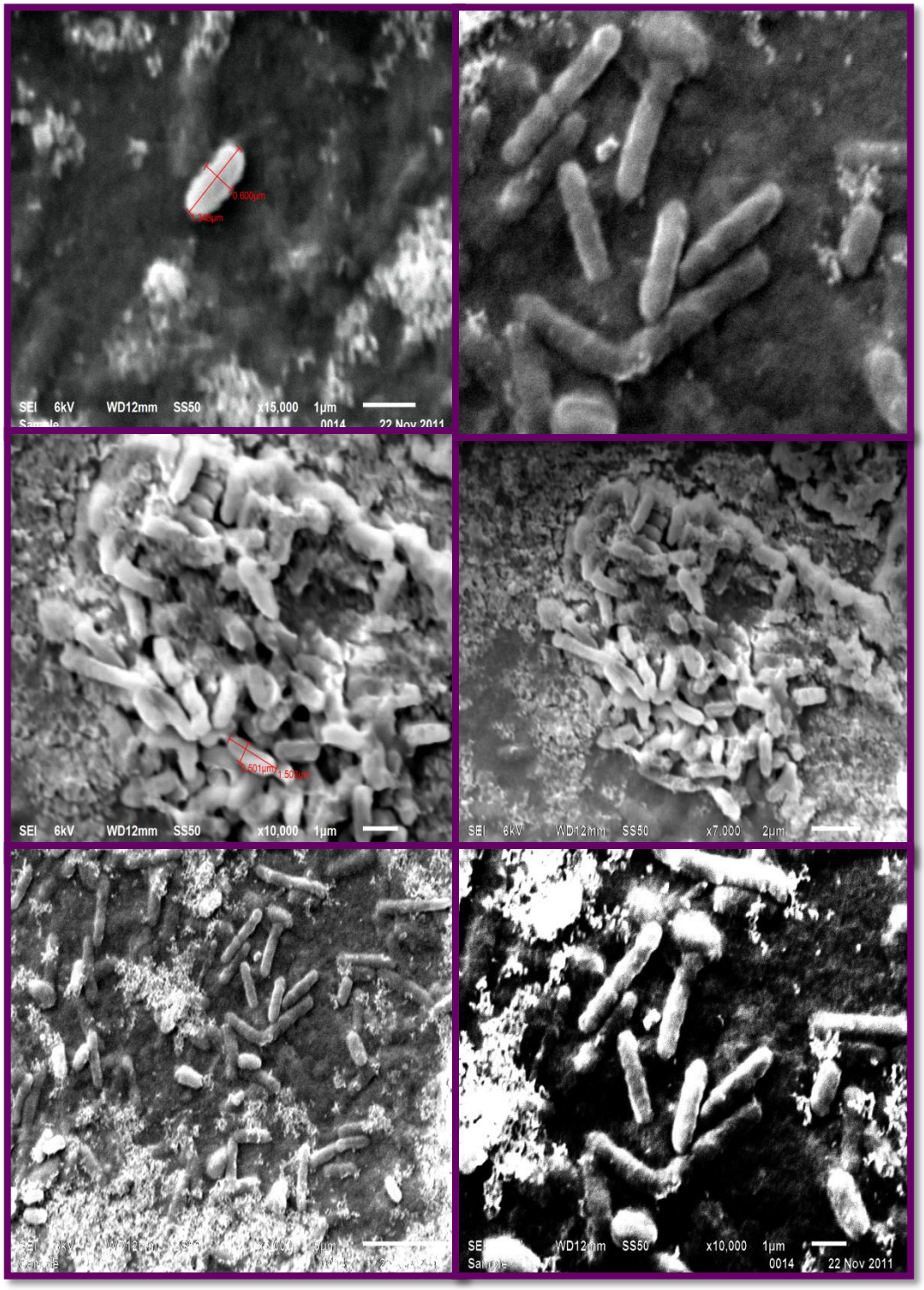


Plate 11

Scan electron microscopy: Straight rod shaped cells with rounded ends of *Erwinia chrysanthemi* at different magnification

Table 4.1: Biochemical, physiological and morphological characterization of isolates of *Erwinia chrysanthemi* isolated from infected sorghum plants

S. N.	*Ech* isolates	Gram Reaction	String formation in 3% KOH	Oxidase test	Deep pit on CPV medium Ψ	Blue pigment on NGM*	Sensitivity to erythromycin (15µg/disk)	Utilization of citrate	Growth at 37°C	Growth on Logan's medium
1.	Pantnagar -1	-	+	-	+	+	+	+	+	+
2.	Pantnagar -2	-	+	-	+	+	+	+	+	+
3.	Pantnagar UTMC-535	-	+	-	+	+	+	+	+	+
4.	Majra Farm	-	+	-	+	+	+	+	+	+
5.	Banjari farm	-	+	-	+	+	+	+	-	+
6.	Chinki Farm	-	+	-	+	+	+	+	+	+
7.	Sailanigot	-	+	-	+	+	+	+	-	+
8.	Tanakpur	-	+	-	+	+	+	+	+	+
9.	Khetalsanda	-	+	-	+	+	+	+	+	+
10.	Kashipur	-	+	-	+	+	+	+	+	+
11.	Haldwani-1 (HLD1)	-	+	-	+	+	+	+	+	+
12.	Kisanpur (Haldwani)	-	+	-	+	-	+	+	+	+
13.	Bajpur	-	+	-	+	+	+	+	+	+
14.	Doraha (Bajpur)	-	+	-	+	+	+	+	-	+
15.	Rudrapur	-	+	-	+	-	+	+	+	+
16.	Sultanpur (Kashipur)	-	+	-	+	-	+	+	+	+
17.	Tanda Kajal	-	+	-	+	+	+	+	+	+
18.	Gadarpur	-	+	-	+	+	+	+	+	+
19.	Barhani	-	+	-	+	+	+	+	-	+
20.	Sitarganj	-	+	-	+	+	+	+	+	+
21.	Nagina	-	+	-	+	+	+	+	+	+

+= Positive reaction, -= Negative reaction,

Ψ Size of deep pit was found variable it may be due to variability

* Most isolates produced blue pigment but some lacked

convex, smooth and entire, or sculptured with irregular margins, depending on the moisture content of the growth medium. Colonies become, round, raised centre and lobed periphery, which later becomes feathery or almost coralloid after 4-8 days. All isolates were morphologically similar in their appearance when incubated at 30°C for 48 h. They formed circular, translucent, slightly convex and entire colonies with regular edges, and mostly produce blue pigment on NGM medium, some isolates failed to produce pigment. The cells were Gram-negative and rod-shaped, with a peritrichous flagella arrangement. On the CVP medium, all isolates had circular, smooth and concave colonies with deep pit.

On the basis of these results, these 21 bacterial isolates were considered to belong to the soft rot group of bacteria of the genus *Erwinia* as it has been established by several investigators **(Kelman and Dickey, 1980)**. These set of biochemical and physiological testing has been recommended by several investigators: gram staining **(Gregeresen, 1978)** and string formation test in 3% KOH **(Suslow, *et al.*, 1982)**, oxidase test **(Cuppels and Kelman, 1974)**, sensitivity to erythromycin at 15µg/ml **(Jensen *et al.*, 1986; Dickey and Kelman, 1988)**, colony morphology on yeast dextrose calcium carbonate agar medium (YDC) **(Goto, 1979; Hseu *et al.*, 2008)**, and Growth at 37°C **(Pérombelon and Hyman, 1986)**. *Ech* is able to grow at all three temperatures; *Ecc* does so at 27 °C and 35.5 °C only, and *Eca* only at 27 °C **(Janse and Spit, 1989; Perombelon and Hyman, 1986)**. The soft rot erwinias are, as previously mentioned, motile straight rods with peritrichous flagella. Their ability to produce large quantities of pectic enzymes distinguishes them from other *Erwinia* species. Like other erwinias they are Gram-negative, facultatively anaerobic and do not form spores. Strains of *Erwinia carotovora* subsp. *atroseptica* usually lack fimbriae **(Christofi *et. al.*, 1979)** and pili. In contrast, in a majority of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* strains such structures are present **(Pérombelon and Kelman, 1980)**. When grown on nutrient agar, the pectolytic erwinias form round and greyish colonies **(Dowson, 1957)**, whereas on selective pectate media (e.g. CVP [**Cuppels and Kelman, 1974**]) they produce deep cavities containing iridescent, translucent and criss-crossed colonies with internal markings **(Lelliott and Stead, 1987)**. This group of bacteria are also

biochemically recognized by their positive catalase and negative oxidase reactions, their utilization of a wide range of carbon sources, polypectate degradation, absence of fluorescent pigment on King's B Medium, and the production of acid from salicin. In the diagnostic procedure for pectolytic erwinias presented by **Pérombelon and Hyman (1986)**, some of the distinguishing biochemical properties of this group and of the organisms *E. chrysanthemi*, *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* are concentrated into one test. Test organisms of the *Erwinia* '*carotovora*' group are recognized by the characteristic cavities that they form on selective CVP (crystal violet pectate) medium **(Cuppels and Kelman, 1974)**. The subsequent specific and subspecific differentiation is obtained by observing the pectolytic activity (on CVP) of the test organism at different temperatures. If cavities are formed at 27°C, the test isolate could be a strain of any of the three erwinias. Cavity formation at 33.5°C would eliminate the possibility of it being *E. carotovora* subsp. *atroseptica*, and at 37°C the only remaining possibility would be *E. chrysanthemi*. *E. chrysanthemi* is also distinguished by its inability to grow when erythromycin **(Graham, 1972)** is added to the CVP medium. **Pérombelon and Hyman (1986)** submit a possible explanation for the lower differential temperatures recorded on CVP than for growth on nutrient agar or nutrient broth. They suggest that the production by the bacteria of pectolytic enzymes is more sensitive to higher temperatures than is their growth on rich media. The CVP medium is highly sensitive. Small numbers of Erwinias present in plant tissue or in mixed cultures with other bacteria may be directly detected, but also identified (employing the differential temperatures and erythromycin). The proposed method is much less time-consuming and less labour-intensive than the regular biochemical tests. The latter are, on the other hand, more accurate, since a large number of features are observed. Difficulties might arise when the depressions formed on CVP are to be interpreted. Deep, cup-shaped cavities and characteristic colony morphology are the two identification marks of the soft rot Erwinias. Other pectolytic bacteria form shallower and wider cavities **(Cuppels and Kelman, 1974)**. However, certain strains or too little inoculum **(Pérombelon and Hyman, 1986)** of soft rot *Erwinia* isolates can also give shallow cavities. Furthermore, the CVP medium is easy to prepare **(O'Neill and Logan, 1975)**. However, the quality of the

polypectate in it must be checked, since it is able to adversely affect the consistency and cavity formation by erwinias **(Pérombelon *et al*., 1987)**. For a specific result, it is essential that incubation chambers do not fluctuate in temperature more than during the time of incubation **(Pérombelon and Hyman, 1986, and Pérombelon *et al*., 1987)**. As the bacterium produce water-insoluble blue pigment (indigoidine) **(Starr *et al*., 1966)** on NGM agar medium, it has been confirmed as *chrysanthemi* species several worker **(Lee and Yu, 2006; Jafari and Taghavi, 2007; Olabiyi, 2010)**. *E. chrysanthemi* is the only species in the genus *Erwinia* that is able to produce a water-insoluble blue pigment, known as indigoidine **(Starr *et al*., 1966)**. Accordingly, the formation of blue pigment, which imparts a blue color to bacterial colonies, can be used as a chemotaxonomic trait for rapid identification of *E. chrysanthemi*. However, the production of blue pigment is influenced by the composition of the growth medium and has become a variable trait among wild-type strains of *E. chrysanthemi* **(Starr *et al*., 1966; Reverchon *et al*., 2002)**. Such instability in pigment phenotype might result from the different growth media used. Thus, *E. chrysanthemi* is not usually differentiated from other *Erwinia* spp. on the basis of blue pigment production, and should be defined by several biochemical and physiological tests **(Verdonck *et al*., 1987)**.

4.3 Screening of biocontrol agents

4.3.1 Testing of antagonistic potential between *T. harzianum* isolates and *Ech* using dual culture method

The antagonistic potential of 21 isolates of *T. harzianum* was evaluated against the pathogen by dual culture assay (Table 4.2, Plate 12). In dual culture test, all the isolates reduced the colony growth of pathogen. Th-39 performed best which gave 90.40 % inhibition of radial growth followed by Th-R (89.6 %), Th-19 (87.7 %), Th-43 (79.6 %) and Th-31(76.6 %), whereas least inhibition was obtained with Th-75 (30.7 %). The isolates, Th-60 and Th-56 was found statistically at par with Th-75. The isolates found effective under *in vitro* conditions were further evaluated for efficacy under glasshouse conditions. Several researchers have successfully employed antagonistic bacteria, streptomyces and yeasts to control plant bacterial diseases **(Alivizatos and Pantazis, 1992; Ozaktan *et al*., 1999)**. One of the alternative control methods of *E. chrysanthemi* is the use of biological control

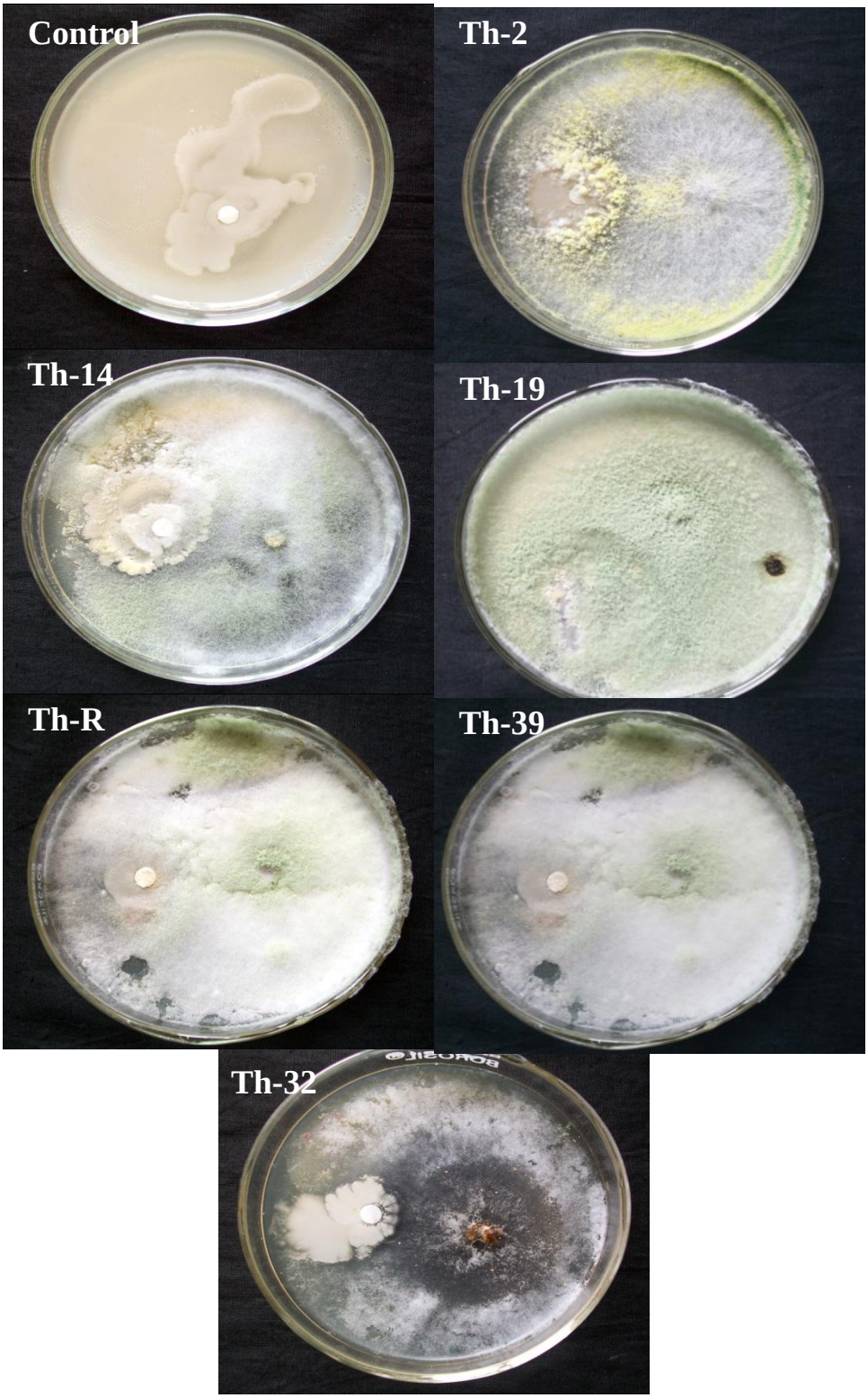


Plate 12
Evaluation of *T. harzianum* isolates against *E. chrysanthemi* using dual culture technique

Table 4.2: Efficacy of *T. harzianum* isolates against *Ech* under *in vitro* condition using dual culture method after 7 days

Treatment	Colony diameter (mm)	Reduction in colony diameter (%)
Th-2	15.0 (22.7)	83.3
Th-4	34.0 (35.6)	62.2
Th-5	41.6 (40.1)	53.7
Th-9	45.6 (42.5)	49.3
Th-10	41.6 (40.1)	53.7
Th-14	15.0 (22.7)	83.3
Th-18	56.0 (48.4)	37.7
Th-19	11.0 (19.2)	87.7
Th-21	40.0 (39.2)	55.5
Th-28	48.3 (44.0)	46.3
Th-31	21.0 (27.2)	76.6
Th-32	15.6 (23.2)	82.6
Th-36	40.6 (39.6)	54.8
Th-37	41.6 (40.1)	53.7
Th-38	53.3 (46.9)	40.7
Th-39	8.6 (17.0)	90.4
Th-43	18.3 (25.3)	79.6
Th-56	61.0 (51.3)	32.2
Th-60	59.3 (50.3)	34.1
Th-75	62.3 (52.1)	30.7
Th-R	9.3 (17.5)	89.6
Control	90.0	0.0
CD at 5 %	5.16	
SEM	1.81	
CV	9.32	

*Figures in parentheses are angular transformed values

agents. *Trichoderma* spp. inhibiting the growth of pathogens by the mechanism of antibiosis has been reported by several workers **(Wilhite *et al*., 2001; Thomashow *et al.* 2002).**

4.3.2 Testing of antagonistic potential between *P. fluorescens* isolates and *Ech* using dual culture method

Using dual culture method antagonistic potential of 12 isolates of *P. fluorescens* was evaluated against the pathogen *E. chrysanthemi.* The results (Table 4.3, Plate 13) showed that the most effective isolates in inhibiting the bacterial colony of *E. chrysanthemi* were Psf-3(91.8 %) and Psf-2 (90.7%). Least inhibition was obtained with Psf-11 (61.8%). *Pseudomonas fluorescens* F113 inhibited the growth of *E. carotovora* subsp. *atroseptica* under *in-vitro* conditions **(Cronin *et al*., 1997). Johnson *et al.* (2008)** has shown that antagonistic microorganism, *Pseudomonas aeruginosa* was found to be more effective in inhibiting the growth of the pathogen followed by *P. fluorescens*. **Snehalatharani and Khan (2009)** have found *P. fluorescens* producing an inhibition zone of 12.0, 11.0, 11.5 and 10.3 mm dia. in I1, I4, I7 and I9 isolates, respectively when tested against *Erwinia carotovora* sub sp *carotovora/ Erwinia chrysanthemi* under *in-vitro* conditions. Similar results were reported by **Shanmugam *et al.* (2003); Singh and Sinha (2005); Johnson *et al.* (2008)**. The ability of *P. fluorescens* to restrict the *in vitro* growth of disease causing microorganisms is well documented **(Thomashow and Weller 1988; Hebbar *et al.* 1991; Rosales *et al.* 1995)**. Several researchers have successfully employed antagonistic bacteria, *Streptomyces* and yeasts to control plant bacterial diseases **(Alivizatos and Pantazis, 1992; Ozaktan *et al*., 1999; Thomson and Gouk, 2003.)**. **Paternoster *et al.* (2010)** reported *Pseudomonas rhizosphaerae* as potential biocontrol agents against *E. amylovora* under *in vitro* conditions**.** Selection of potential biocontrol agents against *E. amylovora,* causal agent of fire blight of apple and pear has been seen based on screening for inhibition on artificial media **(**W**odzinski, *et al*., 1990; Psallidas *et al*., 1993; Bazzi *et al*., 2006)**, which lead to biocontrol agents able to produce antimicrobial compounds and therefore with a higher risk of non-target effects. Although traditional, applications of fluorescent pseudomonads in controlling plant diseases have been limited to seed or root treatments **(Burr *et al*., 1978; Capper and Hiigins 1993, Maurhofer *et al*., 1994; Alstorm 1995)**. Some investigators have applied fluorescent pseudomnads to foliage **(Leeman *et al.* 1995; Liu *et al*., 1995; Hoffland *et al*., 1996)**.

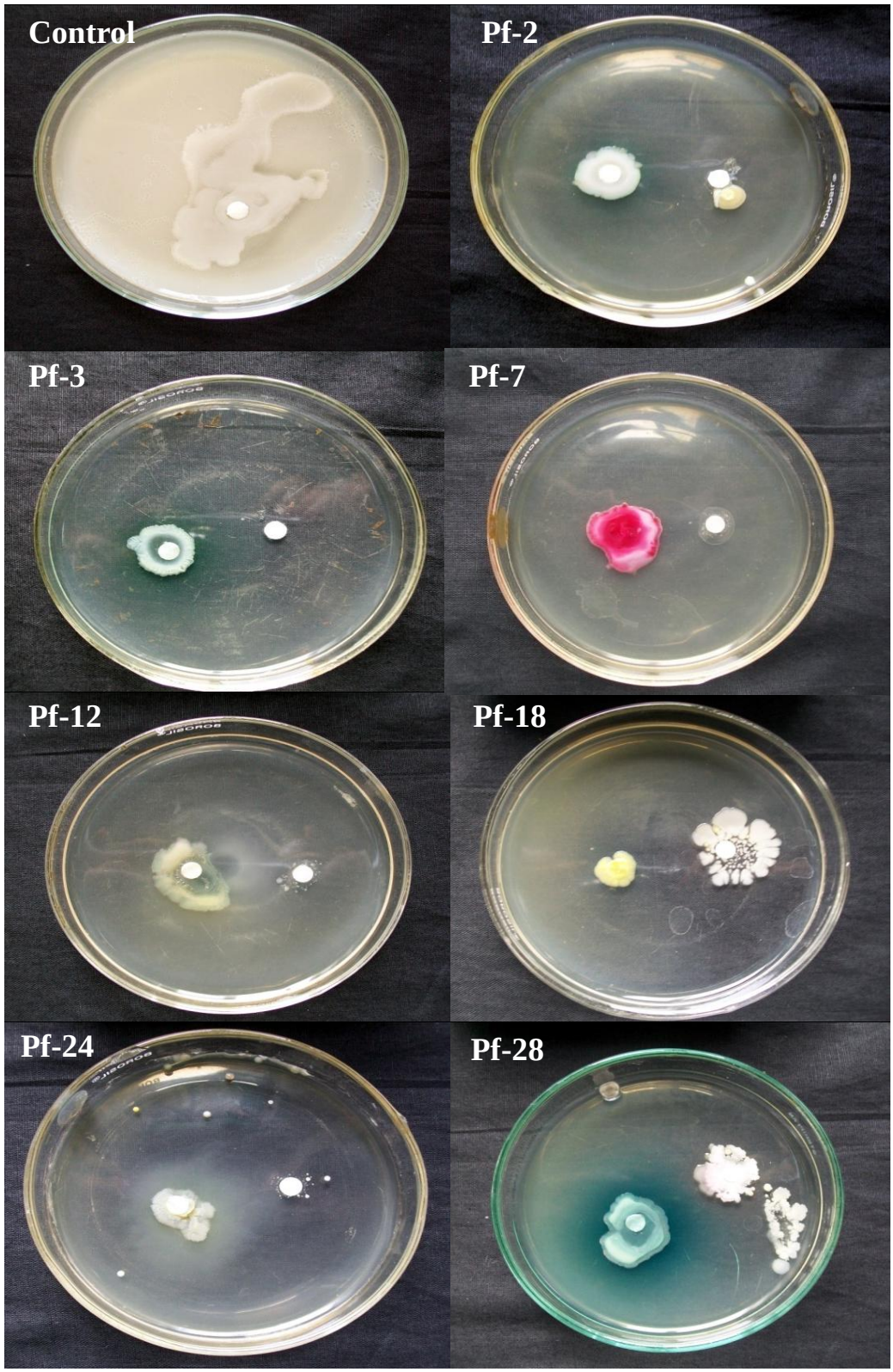


Plate 13
Evaluation of *P. fluorescens* isolates against *E. chrysanthemi* using dual culture technique

Table 4.3: Efficacy of *P. fluorescens* isolates against *Ech* under *in vitro* condition using dual culture method after 5 days

Treatment	Colony diameter of *Ech* (mm)	Reduction in colony diameter (%)
Psf-2	8.3 (16.6)	90.7
Psf-3	7.3 (15.6)	91.8
Psf-4	30.3(33.4)	66.3
Psf-6	23.6(29.1)	73.3
Psf-7	9.0 (17.4)	90.0
Psf-11	34.3(35.8)	61.8
Psf-12	10.6(19.0)	88.2
Psf-18	9.6 (18.0)	89.3
Psf-24	23.3(28.8)	74.1
Psf-25	29.0(32.5)	67.7
Psf-28	15.6(23.2)	82.6
Psf-31	29.0 (32.5)	67.7
Control	90.0 (71.5)	0.0
CD at 5 %	3.19	
CV	7.71	
SEM	1.09	

*Figures in parentheses are angular transformed values

4.4 Screening of chemicals

4.4.1 Screening of chemicals against *Ech in vitro* under using disk diffusion method

In sensitivity test (Table 4.4, Plate 14) of *Ech* to fungicide Blitox-50, Bleaching powder and 4 chemicals, Tetracyclin was found most effected followed by Streptocyclin at all concentrations. Maximum zone of inhibition was observed with Tetracyclin (3.80cm) followed by Streptocyclin (2.84cm), Streptomycin

Table 4.4: Efficacy of chemicals against *Ech* under *in vitro* condition using disk diffusion method

Treatment	Concentration (ppm)	Zone of inhibition (Diameter in cm∗)
Blitox-50	100	1.50
	200	1.77
	400	2.14
Chloramphanicol	100	0.23
	200	0.41
	400	0.62
Tetracyclin	100	2.40
	200	3.27
	400	3.80
Streptomycin	100	1.10
	200	1.57
	400	1.94
Bleaching powder	100	0.54
	200	0.80
	400	1.17
Streptocyclin	100	1.64
	200	1.94
	400	2.84
Control	-	0.00

CD at 5%	
Treatment a	0.22
Treatment b	0.15
a*b	0.38

∗Mean of three replications

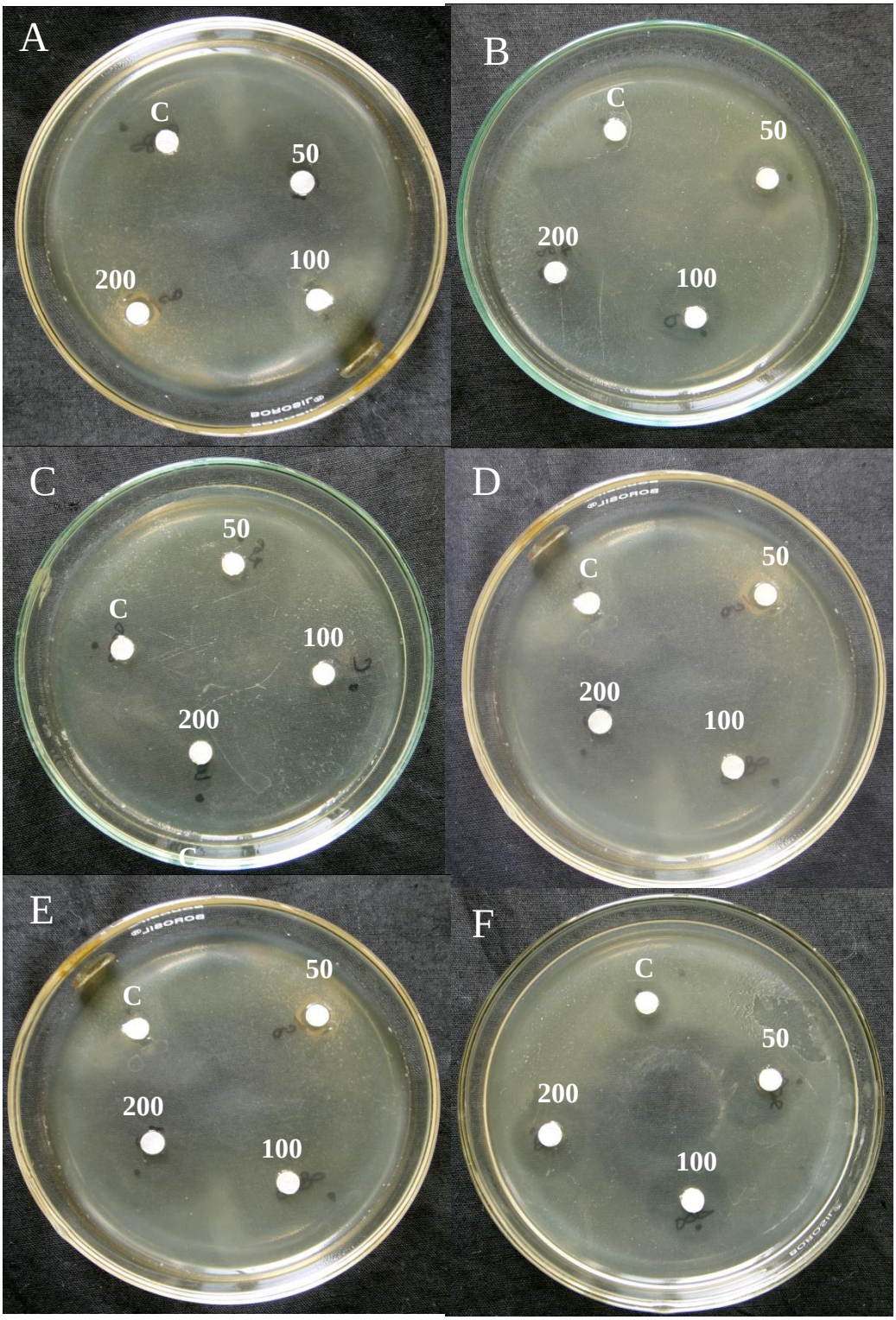


Plate 14

Evaluation of antibiotic against *E. chrysanthemi* using disk diffusion technique A.Blitox-50 , B. Chloramphanicol , C. Tetracyclin, D. Streptomycin, E.Bleaching Powder, F. Streptocyclin

(2.14cm) at 400 ppm concentrations. Least zone of inhibition (0.23cm) around the disk was observed with Chloramphanicol at 100 and 200 ppm. In *vitro* screening for the efficacy of different agrochemicals to inhibit the growth of this bacterium has been shown by several workers. Among them, oxylinic acid was the most effective **(Hseu, *et. al*., 2008)**. In India, a large number of chemicals have been evaluated against the soft rot bacteria both *in vitro* and *in vivo*. A good number of chemicals, Dithiocarbamates, Sulphur fungicides and Potassium permanganate have shown inhibitory effects *in vitro* **(Sabet, 1956; Thompson, 1965; Chakravarti and Rangrajan, 1966; Rangrajan and Chakravarti, 1969; Rangarajan and Chakravarti, 1970; Thind and Payak, 1972; Alberghina, 1974; Shina and Prasad, 1977, Randhawa *et al*., 1979)**. **Singh *et al.* (1980)** tested, chemicals, fungicides and stable Bleaching power against *Erwinia carotovora* subsp. *carotovora in vitro*, and found complete inhibition of bacterium by Streptomycin. **Sani and Prashar (1981)** found that 1000 ppm concentration of stable Bleaching power was inhibitory against *Erwinia carotovora* subsp. *Carotovora*. This pathogen also has been found sensitive to streptomycin, Tetercyclin, Ampicilin and Chloramphanicol but not Penicillin **(Mahmoud, *et al*., 1981)**.

4.5 Genomic diversity analysis

4.5.1 DNA fingerprinting of 21 *Ech* isolates using PCR based assay

PCR based assays i.e. rep- PCR were used to analyse the haplotypic distribution of isolates from different location of district U.S. Nagar (Uttarakhand). Banding patterns generated by the rep-PCR based assays were compared among all *Ech* isolates. All the 21 isolates differentiated into 21 distinct banding patterns (haplotypes) by the rep-PCR technique used. Reproducible DNA fingerprints were generated from total DNA of all isolates listed in Table 4.1. REP and ERIC primers resulted more discriminative than BOX in pointing out variability within *E. chrysanthemi* strains. A cophenetic value of >0.91 and 0.92 was determined for the similarity matrix, respectively, indicating a high goodness-of-fit for the cluster analysis. UPGMA analysis was performed by combining the data obtained from BOX-PCR, REP-PCR and ERIC-PCR. Therefore, the technique could be used with essentially equal efficiency. Representative genomic fingerprints are shown in Plate 15 and the corresponding dendrogram is shown in Fig. 4.1

4.5.1.1 DNA typing

Fingerprints generated by all the primers were compared among all *Ech* isolates. PCR analysis revealed clearly distinct pattern and 21 haplotypes were detected. The amplification of genomic DNA of 21 isolates of *Erwinia chrysanthemi*, followed by gel electrophoresis of resulting PCR products, showed 6 to 22 bands for the whole set of isolates, and discrete bands were scored, ranging in size from 0.25 bp to 4 kb. A total of 294, 357 and 231 clearly resolved bands were selected for composing the binary matrix (showing presence or absence of these bands) used for BOX-PCR, ERIC-PCR and REP-PCR analyzed by Jaccard coefficient and UPGMA, and a dendrogram displaying the distances between the 21 isolates (Fig. 4.1).

4.5.1.2.1 Electrophoretic profile with BOX primer

Box primer generated fingerprint pattern of unique haplotypes of *Ech* isolates collected from 21 locations are shown in (Plate 15A). Amongst the fingerprint largest DNA fragment of approximately 2.5kb and smallest of 100bp was observed. BOX primer amplified a minimum of 6 and a maximum of 21 bands (average 17-21 bands per isolates). Thirty two different band position and a total of 294 bands were scored. Some of the haplotytes had nearly identical box fingerprints (Plate 15A, lanes 6, 9, 18 and 20) and some had overall unique profiles but some common bands of equal mobility were observed (Plate 15A, lanes 10, 15, 16, 17 and 21). Haplotypes having multiple bands of apparent equal mobility suggested that these haplotypes had a common evolutionary history. For example, the four haplotypes of *Ech* (Plate 15A. lane 6, 9, 18 and 20) shared multiple comigrating bands but were different for the remainder of the bands generated. Haplotypes 6 and 9 were found to be highly similar but differed on the basis of 2 different band positions. Similarly, two other haplotypes (Plate 15A, lanes 18 and 20) shared most of the bands of equal mobility but differed on the basis of two band positions.

4.5.1.2.2 Cluster and Bootstrap analysis

UPGMA Cluster analysis of the fingerprints generated by BOX primer revealed the presence of four groups at 58% similarity level (Fig.4.1a). Cluster A consisting of 17 haplotypes was found the biggest. Cluster C and D were represented

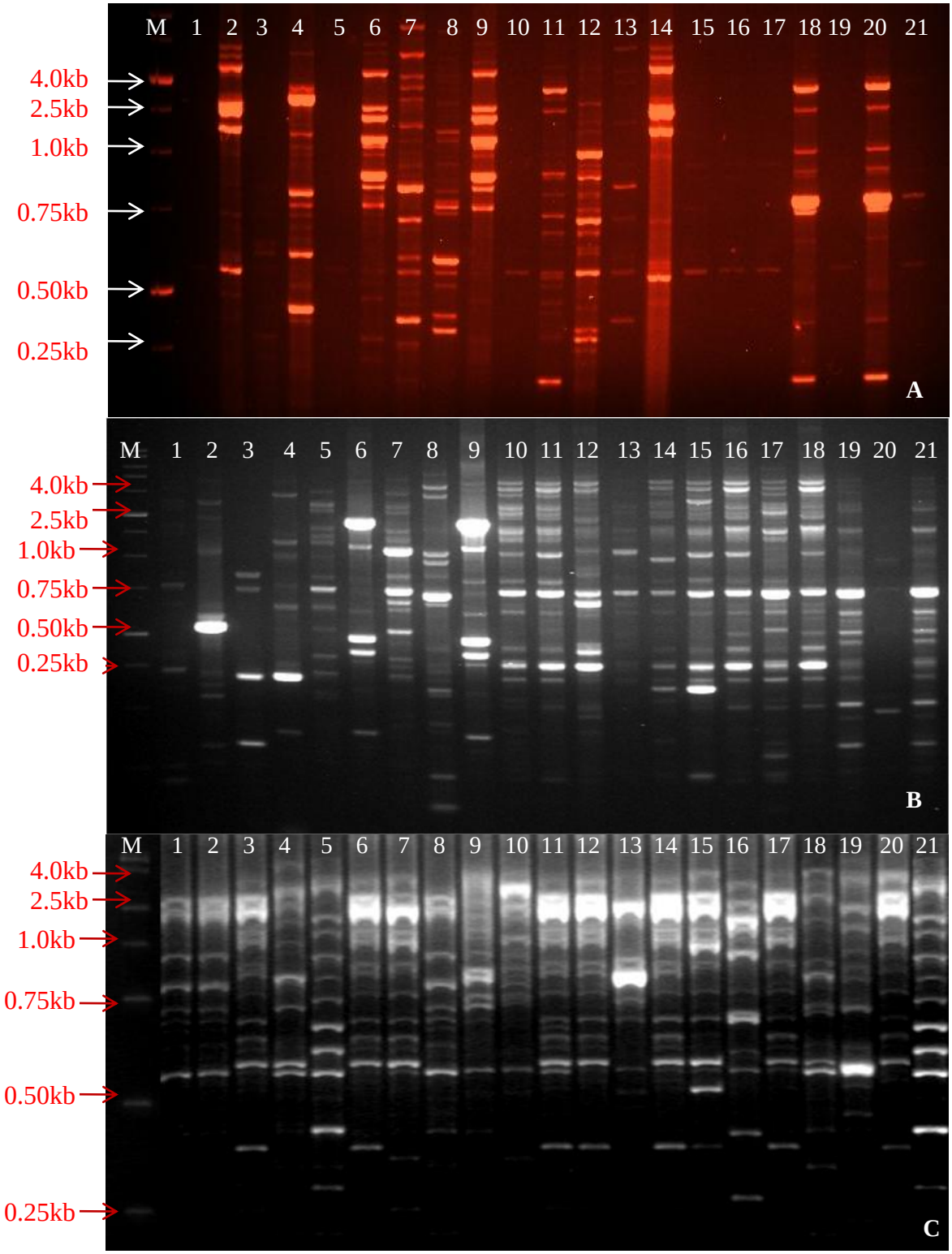
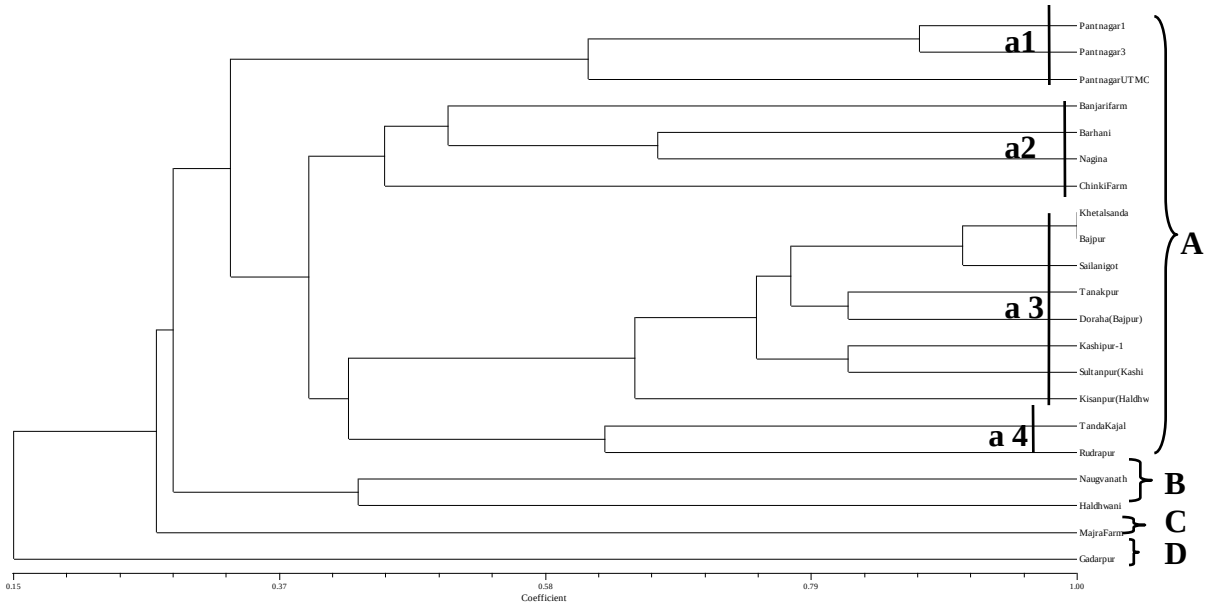
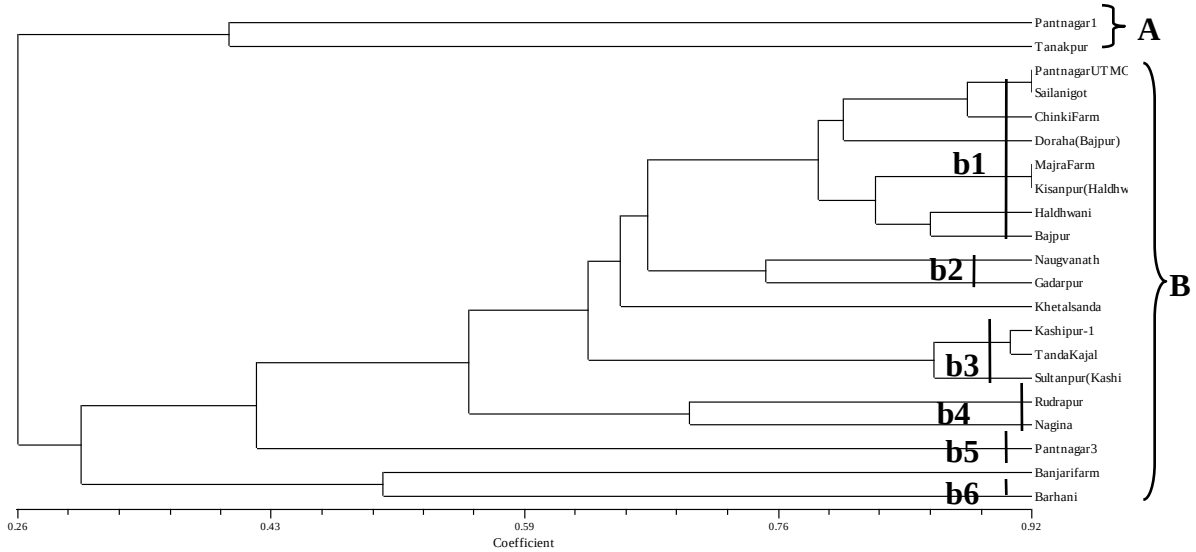


Plate 15

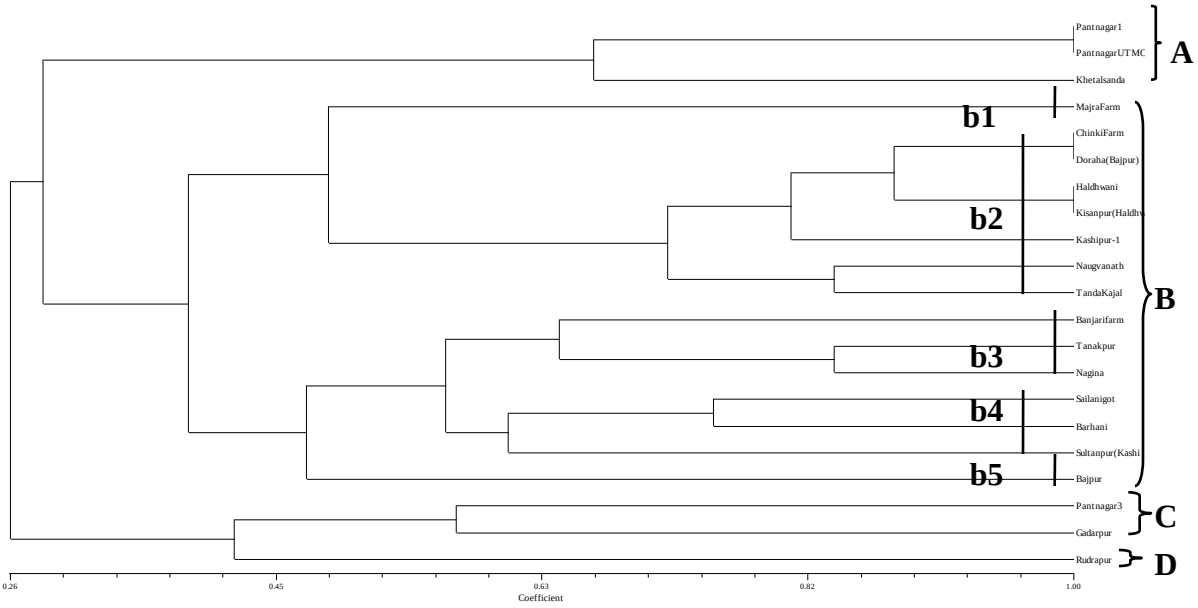
rep-PCR profiles [generated by A, BOX; B, ERIC; C, REP Primer (REP1 and REP2)] of *Ech.* M, molecular marker size (1-kb ladder). Sizes (in kilo base pairs) are indicated on the left. Isolates are indicated above by lane numbers.



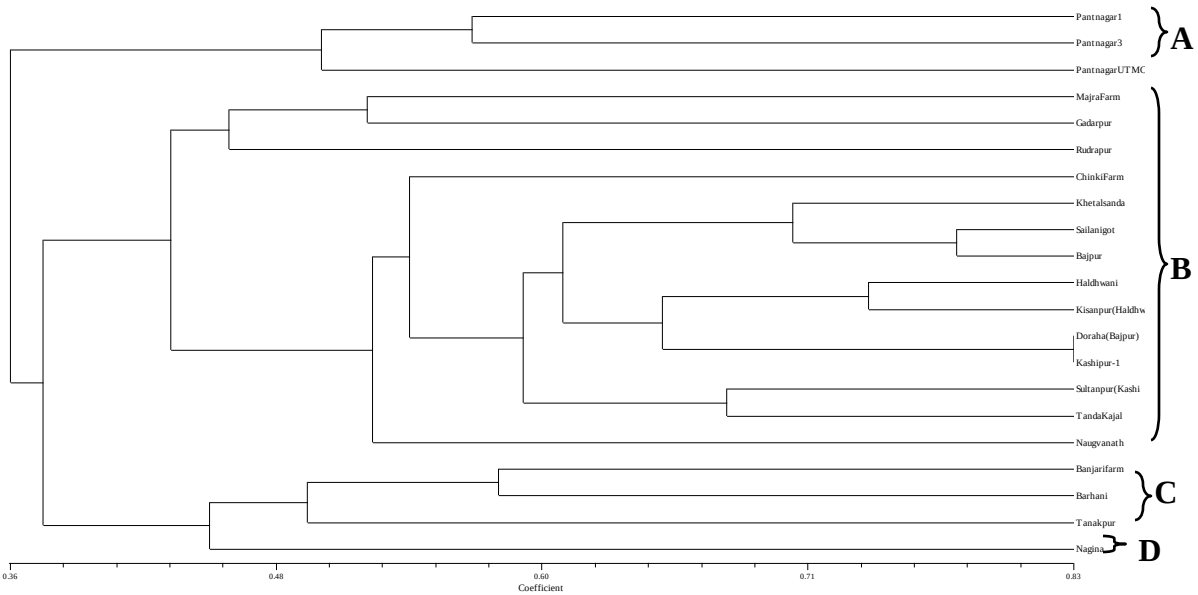
A) BOX-PCR



B) ERIC-PCR



C) REP-PCR data



D) Combined Rep-PCR data

Fig. 4.1: Dendogram constructed by UPGMA method, of unique haplotypes of *Ech* using (a) BOX (b) ERIC (c) REP-PCR data (d). Combined rep-PCR data. Bootstrap P values are indicated at the corresponding node of each cluster

by only single haplotype. Cluster B was represented by two haplotypes, showed 43% genetic similarity with each other. Cluster A was further divided into four sub clusters i.e. a1, a2, a3 and a4. Subcluster a1 and a2 showed 63% similarity to each other and consisted of three and four haplotypes, respectively. A single representative of cluster C showed approximately 25% similarity to cluster D. Only a2 A was found to be robust with a bootstrap P value of 90.6 to 100 % (Subcluster). Cluster D was not consistently grouped having low bootstrap value.

4.5.1.2.3 Similarity coefficient

Value of similarity coefficient varied from a minimum of 15% to a maximum of 100%. Maximum similarity (100%) was found between haplotypes 8 and 13 and a minimum of 15% was found between haplotypes 4 and 18.

4.5.1.3.1 Electrophoretic profile with ERIC primer

The amplification of genomic DNA of 21 isolates of *E. chrysanthemi*, followed by gel electrophoresis of resulting PCR products, showed 13 to 23 bands for the whole set of isolates, and a total of 357discrete bands were scored. The largest DNA fragment detected was approximately 4kb and the smallest was 100bp. Most of the DNA fragments generated by ERIC PCR were similar among the members of each group. However, distinct DNA polymorphisms in the region from 500 bp to approximately 4 kb was identified among different haplotypes. ERIC primer amplified a minimum of 8 and a maximum of 24 bands (average 17-21 bands per isolates). Some of the haplotytes had nearly identical ERIC-PCR fingerprints (Plate 15B, lanes 6, 9, 10, 11, 15, 16, 17, 18, 19 and 21) and some had overall unique profiles but some common bands of equal mobility were observed (Plate 15B, lanes 10, 15, 16, 17 and 21). Haplotypes having multiple bands of apparent equal mobility suggested that these haplotypes had a common evolutionary history. For example, the four haplotypes of *Ech* (Plate 15B, lane 6, 9, 18 and 20) shared multiple comigrating bands but were different for the remainder of the bands generated. Haplotypes 6 and 9 were found to be highly similar but differed on the basis of 2 different band positions. Similarly, two other haplotypes (Plate 15B, lanes 18 and 20) shared most of the bands of equal mobility but differed on the basis of two band positions. Isolates in group A displayed maximum five bands in this region and a

very intense first band position was observed while Group B and C isolates displayed only three bands. Representatives of Group F displayed a maximum of four and a minimum of three band positions in this region. Single representative of groups C and G displayed one and two bands in the same region. Members of group A also displayed a continuous stretch of four to five bands in the region between 350 to 250 bp. BOX patterns of Group A isolates also displayed a stretch of 5 bands slightly above from that region which were of equal mobility. Isolates having bands of equal mobility clearly indicated that they had a common evolutionary heritage.

4.5.1.3.2 Cluster and bootstrap analysis

Cluster analysis of ERIC patterns generated binary data showed two clusters at 64% similarity level (Fig 4.1B). Cluster B was the biggest and composed of six haplotypes. Cluster A was consisted of two haplotypes. Cluster B was further subdivided into six sub clusters, b1, b2 b3 b4 b5 and b6, which were related to each other at approximately 80% similarity level. Cluster B was found only robust with a bootstrap P value of 92%. Other groups were not robust.

4.5.1.3.3 Similarity coefficient

Jaccards similarity cofficients for all the pairwise combinations showed a maximum similarity of 92% between representatives of cluster B (b1) i.e. 2 and 9, and between 4 and 12. Similarly, a maximum similarity of 90 % was found between the representatives of cluster (b 4) i.e. 15 and 17. A minimum similarity of 32 % was found between haplotypes 1 and 10.

4.5.1.4.1 Electrophoretic profile with REP primer

The number of scorable bands amplified with REP primers ranged from a minimum of 14 and a maximum of 20 bands per isolates (average 16 bands per isolates). All bands are well-separated in agarose gel. A total of 231 bands were scored. The largest DNA fragment detected was approximately 4kb and the smallest was approximately 100bp. There were 34 different band positions scored in the PCR-based fingerprints. The sizes and distribution of the REP PCR products of six haplotypes (Plate 15C. lanes 3, 5, 7, 11, 15, 17, 20 and 21) were found to be highly similar i.e only minor differences were observed, suggesting that these isolates were

very closely related. Similarly, pattern of two haplotypes (lane 5 and 21) were almost found similar with product size of approximately 4.0 kb differentiated these patterns.

4.5.1.4.2 Cluster and bootstrap analysis

Cluster analysis grouped the isolates into three clusters (A, B and C) at the demarcation of 67% genetic similarity level (Fig 4.1C). The major cluster B comprised of 15 haplotypes. The members of this cluster showed approximately 82 to 100% genetic similarities with each other. Cluster A and C was further subdivided into two minor clusters, showing approximately 65% similarity to each other and was represented by three haplotypes in each. Cluster B consisted of three haplotypes and showed a genetic similarity of approximately 32% with a single representative of cluster A. Clusters A and B was found to be robust with a bootstrap value of 100%. Other clusters with lower bootstrap P values were not found to be robust.

4.5.1.4.3 Similarity coefficient

Jaccard similarity coefficient for all the pairwise combinations showed maximum similarity of 82 to 100% among the members of cluster B and a minimum of 28% was found between haplotypes 21 and 16.

4.5.1.4.1 Combined cluster analysis

A separate phenogram for combined rep-PCR was also prepared and the reliability of the groups was measured by INTYSIS program. Phenograms generated by the PCR analysis and combined cluster analysis detected the same type of grouping. With combined data three clusters were formed at 65% similarity level. Cluster A consisted of three haplotype, whereas, cluster B and C contained four and two haplotypes, respevtively. The clusters B was found highly robust with a bootstrap P value of 100% and for cluster A and C were 55, 60% respectively in combined rep-PCR analysis. Combined dendogram from the data obtained from the rep-PCR analysis detected the same kind of grouping, which were found to be highly reliable (Fig. 4.1 D).

4.5.1.4.2 Similarity coefficient

Jaccards similarity coefficient was calculated from the combined data of the rep-PCR analysis. Maximum similarity (100 %) was found between haplotypes 14

and 15. Minimum similarity (36%) was found between haplotypes 10 and 21**.** Although bacteria are traditionally identified by phenotypic descriptions, it is not possible to obtain from nutritional studies a battery of discriminating substrates to the genomospecies as related by **Gardan *et al.* (1999)**. Rep-PCR generates DNA fingerprints that allow the discrimination of bacterial strains **(Versalovic *et al*., 1991, 1994)**. The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive sequence elements dispersed throughout the bacterial genome. Palindromic units, or REP elements, and ERIC sequences are the most commonly used targets for DNA typing **(Gilson *et al*., 1984; Hulton *et al*., 1991** and **Stern *et al*., 1984)**. Rep-PCR has been demonstrated to be a useful typing technique for a variety of bacteria. Interspersed noncoding repetitive DNA sequences have been preferentially found in gram-negative bacteria **(Versalovic *et al*., 1991)**. Linkage between rep-PCR results and the geographic origin of bacterial strains has been recognized in various studies **(Scortichini *et al*., 2001; Mkandawire *et al*., 2004). Louws *et al*. (1994)** believe that one of the important reasons for this phenomenon is that the selection for one geographically suitable area can have influence on the genetic map of bacterium and also dispersion of these repetitive units in the genome of bacterium. **McManus and Jones (1995)** have been also determined genetic fingerprint of 189 strains of *Erwinia amylovora* isolated from tree-fruit crops and *Rubus* spp. by using rep-pPCR technique. **Bielsa *et al*. (2012)** have been also characterize *Erwinia* spp. from pome fruit trees to pathogenic and non-pathogenic species by using rep-pPCR technique. There was a considerable variation in gene electrophoretic clusters among the strains. These findings agree with Louws justification on Rep-PCR and its high efficiency towards discrimination of pathogen population diversity **(Louws *et al*., 1994)**. The BOXA1R primer used in rep-PCR is complementary to one of three subunits in the BOX element, a conserved repetitive sequence present in high copy number on the chromosomes of both gram-negative and gram-positive organisms **(Martin *et al*., 1992 and Versalovic *et al*., 1994)**. As such, it can be used to generate specific genetic fingerprints that can indicate the homogeneity or heterogeneity of a given bacterial population. Failure to detect a common band for all *E. chrysanthemi* strains in this study was not unexpected, because the genomic heterogeneity of this species

has been documented **(Boccara *et al*., 1991; Norman *et al*., 2003 and Kaneshiro *et al*., 2008)**. By using rep-PCR, similar results were obtained by **McManus and Jones (1995).** Also in their study all the strains from *Rubus* spp. (*Rosoideae*) clustered separately from the strains from Maloideae, even if ARDRA analysis carried out with HaeIII did not reveal any diversity in the restriction pattern between the two subgroups. In the present work, the three strains from *Rubus* spp. assessed clustered differently upon UPGMA analysis. It is worth noting that by using REP primer any difference between NCPPB 2292 and NCPPB 2293 was observed. Conversely, the utilization of ERIC primer allowed to discriminating between the two strains. The utility of a recently developed method to classify bacteria on the basis of their genomic fingerprint patterns was investigated, using collections of *E. chrysanthemi*. The genomic fingerprinting method employed is based on the use of DNA primers corresponding to naturally occurring interspersed repetitive elements in bacteria, such as the REP, ERIC and BOX elements, and the PCR reaction (rep-PCR). The results show that rep-PCR fingerprinting is a highly reproducible and simple method to distinguish closely related strains, to deduce phylogenetic relationships between strains and to study their diversity in a variety of ecosystems. Ninety-three strains obtained from 12 plant genera and different geographical locations were examined by repetitive-sequences PCR using Enterobacterial Repetitive Intergenic Consensus, BOX and Repetitive Extragenic Palindromic primer sets. The strains from *Maloideae* exhibit a wider genetic variability. The RFLP analysis of a fragment of the pEA29 plasmid would not seem a reliable method for typing *E. amylovora* strains **(Barionovi *et al*., 2006)**. **Sahilah *et al*. (2008)** generated the dendrogram from the ERIC-PCR fingerprinting showed that the *E. chrysanthemi* strains formed 4 clusters and 7 single isolates at 80% similarity level. In comparison ERIC-PCR fingerprinting, restriction fragment length polymorphism (RFLP) analysis for 16 strains of *E. chrysanthemi* with *Hinf* I and *Hae*III endonuclease, 2 and 4 restriction profiles were obtained, respectively. He also found that ERIC-PCR fingerprinting method is more discriminating and useful for the determination of the *E. chrysanthemi* strains relatedness. The combinations of the four techniques were able to differentiate the 16 *E. chrysanthemi* strains into 14 genome types, suggesting a wide diversity of strains examined **(Sahilah *et al*., 2008)**. Long after **Louws *et al.***

(1994) claimed BOX analysis could discriminate pathovars of *P. syringae*, it now appears that the authors were dealing with three different genomospecies: pv. *morsprunorum* (genomospecies 2), pv. *syringae* (genomospecies 1), and pv. *tomato* (genomospecies 3). According to **Louws *et al.* (1995)** BOX-PCR was able to distinguish strains A and B of *X. campestris* pv. *vesicatoria*, separated by DNA-DNA hybridization by **Stall *et al.* (1994)**, and further named *X. axonopodis* pv. *vesicatoria* (ex A) and *X. vesicatoria* (ex B) **(Vauterin *et al.*, 1995)**.

4.6 Glasshouse experiments

4.6.1 Evaluation of inoculation techniques

Results presented in Table 4.5 and Plate 16 reveal that all methods produced stalk rot symptoms with variable and non-uniform pattern, except the root tip cut & dip method in which all inoculated plants showed most typical, reliable and uniform disease development. The disease mainly affected sorghum stem showing water-soaked symptoms that later turned reddish dark brown color. The infected stem pith disintegrated and showed slimy soft-rot symptoms with foul-smell and eventually the whole plant wilted. The apical leaves of wilted plant gave ashy appearance. Lower leaves and Typical stalk rot type of symptoms were first observed in root tip cut & dip method; water-soaked lesions appeared at the crown or soil line of stalk on 3rd day of inoculation although the upper portion remained asymptomatic. As the disease advanced, all the plants collapsed on 4th day of inoculation. However, symptoms started appearing after 4th days of inoculation in other methods. Highest stalk rot severity (92.40 %) with typical rotting, wilting and uniform type disease development was found in case of root tip cut methods followed by stem injection (85.96%), leaf-whorl (80.60%), and tooth-pick (74.76%) method of inoculation. Partial rotting was encountered with midrib injection (29.22) and cotton wool method (22.57%) over control. Inoculation with bacterial suspension in leaf-whorl and through hypodermic needle in punctures at the base of stalks of sorghum plants has been used (**Zummo, 1969; Jensen, 1986; Saxena *et al.*, 1990**). A rapid whorl inoculation technique recommended by **Hartman and Kelman (1973)** in corn for *Erwinia* soft rot was successfully used in sorghum plants also **(Hepperly *et al.*, 1987)**. Suspension of bacterium cells have also been infiltrated into leaf tissues of maize plants and stems below the first leaf whorl with a syringe **(Goszczynska *et al.*, 2007)**. Results showed

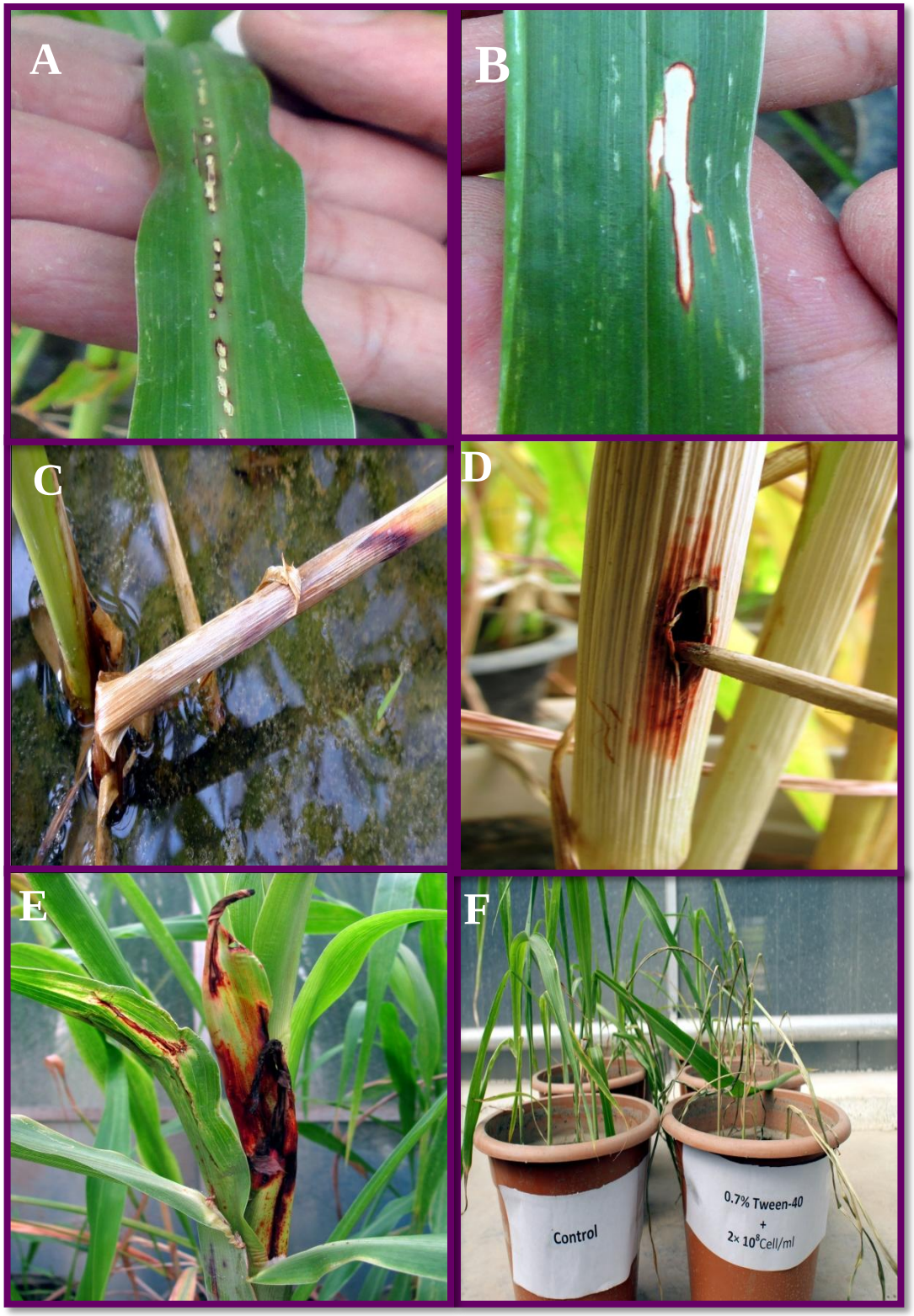


Plate 16

Symptom development of Erwinia stalk rot of sorghum by artificial inoculation methods A. Midrib injection method, B. Cotton wool method, C. Stem injection method, D. Tooth-pick method, E. Leaf-Whorl inoculation method, F. Root tip cut & dip method

varying extent of disease development by stem injection method that received different concentrations of test pathogen in sorghum and other crops by various investigators **(Anderson and Gardner, 1999; Kutama *et al*., 2011; Sobowale, 2011)**. Artificial inoculation by stem pricking method, midrib injection method, leaf pricking method of inoculation with *Ech*, has been found effective in symptom development of bacterial stalk rot of sorghum **(Hseu *et al*., 2008)**. The toothpick inoculation technique has been used to screen germplasm against sorghum and maize pathogens **(Bramel-Cox *et al*., 1988; Bramel-Cox and Claflin, 1989; Clements *et al*., 2003; Tesso *et al*., 2009; Sobowale, 2011)**. Inoculation via root wounding with a bacterial (*Ralstonia solanacearum*) water suspension in Kahili ginger (*Hedychium gardnerianum*), an invasive weed, has been shown by **Anderson and Gardner (1999)**. The root tip cut & dip method of inoculation used in this investigation does simulate most probable natural condition of infection to occur in many respects and is an efficient means of getting consistent and uniform infection of sorghum plants with *Ech*. In natural conditions, roots get damaged when growing or penetrating the soil layer, to obtain required nutrients material. This damaged or wounded point can serve as mode of entry for the bacterium, as most do so. As this method gives uniform disease development in a quick time, it can also be used for determination of virulence and races of the pathogen in root rot type of diseases and plants in which transplanting is possible.

Table 4.5: Percent disease severity in artificial inoculation methods after 7 days of inoculation

Methods of artificial inoculation	Disease severity (%)
1.Midrib injection method	29.22(32.95)
2.Cotton wool method	22.57(27.68)
3.Tooth-pick method	74.76(59.78)
4.Stem injection method	85.96(68.06)
5.Leaf-Whorl inoculation method	80.60(64.15)
6.Root tip cut & dip method	92.40(73.88)
7.Control	00.00
CD at 5%	4.87
SEM	1.50

*Figures in parentheses are angular transformed values

4.6.2 Testing of *E. chrysanthemi* isolates for their aggressiveness and virulence variability analysis

Measurements of disease severity revealed existing variability in virulence and aggressiveness for each *E. chrysanthemi* isolates (Table 4.6). All isolates of stalk rot bacterium proved to be highly pathogenic, causing a rapid soft rot of parechymatous tissues on all inoculated plants or plant parts (leaf whorl and stem) water-soaked and discoloration of tissues (wet, glassy, yellow to brown steam lesions) around the inoculation court on sorghum plants were evident after 4 days of inoculation. Soft-rot symptoms produced by different isolates varied in lesions size (or necrotic areas covering much of the surface of stems). After 5-10 days, extensive invasion of stem tissues were evident and plants toppled over. Symptoms in general resembled those observed in field conditions. Leaf whorl-inoculated plants develop symptoms after 5 days. Symptoms were curling, yellowing, and wilting of leaves, red discoloration of main vein, sometimes wet, glassy to brown necrotic leaf spots, later with a white, sunken necrotic centre malformation of newly formed leaves, and finally a total yellow-brown necrosis and death of plant. When infected leaves were pulled, they separated from stalk at the point of intense bacterial rot near the stem's apex. The disjointed apex appeared as a mushy foul smelling whip. The following three stages of symptoms were observed: (a) flaccid leaves; (b) greasy leaves and finally (c), withered leaves. Stalk appeared to proceed from the apical center, move downward and laterally. Significant differences in disease levels were observed among several of the strains on sorghum plant. It is evident from data shown in Table 4.6 Plate 17, that after 5 days of inoculation maximum lesion length (29.50mm) was produced by isolate *Ech*-20 followed by *Ech*-18 (29.00mm), *Ech*-2(28.66mm) ≈ *Ech*-21(28.66mm), *Ech*-11(28.00mm), *Ech*-17(27.66mm), *Ech*-16 (24.66mm) and *Ech*-15 (23.66mm). Least lesion length (12.33mm) was produced by isolate *Ech*-1. None the isolates of *E. chrysanthemi* were able to produce top rot and lodging type of symptoms after 5 days of inoculation. After 10 days of inoculation, maximum lesion length (55.00mm) was produced by isolate *Ech*-18 followed by *Ech*-16 (51.66mm), *Ech*-21(48.33mm), *Ech*-20 (43.00mm) and *Ech*-1(41.00mm). Least lesion length (17.00mm) was produced by isolate *Ech*-10. Maximum severity (62.50%) of top rot was observed with *Ech*-12 followed by *Ech*-10 (50.00%). Four isolates of *Ech* viz. *Ech*-3, 8, 11 and 20 were able to produce equal amount disease severity (37.50%).

Plate 17

• Aggressiveness of 21 isolates of *E. chrysanthemi* under glasshouse conditions after 5 days of inoculation

•Wet, glassy to brown necrotic leaf whorl, and wet, glassy, yellow to brown steam lesions with a white, cracked necrotic centre on stem tissue. Control (22) plant inoculated with sterilize water.

Maximum percentage (75.00%) of lodging was caused by *Ech*-12 after 10 days of inoculation. Some isolates were not able to produce top rot and lodging type of symptoms after even10 days of inoculation. The rate of symptoms (disease) progress was found approximately static after 10 days of inoculation; it may be due to saturation of infection sites, negative competition among bacterial cells for occupying more space and nutrients. Some *E. chrysanthemi* isolates was found not able to produce top rot and lodging type of symptoms even after 20 days (Plate 18) of inoculation viz. *Ech*-4, *Ech*-5, *Ech*-9, *Ech*-13, *Ech*-14, *Ech*-15, *Ech*-16, *Ech*-17, *Ech*-19 and *Ech*-20. In virulence variability analysis, most of the *Ech* isolates were found least virulent in producing disease except *Ech*-12 (53.37 % disease severity) which was found most virulent. Four isolates (*Ech*-3, 8, 10 and 11) were found moderately virulent. Some isolates (*Ech*-15,16 and17) were not able to produce typical symptoms of top rot and lodging even after 20 days of inoculation, therefore, they cannot be categorized for virulence reaction but were found moderately aggressive (*Ech*-15 and *Ech*-16) and most aggressive (*Ech*-17). In aggressiveness analysis, only five isolates (Ech-1, 17, 18, 20 and 21) were found most aggressive, rest other isolates were found moderately aggressive (Table 4.7). Similarly, some isolates were least virulent (percept disease severity) but found most aggressive (host tissue colonization) viz. *Ech*-1, 18, 19, 20 and 21. In Plant Pathology the definitions proposed by **Vanderplank (1968)** had a long-standing influence: the quantitative negative effect of a pathogen on its host was named aggressiveness, while the term virulence was used to describe the capacity of a pathogen to infect a particular host genotype, what in evolutionary biology is usually termed infectivity **(Gandon *et al*., 2002; Tellier and Brown, 2007)**. Restricted gene flow in both the parasite and the host, resulting in genetic differentiation of their populations, is a prerequisite for local adaptation, although gene flow between parasite subpopulations increases the potential for local adaptation, as long as migration does not homogenize populations **(Gandon *et al*., 1996; Gandon and Michalakis, 2002; Kawecki and Ebert, 2004; Morgan *et al*., 2005)**. Soft rot results from the general disorganization of plant tissues following the degradation of pectin, the major component of primary cell walls. Bacterial soft rots are diseases difficult to control because of the ubiquity of the soft-rot Erwiniae. They are widespread in surface water (**Cother *et al*., 1992)**; they are competitive

Table 4.6: Screening 21 isolates of *Ech* for their aggressiveness and virulence variability analysis under glass house conditions using stem and leaf whorl inoculation methods

Isolates	Progress of disease (day after inoculation)											
	5 days			10days			15 days			20days		
	Lesion Size (mm)	Top rot (%)	Lodging (%)	Lesion Size (mm)	Top rot (%)	Lodging (%)	Lesion Size (mm)	Top rot (%)	Lodging (%)	Lesion Size (mm)	Top rot (%)	Lodging (%)
1.Pantnagar -1	12.33	00.00	00.00	41.66	12.33	25.33	47.33	14.73	27.93	69.33	16.10	28.60
2.Pantnagar -2	28.66	00.00	00.00	38.00	00.00	37.83	40.33	00.00	40.50	43.00	00.00	41.60
3.Pantnagar UTMC-535	23.00	00.00	00.00	40.00	37.83	37.83	45.00	40.60	42.70	45.80	42.66	44.80
4.Majra Farm	14.33	00.00	00.00	40.00	12.50	00.00	35.66	16.50	00.00	42.00	17.50	00.00
5.Banjari farm	18.66	00.00	00.00	34.33	00.00	12.50	36.00	00.00	15.90	38.00	00.00	17.53
6.Chinki Farm	23.00	00.00	00.00	30.66	12.50	12.50	36.00	14.60	18.60	36.80	16.20	19.60
7.Sailanigot	12.66	00.00	00.00	35.00	25.00	12.63	30.66	27.33	19.16	37.70	29.83	20.96
8.Tanakpur	15.33	00.00	00.00	22.33	37.43	25.00	41.66	41.50	26.40	42.40	42.80	27.43
9.Khetalsanda	20.33	00.00	00.00	40.33	12.63	00.00	38.00	14.70	00.00	41.30	17.63	00.00
10.Kashipur	15.33	00.00	00.00	17.00	50.00	12.50	36.70	54.80	18.50	48.66	57.26	19.30
11.Haldwani-1 (HLD1)	28.00	00.00	00.00	27.66	37.50	12.50	46.00	39.50	20.50	47.80	41.63	22.60
12.Kisanpur (Haldwani)	17.66	00.00	00.00	31.00	61.93	75.00	45.33	66.00	77.60	49.40	67.30	78.50
13.Bajpur	19.33	00.00	00.00	39.66	00.00	12.16	44.33	00.00	16.30	48.40	00.00	20.20
14. Doraha (Bajpur)	15.66	00.00	00.00	31.00	25.00	00.00	50.33	30.80	00.00	53.26	29.80	00.00
15.Rudrapur	23.66	00.00	00.00	22.66	00.00	00.00	34.66	00.00	00.00	49.80	00.00	00.00
16.Sultanpur (Kashipur)	24.66	00.00	00.00	51.66	0 0.00	00.00	57.66	00.00	00.00	61.26	00.00	00.00
17. Tanda Kajal	27.66	00.00	00.00	35.33	12.50	00.00	53.00	16.70	00.00	58.59	18.50	00.00
18. Gadarpur	29.00	00.00	00.00	55.00	25.00	12.10	51.50	28.30	15.70	69.33	30.63	16.00
19.Barhani	19.66	00.00	00.00	37.66	25.00	00.00	37.00	28.50	00.00	46.80	28.30	00.00
20. Sitarganj	29.50	00.00	00.00	43.00	37.86	00.00	50.00	40.70	00.00	52.59	41.23	00.00
21. Nagina	28.66	00.00	00.00	48.33	12.40	12.93	58.66	17.70	15.30	61.46	18.10	18.80
Control	-	-	-	-	-		-	-		-	-	-
CD at 5%	13.12			7.85	4.21	3.49	15.71	3.80	4.09	7.69	3.80	4.97

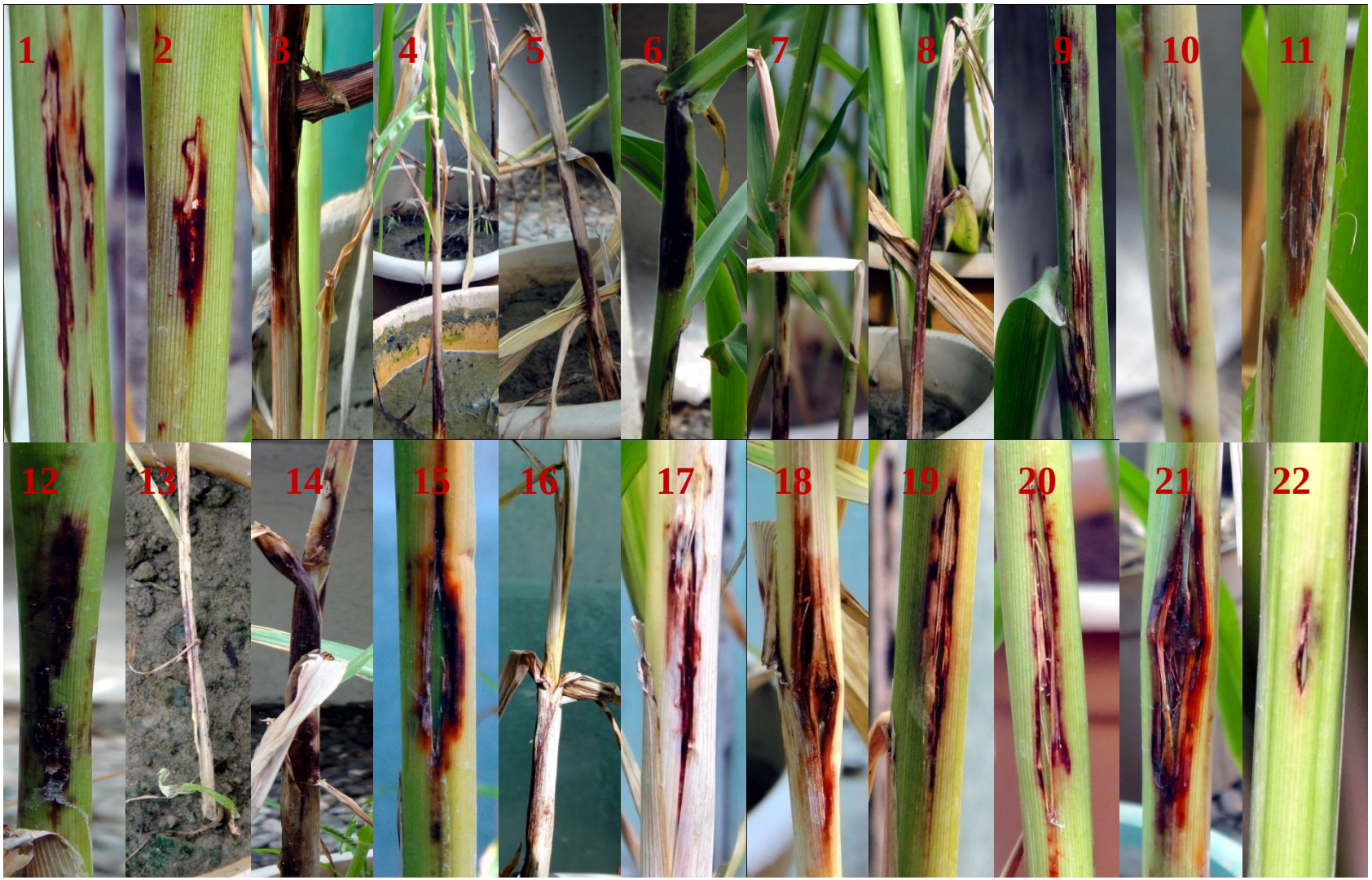


Plate 18

- Aggressiveness of 21 isolates of *E. chrysanthemi* under glasshouse conditions after 20 days of inoculation
- Wet, glassy, yellow to brown, later with a white, sunken necrotic steam lesions and lodging of plants
- Control (22) plant inoculated with sterilize water.

Table 4.7: Aggressiveness of *E. chrysanthemi* isolates on sorghum plants, assessed by the stem & leaf-whorl inoculation method

Isolates	Aggressiveness and virulence variability analysis					
	Mean				Rating	
	Lesion* size (mm)	Top* rot (%)	Lodging* (%)	Top rot + Lodging (%)	Virulence	Aggressiveness
1.Pantnagar -1	42.66	10.80	20.50	15.66	Least virulent	Most aggressive
2.Pantnagar -2	37.49	00.00	30.16	15.08	Least virulent	Moderately aggressive
3.Pantnagar UTMC-535	38.45	30.27	31.79	31.03	Moderately virulent	Moderately aggressive
4.Majra Farm	32.99	11.65	00.00	05.82	Least virulent	Moderately aggressive
5.Banjari farm	31.74	00.00	11.95	05.97	Least virulent	Moderately aggressive
6.Chinki Farm	31.61	10.82	13.01	11.91	Least virulent	Moderately aggressive
7.Sailanigot	29.00	20.00	13.40	16.70	Least virulent	Moderately aggressive
8.Tanakpur	30.43	30.47	19.98	25.22	Moderately virulent	Moderately aggressive
9.Khetalsanda	34.99	11.15	00.00	05.57	Least virulent	Moderately aggressive
10.Kashipur	29.42	40.61	12.82	26.71	Moderately virulent	Moderately aggressive
11.Haldwani-1 (HLD1)	37.36	29.60	14.42	22.01	Moderately virulent	Moderately aggressive
12.Kisanpur (Haldwani)	35.84	48.95	57.80	53.37	Most virulent	Moderately aggressive
13.Bajpur	37.93	00.00	13.26	06.63	Least virulent	Moderately aggressive
14. Doraha (Bajpur)	37.56	20.88	00.00	10.44	Least virulent	Moderately aggressive
15.Rudrapur	32.69	00.00	00.00	00.00	-*	Moderately aggressive
16.Sultanpur (Kashipur)	48.81	00.00	00.00	00.00	-*	Moderately aggressive
17. Tanda Kajal	43.72	11.94	00.00	00.00	-*	Most aggressive
18. Gadarpur	51.33	21.05	11.12	16.08	Least virulent	Most aggressive
19.Barhani	35.28	20.04	00.00	10.02	Least virulent	Moderately aggressive
20. Sitarganj	43.77	29.95	00.00	14.97	Least virulent	Most aggressive
21. Nagina	49.27	11.99	12.52	12.25	Least virulent	Most aggressive

-* No symptoms (top rot and lodging) produced even after 20 days of inoculation

* Means of all four days (5,10, 15 & 20) data

saprophytes in the rhizosphere **(Stanghellini, 1982)**; and they can aggressively utilize pectate as a carbon source **(Burr and Schroth, 1977)**. The virulence of the pectinolytic Erwiniae is mainly correlated with their ability to produce and secrete cell-wall degrading enzymes mainly pectate lyases (Pel) **(Collmer and Keen, 1986; Hugouvieux-Cotte-Pattat *et al.*, 1996)**. In *E. chrysanthemi*, production of these virulence factors, particularly the pectate lyases, is tightly regulated and responds to several environmental and metabolic stimuli, including growth phase-dependent induction, the presence of pectin or plant extract and catabolic repression **(Hugouvieux-Cotte-Pattat *et al.*, 1996)**. Several regulators (KdgR, PecS, PecT, CRP, H-NS and ExpR) modulating the expression of virulence genes in E. chrysanthemi have been characterized previously **(Reverchon *et al.*, 1991, 1994 and 1997; Praillet *et al.*, 1996; Surgey *et al.*, 1996; Nasser *et al.*, 2001)**. The evolution of virulence may determine important phenomena such as the emergence and re-emergence of pathogens, host switch and host range expansion, and overcoming host resistance, which may compromise the success of control strategies for infectious diseases of people and domestic animals and plants **(Soledad and Arenal 2008)**. Moreover, virulence evolution may also modulate the important role of pathogens in shaping ecosystem composition and dynamics **(Bull, 1994; Ebert and Hamilton, 1996; Frank, 1996; Read, 1994)**.

4.7 Management

4.7.1 Biological and chemical control

4.7.1.1 Pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost and chemicals 15 DAS

In pre-plant soil drenching with vermicompost colonized bioagents experiment (Table 4.8, Plate 19), maximum seed germination was recorded with Th-2 (88.53%) followed by Th-R (84.83%), Th-19 (82.70%) and Psf-24 (82.50%). Among chemicals maximum seed germination was observed with Blitox-50 (79.46%) followed by Tetracyclin (77.13%) and Bleaching powder 73.10%). Least seed germination was recorded with vermicompost (68.46%) while it was found statistically at par with treatment Psf-31(70.43%) and Chloramphanicol (70.58%). Maximum increase in root length was obtained with Th-2 (4.50cm) followed by Psf-18 (4.20 cm), Th-R (3.83cm), Psf-3(3.60cm) and Psf-24 (3.50cm). Maximum increase in plant height was recorded with Th-39 (18.0cm) followed by (16.43cm)

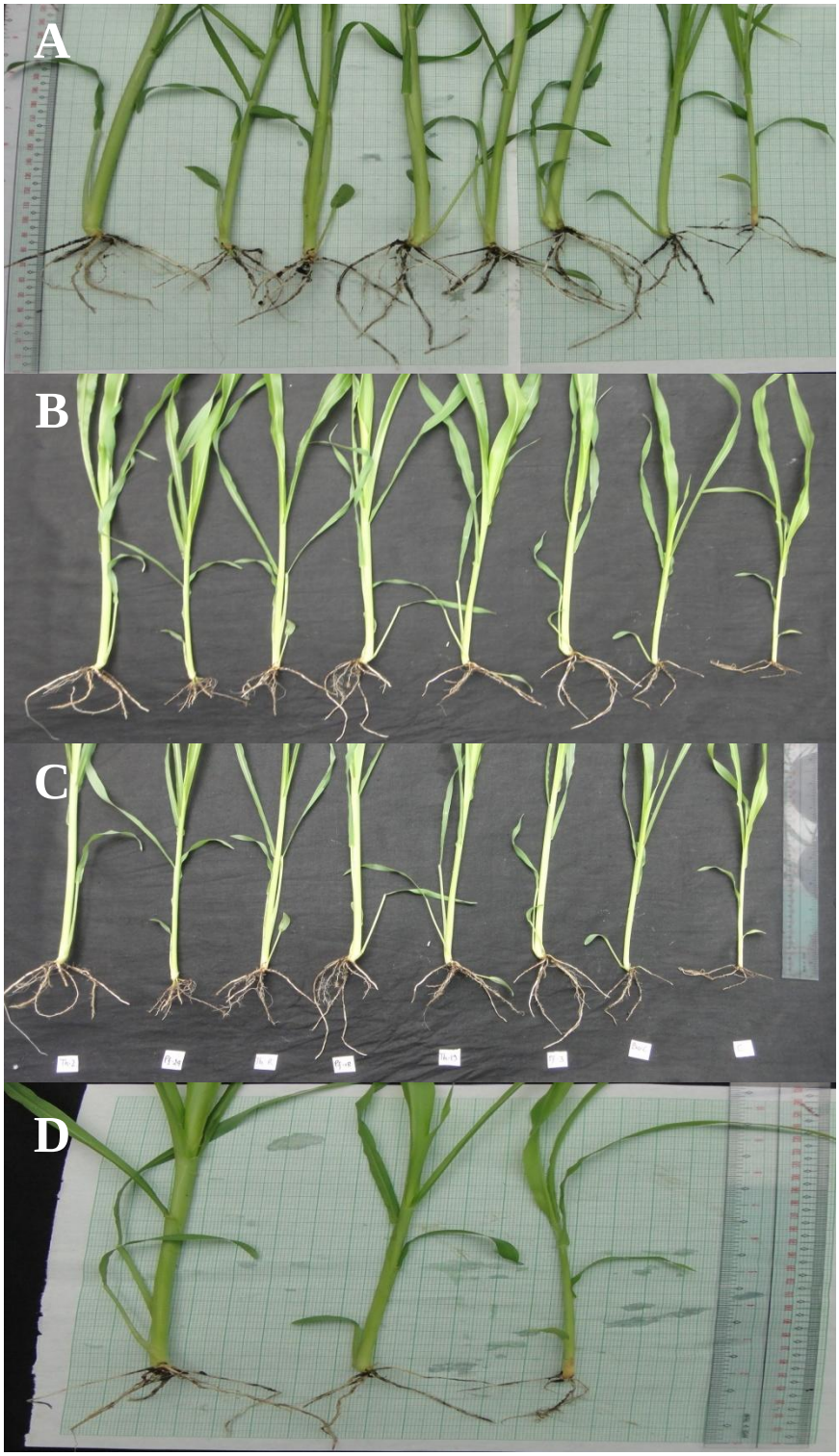


Plate 19

Effect of pre-plant soil drenching on root & shoot length

A. *T. harzianum* colonized vermicompost

B. *P. florescens* colonized *v*ermicompost

C. Chemicals

D. Comparison of three treatments

Table 4.8: Effect of pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates colonized vermicompost and chemicals on seed germination, stem diameter, plant height and root length of stalk rot under glass house conditions 15 DAS

Treatment	Seed germination (%)	Stem diameter (cm)	Root length (cm)	Plant height (cm)
Th-2	88.53	0.63	4.50	11.46
Th-14	80.43	0.50	3.16	14.33
Th-19	82.73	0.60	2.86	15.63
Th-31	73.86	0.43	1.93	10.73
Th-32	74.76	0.43	2.20	11.43
Th-39	81.03	0.60	3.16	18.03
Th-R	84.83	0.73	3.80	14.33
Psf-3	79.30	0.60	3.66	13.70
Psf-7	74.26	0.53	2.93	11.80
Psf-12	80.90	0.43	2.80	14.86
Psf-18	80.96	0.70	4.20	12.36
Psf-24	82.50	0.63	3.53	15.50
Psf-31	70.43	0.60	2.70	12.33
Vermicompost	68.46	0.53	2.43	12.60
Blitox-50	79.46	0.43	2.30	16.40
Chloramphanicol	70.58	0.36	1.70	10.43
Tetracyclin	77.13	0.46	1.76	14.23
Streptomycin	71.46	0.53	1.70	13.83
Bleaching Powder	73.10	0.40	2.06	15.33
Streptocyclin	72.16	0.43	2.16	14.06
Control	65.80	0.40	1.40	10.23
CD at 5 %	2.82	0.16	0.32	2.56

and Th-19 (15.66cm). *T. harzianum* has been studied and selected for disease suppressive properties on a variety of vegetable species and other crops **(Bennett, 1997)**. Efficacy of *Trichoderma* for improving the seed germination, growth and yield of several crops plants has been well established by various workers (**Chang *et al.*, 1996, Inbuar *et al.*, 1994, Harman *et al.*, 2004)**. **Singh *et al.* (2004)** reported several potent isolates of *T. harzianum* and *T. viride*, very effective against red rot of sugarcane pathogen and found the effective for improving them growth and yield of sugarcane plant and ratoon. The soil fungus *T. harzianum* is active against a range of economically important aerial and soil borne plant pathogens and is successfully used as a biopesticides in greenhouse and field applications **(Krause *et al.*, 2001; Tondje *et al.*, 2007)**. It is generally believed that field/greenhouse assays are the most reliable methods for testing the beneficial microorganisms, even though they do not always give positive correlated results with each other. **Yesim *et al.* (2003)** in their glasshouse experiment investigated some antagonists for controlling *Erwinia chrysanthemi*, the causal agent of soft rot on tomato abilities to prevent the disease development. Eight out of 13 selected isolates reduced the disease development between 89% and 33%.

4.7.1.2 Pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost

It is evident from the data presented in the Table 4.9 (Plate 20) that all the treatments significantly reduced the disease severity over control. Maximum reduction in disease severity was observed with Psf-24(34.08%) followed by Th-14 (32.94%) and Th-39 (30.45%). Least reduction in disease severity was observed with vermicompost (17.58%). Maximum increase in plant height was observed with Th-2 (89.16cm) however Thh-14 (85.53cm), Th-R (85.03cm) and Th-39 (84.50 cm). Th-19 (82.90cm) and Psf-24 (3.53cm) were found statistically at par with Th-2 in increment of plant height. Least increase in plant height was observed with vermicompost (70.60cm). Similarly maximum increase (1.20cm) in stem diameter was found with Th-2.

4.7.1.3 Pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates colonized vermicompost and one foliar spray

The results of pot study (Table 4.10) showed that pre-plant soil drenching with vermicompost colonized bioagents and one foliar spray significantly reduced

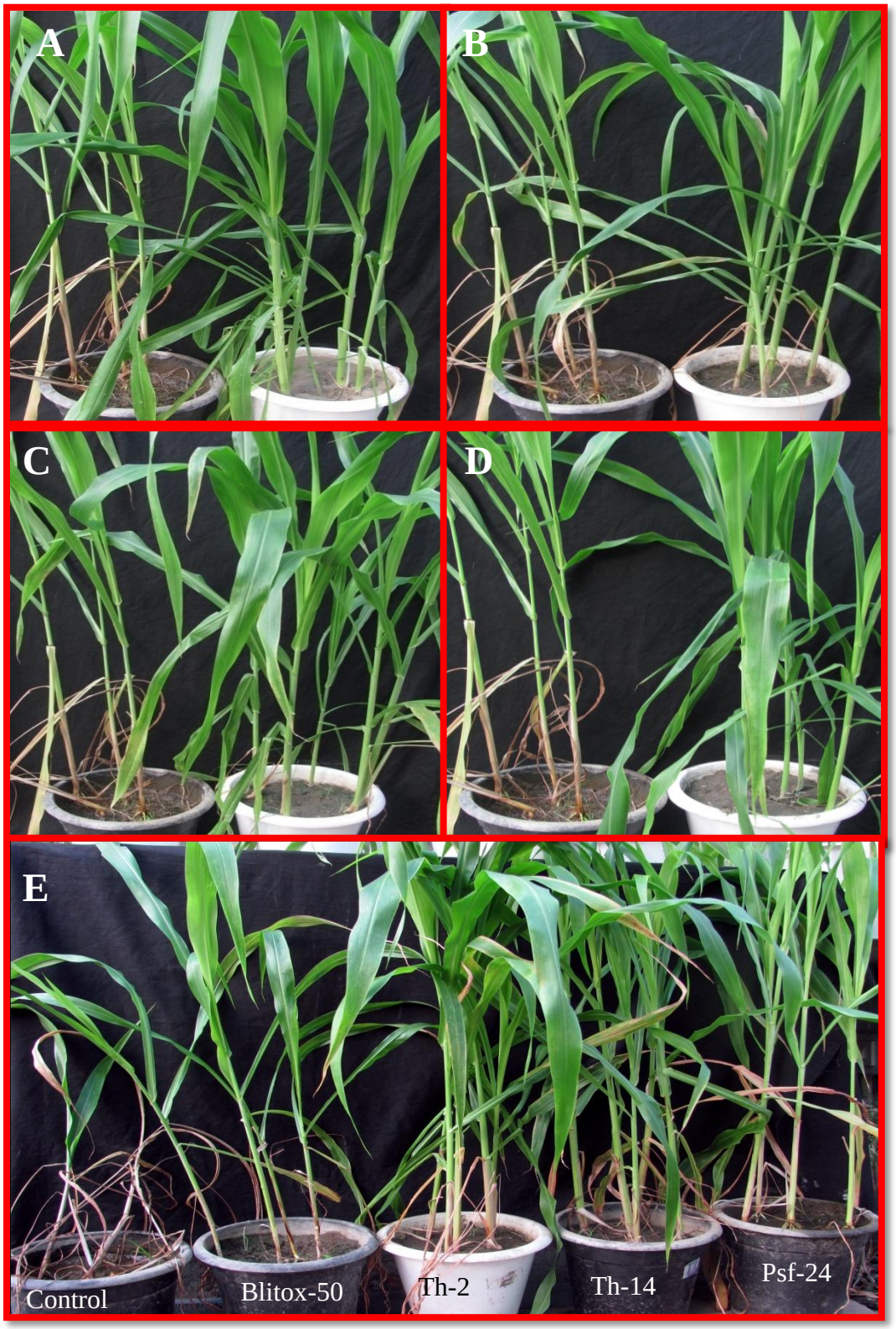


Plate 20

Effect of pre-plant soil drenching with bioagents colonized vermicompost & chemicals
A. Th-2 B. Th-14, C. Psf-24, D.Blitox-50 , F. Comparison of all treatment with control

Table 4.9: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	1.20	89.16	31.23	33.30	32.26	28.91
Th-14	0.70	85.53	28.60	32.26	30.43	32.94
Th-19	1.16	82.90	32.73	35.33	34.03	25.01
Th-31	0.80	74.26	35.53	38.46	36.99	18.48
Th-32	0.70	78.56	34.13	37.70	35.70	21.33
Th-39	1.10	84.50	29.70	33.43	31.56	30.45
Th-R	1.16	85.03	31.43	34.43	32.93	27.43
Psf-3	1.06	81.66	30.06	32.66	31.66	30.23
Psf-7	1.06	81.30	32.56	33.50	33.03	27.21
Psf-12	0.86	78.73	30.53	34.33	32.43	28.53
Psf-18	0.86	79.16	34.33	38.26	36.29	20.03
Psf-24	1.13	82.39	29.53	30.30	29.91	34.08
Psf-31	0.76	71.73	34.53	37.90	36.21	20.20
Vermicompost	0.73	70.60	35.60	39.20	37.40	17.58
Control	0.70	66.43	42.40	48.36	45.38	-
CD at 5 %	0.21	7.29	5.58	8.01		

Table 4.10: Effect of pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates colonized vermicompost and one foliar spray on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	1.20	91.83	28.20	29.23	28.20	37.85
Th-14	0.80	88.83	26.76	30.06	28.41	37.39
Th-19	0.83	88.00	34.26	31.80	33.03	27.21
Th-31	0.83	79.60	30.90	36.50	33.70	25.73
Th-32	0.73	84.93	32.50	36.66	34.58	23.79
Th-39	1.10	88.66	26.20	28.60	27.40	39.62
Th-R	1.26	93.16	28.83	30.80	29.81	34.31
Psf-3	1.10	85.36	30.30	30.73	30.51	32.76
Psf-7	1.16	85.70	30.53	32.63	31.58	30.40
Psf-12	0 .86	82.73	29.66	31.33	30.49	32.81
Psf-18	0.89	81.83	33.60	34.66	34.13	24.79
Psf-24	1.23	84.63	27.53	27.00	27.26	39.92
Psf-31	0.73	82.33	30.40	31.20	33.45	26.28
Vermicompost	0.73	73.50	32.56	37.93	35.24	22.34
Control	0.70	70.60	42.06	48.03	45.04	-
CD at 5 %	0.26	6.54	2.47	6.93		

the disease severity over control. Maximum reduction in disease severity (39.92 %) was observed with Psf-24 followed by Th-39 (39.62 %), Th-2 (37.85%) and Th-14 (37.39%). Least reduction in disease severity (22.34 %) was observed with vermicompost. Maximum plant height (93.16cm) was observed with Th-R followed by Th-2(91.83cm) and Th-39 (88.66cm).

4.7.1.5 Pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates colonized vermicompost and two foliar sprays

Application of the bioagents colonized vermicompost and two foliar sprays significantly reduced the disease severity of the stalk rot compared with that of the control (Table 4.11). Maximum reduction in disease severity (40.03%) was observed with the isolate Th-2 followed by Th-14(38.49 %), Th-19(34.35%), Th-39 (33.38%) and Th-R (32.65%) as compared to control. Least reduction in disease severity (20.86 %) was observed with vermicompost. Maximum 107.80cm plant height was observed with Th-39 followed by Psf-3(103.80cm) and Th-2 (102.23cm). Stem diameter was found to be maximum in treatment Psf-24 (2.00cm) followed by Th-2 (1.90cm), Th-14 (1.86cm) and Th-R (1.83cm). Various scientists from across the world have reported the non-pathogenic fluorescent pseudomonads as effective biological agents against species of *Erwinia* and other pathogens **(Singh and Sinha, 2005). Shanmugam *et al.* (2003)** identified that the mixture of *Pseudomonas fluorescens* and *P. putida* was more effective than either *Pseudomonad* alone in inhibiting the growth of *Erwinia* spp. Application of fluorescent pseudomonads reduced soft rot severity, improved plant growth or increased tuber yield in field trials **(Kloepper, 1983; Xu and Gross, 1986 and Kloepper *et al*., 2004)**. **Hajhamed *et al*. (2007)** found that all tested bioagents significantly reduced severity (upto 83.5 %) of bacterial soft rot of potato caused by *Erwinia carotovora* subsp. *carotovora*. **Cronin *et al*. (1997)** found *P. fluorescens* F113 a promising biocontrol agent against the potato soft rot agent *E. carotovora* subsp. *atroseptica* and suggested that the pseudomonad's ability to produce DAPG is a key factor in its inhibition of the pathogen. **Tiwari *et al.* (2008)** reported that growth promoting performance of *P. fluorescens* was recorded superior with FYM amended soil in rice field.

Table 4.11: Effect of pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates colonized vermicompost and two foliar sprays on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	1.90	102.23	23.86	30.56	27.21	40.03
Th-14	1.86	100.40	25.10	30.73	27.91	38.49
Th-19	1.33	91.43	26.73	32.86	29.79	34.35
Th-31	1.16	81.20	30.30	37.73	34.01	25.05
Th-32	1.76	79.96	27.06	30.66	34.29	24.43
Th-39	1.20	107.80	27.86	32.60	30.23	33.38
Th-R	1.83	93.06	28.33	32.80	30.56	32.65
Psf-3	1.36	103.80	30.96	33.73	32.34	28.73
Psf-7	1.46	100.00	33.26	32.80	33.80	25.51
Psf-12	1.13	89.76	30.80	33.00	31.90	29.70
Psf-18	1.70	89.20	32.26	36.33	34.29	24.43
Psf-24	2.00	90.50	30.86	33.63	32.24	28.95
Psf-31	0.86	77.73	32.50	36.66	34.58	23.79
Vermicompost	1.12	75.03	31.56	40.26	35.91	20.86
Control	1.00	72.13	42.40	48.36	45.38	-
CD at 5 %	0.52	5.93	2.78	3.48		

4.7.1.6 Pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates and chemicals

Maximum reduction in disease severity (24.01%) was observed with Th-2 followed by Th-R (23.33 %), Th-39 (22.10 %) and Psf-39 (21.57%) as compared to control (Table 4.12). Maximum plant height (71.03cm) was observed with Th-2 followed by Th-14 (67.76cm), Blitox-50(65.36cm), Th-39 (63.70cm) and Psf-12(61.76cm). Plants roots have been colonized by selected strains of nonpathogenic fluorescent *Pseudomonas* spp. develop an enhanced level of protection against pathogen attack **(Van Loon *et al.*, 1998).** Many biocontrol fluorescent pseudomonads protect plants from soil-borne diseases by the production of antimicrobial secondary metabolite(s) **(Haas and Keel, 2003; Morrissey *et al*., 2004; Haas and Défago, 2005).**

4.7.1.7 Pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates, chemicals and one foliar spray

The results Table 4.13 indicate that to maximum reduction (30.25%) in disease severity was noted with Th-R followed by Th-2(29.57%), Th-14 (28.86%), Th-39(26.44%) and Psf-7(26.22%). In case of chemicals maximum reduction (23.68%) in disease severity was observed with Blitox-50. Least reduction in disease severity (13.19 %) was observed with Chloramphanicol. Maximum plant height (72.36cm) was observed with Th-2 followed by Th-14 (69.43cm), Blitox-50 (67.03cm), Th-39(66.36) and Th-R (65.06cm). Not all isolates showing pathogen inhibition *in vitro* provide disease control or are rhizosphere competent. Biocontrol efficacy of pseudomonads is often related to their density in the rhizosphere. The bacterial population size was indicated to be the limiting step for biocontrol **(Weller, 1988)**. In several field trials the ability of the introduced biocontrol agent to establish itself effectively in the rhizosphere was essential for biocontrol **(Schippers *et al*., 1987; Weller, 1988; Bull *et al*., 1991; Raaijmakers *et al*., 1999)**.

4.7.1.8 Pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates, chemicals and two foliar sprays

It is evident from the data presented in the Table 4.14 that all the treatments significantly reduced the disease severity over control. Maximum reduction in disease severity was observed with Th-2 (32.04%) followed by Th-14 (29.70%), Th-R (29.48%), Psf-7 (27.30), Blitox-50 (27.17%), Th-19 (24.85%) and Th-39 (23.20%).

Table 4.12: Effect of pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates and chemicals on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	0.90	71.03	29.96	35.60	32.78	24.01
Th-14	0.96	67.76	33.10	35.50	34.30	20.66
Th-19	1.00	58.80	30.96	37.63	34.29	20.69
Th-31	0.76	49.56	33.80	40.16	36.98	14.76
Th-32	0.80	48.59	32.80	41.60	37.20	14.27
Th-39	1.00	63.70	31.00	36.30	33.65	22.10
Th-R	1.00	62.40	29.13	37.06	33.09	23.33
Psf-3	0.73	54.93	33.26	40.90	37.08	14.15
Psf-7	0.70	58.80	30.33	37.46	33.89	21.57
Psf-12	0.70	61.76	31.66	36.46	34.06	21.19
Psf-18	0.80	52.40	32.00	38.86	35.43	18.17
Psf-24	0.70	56.40	37.43	36.26	36.84	15.07
Psf-31	0.76	48.76	33.73	39.46	35.09	18.92
Blitox-50	1.10	65.36	30.46	38.40	34.43	20.38
Chloramphanicol	0.96	47.70	34.63	43.46	39.04	10.22
Tetracyclin	0.83	57.46	31.90	38.10	35.00	19.12
Streptomycin	0.76	49.10	35.90	43.70	39.00	10.31
Bleaching Powder	0.83	51.76	31.20	40.70	35.95	17.03
Streptocyclin	0.66	50.63	32.50	39.96	36.23	16.41
Control	0.63	47.66	38.63	48.73	43.68	-
CD at 5 %	0.30	4.90	8.17	8.63		

Table 4.13: Effect of pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates, chemicals and one foliar spray on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	0.83	72.36	27.93	32.60	30.26	29.57
Th-14	0 .80	69.43	27.46	33.70	30.58	28.86
Th-19	0.73	63.80	30.46	37.50	33.98	21.37
Th-31	0.83	51.56	32.50	39.96	36.96	14.80
Th-32	0.70	49.59	31.50	40.46	35.98	16.96
Th-39	1.10	66.36	28.50	34.86	31.68	26.44
Th-R	1.26	65.06	27.30	32.60	29.95	30.25
Psf-3	1.10	57.59	27.60	36.56	32.56	24.05
Psf-7	1.16	62.13	28.73	34.83	31.78	26.22
Psf-12	0.86	62.43	30.76	35.73	33.24	23.00
Psf-18	0.89	54.06	31.53	37.40	34.46	20.31
Psf-24	1.23	60.73	29.80	38.23	34.01	21.30
Psf-31	0.73	51.43	33.76	39.80	36.78	15.20
Blitox-50	1.20	67.03	29.93	35.93	32.93	23.68
Chloramphanicol	0.96	53.03	34.56	40.83	37.69	13.19
Tetracyclin	0.83	63.80	31.96	37.93	34.94	19.25
Streptomycin	0.76	53.26	33.83	39.93	36.93	14.87
Bleaching Powder	0.66	55.10	32.60	38.96	35.78	17.40
Streptocyclin	1.00	54.30	30.60	37.60	34.10	21.11
Control	0.63	47.66	38.63	48.73	43.68	-
CD at 5 %	0.23	8.69	6.06	7.88		

Table 4.14: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates, chemicals and two foliar sprays on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	0.89	77.73	26.56	31.83	29.14	32.04
Th-14	0.96	76.56	26.90	33.50	30.20	29.70
Th-19	1.00	68.73	29.43	35.40	32.40	24.85
Th-31	0.89	52.33	33.13	38.70	35.91	17.12
Th-32	0.76	52.80	30.70	39.90	35.30	18.46
Th-39	1.03	70.56	27.70	38.60	33.15	23.20
Th-R	1.20	73.90	27.40	33.20	30.30	29.48
Psf-3	1.10	58.53	28.10	34.90	31.50	26.84
Psf-7	1.10	71.40	29.33	33.26	31.29	27.30
Psf-12	0.96	65.46	29.70	37.80	33.56	22.30
Psf-18	0.89	59.40	30.60	35.80	33.20	23.09
Psf-24	1.13	63.96	30.20	38.40	34.20	20.89
Psf-31	0.83	54.23	31.90	39.40	35.65	17.69
Blitox-50	1.13	65.46	28.90	33.80	31.35	27.17
Chloramphanicol	0.96	55.40	31.10	39.80	35.34	18.37
Tetracyclin	0.93	66.36	30.60	39.10	34.85	19.45
Streptomycin	0.83	55.16	32.93	38.60	35.76	17.45
Bleaching Powder	0.80	55.03	32.20	37.30	34.75	19.67
Streptocyclin	0.96	57.96	29.90	36.86	33.38	22.69
Control	0.70	50.43	38.63	48.73	43.68	-
CD at 5 %	0.26	6.27	7.21	8.13		

Stem diameter (1.20cm) was found to be maximum with Th-R (1.20cm). The present study demonstrates that soil drench treatment with bioagent (Psf-24, Th-14, Th-39 and Psf-3) could reduce stalk rot severity in artificially inoculated sorghum plants.

4.7.1.9 One foliar spray with bioagents and chemicals

The data presented in Table 4.15 revealed maximum decrease (17.14%) in disease severity with Th-2 followed by Th-14(16.06%), Psf-7 (14.96%), Th-19 (14.91%), Blitox-50 (14.85%), Psf-3 (14.72%) and Th-39 (14.25%). Least reduction in disease severity (7.02 %) was observed with Chloramphanicol. Maximum plant height (58.90cm) was observed with Th-R. However Th-32, Th-14, Th-19 were statistically at par with Th-R. No statistically significant difference was observed among the all treatment pertaining to increase in stem diameter.

4.7.1.10 Two foliar sprays with bioagents and chemicals

The data presented in Table 4.16 revealed maximum decrease (18.73%) in disease severity with Th-R followed by Psf-3 (18.17 %), Th-2 (17.95%), Blitox-50 (17.58%) and Psf-7 (17.29%). Least reduction in disease severity (10.42%) was observed with Chloramphanicol. Maximum increase (59.16cm) in plant height was observed with Th-2 followed by (59.03cm), Th-39(58.00cm) and (57.43cm). However six treatments were found statistically at par pertaining to increase in plant height viz. Th-32(47.70 cm), Psf-31 (47.43cm), Tetracyclin (47.26cm), Psf-12 (43.50cm), Streptomycin (44.46cm) and Chloramphanicol (41.83cm).

4.8 Field Experiments

4.8.1 Artificial inoculation

Results presented in Table 4.17 (Plate 21) reveal that all three methods produced stalk rot symptoms All isolates of stalk rot bacterium proved to be highly pathogenic, causing a rapid soft rot of parechymatous tissues on all inoculated plants or plant parts (leaf whorl and stem). Water-soaked and discoloration of tissues (wet, glassy, yellow to brown steam lesions) around the inoculation court on sorghum plants was evident after 4 days of inoculation. After 10 days, extensive invasion of stem tissues were evident and plants toppled over. Leaf whorl-inoculated plants develop symptoms after 5 days. Symptoms were curling, yellowing, and wilting of

Table 4.15: Effect of one foliar spray with *T. harzianum, P. fluorescens* isolates and chemicals on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	0.83	56.43	31.20	40.60	35.90	17.14
Th-14	0.96	54.66	32.43	40.36	36.39	16.06
Th-19	0.83	54.66	33.56	40.26	36.91	14.91
Th-31	0.80	50.33	35.30	40.23	37.76	13.04
Th-32	0.76	47.93	34.50	42.36	38.51	11.39
Th-39	0.80	56.50	31.66	42.76	37.21	14.25
Th-R	0.90	58.90	34.56	40.30	37.43	13.77
Psf-3	0.86	54.73	32.40	41.60	37.00	14.72
Psf-7	0.93	52.66	33.46	40.33	36.89	14.96
Psf-12	0.96	54.63	33.50	40.73	37.11	14.47
Psf-18	0.80	55.56	33.43	42.23	37.83	12.89
Psf-24	1.00	55.16	34.60	43.60	39.10	10.09
Psf-31	0.96	48.59	35.80	44.53	40.16	7.75
Blitox-50	0.93	48.86	33.43	40.46	36.94	14.85
Chloramphanicol	0.90	45.46	35.46	45.53	40.49	7.02
Tetracyclin	0.83	47.59	34.70	45.03	39.86	8.41
Streptomycin	0.76	41.80	34.66	42.50	38.58	11.23
Bleaching Powder	0.90	43.26	33.50	42.20	37.85	12.84
Streptocyclin	0.83	48.30	34.16	42.50	38.33	11.78
Control	0.76	41.26	38.63	48.73	43.68	-
CD at 5 %	0.29	6.69	7.72	7.89		

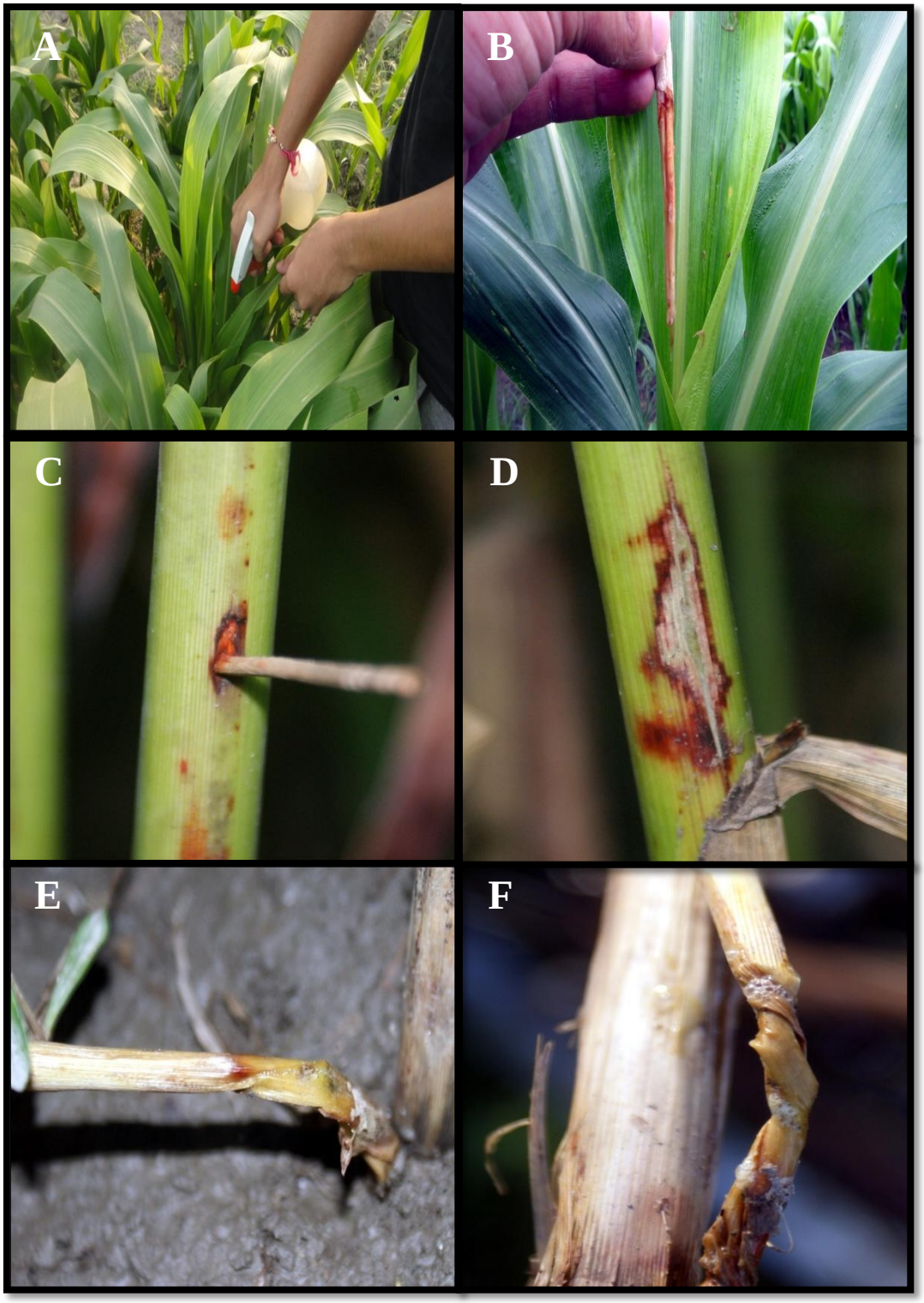


Plate 21

Inoculation & initiations of typical Erwinia rot symptoms after 5 days of inoculation under field conditions . A. Leaf-whrol inoculation B. Pulled out shoot, C Tooth-pick, D. Inject inoculated stalk, E. Lodged stalk, F. Twisted stalk with bacterial ooze

Table 4.16: Effect of two foliar sprays with *T. harzianum, P. fluorescens* isolates and chemicals on stem diameter, plant height and disease severity of stalk rot under glass house conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)
			45DAS	65DAS	Mean	
Th-2	0.83	59.16	30.50	40.56	35.53	17.95
Th-14	0.80	54.56	32.33	40.70	36.51	15.79
Th-19	0.83	58.00	31.13	41.26	36.19	16.50
Th-31	0.86	49.10	30.90	43.80	37.35	13.94
Th-32	0.80	47.70	34.60	42.90	38.75	10.86
Th-39	0.83	54.93	31.60	40.50	36.05	16.81
Th-R	0.93	59.03	30.56	39.80	35.18	18.73
Psf-3	0.96	52.60	30.00	40.86	35.43	18.17
Psf-7	0 .93	54.73	31.87	39.80	35.83	17.29
Psf-12	0.96	43.50	30.77	41.40	36.08	16.74
Psf-18	0.80	53.00	31.53	42.50	37.01	14.69
Psf-24	1.03	57.43	31.64	43.59	37.61	13.37
Psf-31	0.86	47.43	33.70	43.80	38.75	10.86
Blitox-50	1.06	53.63	30.90	40.50	35.70	17.58
Chloramphanicol	0.86	41.83	33.00	44.90	38.95	10.42
Tetracyclin	0.76	47.26	32.80	44.59	38.69	10.99
Streptomycin	0.83	44.46	34.90	42.10	38.50	11.41
Bleaching Powder	0.83	48.63	33.53	42.80	38.16	12.16
Streptocyclin	0.80	51.67	33.80	43.40	38.60	11.19
Control	0.76	51.59	38.63	48.73	43.68	-
CD at 5 %	0.29	5.95	7.06	7.79		

Table 4.17: Evaluation of artificial inoculation methods and disease assessment after 7 days of inoculation under field condition.

Method of artificial inoculation	Disease severity (%)
1.Tooth-pick method	34.35
2.Stem injection method	38.85
3.Leaf-Whorl inoculation method	41.65
4.Control	00.00
CD at 5%	4.12
SEM	1.19
CV	7.06

leaves, red discoloration of main vein, sometimes wet, glassy to brown necrotic leaf spots, later with a white, sunken necrotic centre malformation of newly formed leaves, and finally a total yellow-brown necrosis and death of plant. When infected leaves were pulled, they separated from stalk at the point of intense bacterial rot near the stem's apex. The disjointed apex appeared as a mushy foul smelling whip. Maximum disease severity (41.65%) was recorded with leaf whorl inoculation methods followed by stem injection (38.85%) and tooth pick methods (34.35%). As the result was found consistent with glasshouse experiment, out of these three methods of inoculation leaf whorl inoculation stem and injection methods can be considered as best and feasible. Inoculation with bacterial suspension in leaf-whorl and through hypodermic needle in punctures at the base of stalks of sorghum plants has been used **(Zummo, 1969; Jensen, 1986; Saxena, *et al*., 1990)**. A rapid whorl inoculation technique recommended by **Hartman and Kelman (1973)** in corn for *Erwinia* soft rot was successfully used in sorghum plants also **(Hepperly, *et al*., 1987)**.

4.9 Management

4.9.1 Biological and chemical control

4.9.1.1 Pre-plant soil drenching with bioagents colonized vermicompost

The data presented in Table 4.18 (Plate 22, 23, 24) revealed a statistically significant reduction in disease severity over control. Maximum seed germination

Plate 22

A. Variation in growth of sorghum plants in experimental plots with different treatment
B. Improper seed germination in control plot (above in circle & below close-up)

Plate 23

Effect of bioagents colonized vermicompost on disease severity

A.Control, B. Vermicompost, C.Th-19, D.Th-2, E..Th-R, F.,Th-39, G.Th-14, H.Th-19

Plate 24

Effect of bioagents colonized vermicompost on disease severity

A. Control, B. Psf-24, C.Psf-7, D. Psf-12, E.Psf-18, F. Psf-3

Table 4.18: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Seed germination (%)	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
				45DAS	65DAS	Mean		
Th-2	88.66	1.60	231.36	23.70	32.70	28.20	32.37	357.5
Th-14	86.83	1.70	235.50	24.50	33.90	29.20	29.97	330.0
Th-19	87.46	1.43	224.86	25.90	37.60	31.75	23.86	310.0
Th-39	84.73	1.30	231.40	26.40	39.10	32.75	21.46	330.0
Th-R	87.26	1.50	230.50	24.90	34.10	29.50	29.25	320.0
Psf-3	82.76	1.46	221.83	25.40	36.10	30.75	26.25	300.0
Psf-7	83.50	1.33	225.46	25.90	36.30	31.00	25.65	277.5
Psf-12	85.76	1.33	222.76	26.90	38.90	32.90	21.10	285.0
Psf-18	80.90	1.30	220.70	27.10	38.40	32.75	21.46	272.5
Psf-24	81.73	1.50	226.80	26.60	35.40	31.00	25.65	335.0
Vermicompost	77.63	1.20	224.70	27.50	39.60	33.55	19.54	265.0
Control	68.03	1.06	215.90	33.80	49.60	41.70	-	222.5
CD at 5 %	9.76	0.32	9.82	1.74	4.12			4.47

(88.66%) was recorded with Th-2 followed by Th-19 (87.46%) Th-R (87.26%) and Psf-7 (83.50%). Maximum reduction (32.37%) disease severity was recorded with Th-2 followed by Th-14 (29.97%), Th-R (29.25%) and Psf-3 (26.25%). Least reduction in disease severity (19.54%) was recorded with vermicompost. Maximum plant height (235.50cm) was recorded with Th-14 followed by Th-39 (231.40cm) and Th-2 (231.36cm). The maximum fresh biomass weight (357.5q/h) was observed with Th-2 followed Psf-224 (370.0q/h), Th-14 (330.0q/h) and Th-39 (330.0q/h). Biological control could have an important role in the management of bacterial wilt **(Akiew *et al.*, 1993)**. Several strains of *Pseudomonas fluorescens* have been reported to suppress soil borne diseases caused by pathogens **(O'Sullivan and O'Gara, 1992)**. *P. fluorescens* encompasses a group of common, Gram negative, rod shaped, non pathogenic saprophytes that colonize soil, water and plant surface environments. Since they are well adapted in soil, *P. fluorescens* strains are being investigated extensively for use in biocontrol of pathogens in agriculture. It is known to enhance plant growth and yield and reduce severity of many diseases **(Hoffland *et al.*, 1996; Wei *et al.*, 1996)**. The cell suspensions of *P. fluorescens* should be immobilized in certain carriers and should be prepared as formulations for easy application, storage, commercialization and field use. The potential *P. fluorescens* formulations are formulated using different organic and inorganic carriers either through solid or liquid fermentation technologies **(Nakkeeran *et al.*, 2005)**. **Chakravarty and Kalita (2011)** have reported that vermicompost and farmyard manure used as substrate carrier in conjunction with carboxymethyl cellulose as adhesive in the formulations provided better nutrient sources and congenial microenvironment required for proper growth and subsequent longer shelf life of *P. fluorescens.* **Bora and Deka (2007)** also found that the biopesticide combination of vermicompost, *P. fluorescens,* carboxymethyl cellulose and mannitol showed best shelf life as it maintained highest population recovery at different DAS. **Islam and Toyota (2004)** reported higher microbial activity in Farmyard manure due to increased rates of CO2 evolution and high dehydrogenase activity which was the key factor in the suppression of bacterial wilt of tomato.

4.9.1.2 Pre-plant soil drenching with *T. harzianum* and *P. fluorescens* colonized vermicompost and one foliar spray

The data presented in Table 19 revealed that all the treatments resulted in statistically significant reduction in disease severity over control. Maximum reduction (36.45%) in disease severity was recorded with Th-14 followed by Th-2 (36.11%), Th-R (32.13%) and Th-39 (31.51%). Least reduction (18.46%) in disease severity was recorded with vermicompost. Maximum plant height (242.46cm) was recorded with Th-14 followed by Th-2 (238.80cm) and Th-39 (234.76cm). Least plant height (227.70cm) was obtained with Psf-18. The maximum fresh biomass weight (447.5q/h) was observed with Th-14 followed by Th-2 (402.5q/h) and Th-19 (370.0q/h). Vermicompost was found to be least fresh biomass weight (315.0q/h).Maximum stem diameter (2.10cm) was found with Th-14.

4.9.1.3 Pre-plant soil drenching with *T. harzianum* and *P. fluorescens* colonized vermicompost and two foliar sprays

The data presented in Table 20 all the treatments resulted in statistically significant reduction in disease severity over control. Maximum reduction in disease severity (40.26%) was recorded with Th-2 followed by Th-14 (37.05%) and Th-R (34.86 %). Least reduction in disease severity (23.74%) was recorded with Psf-18. Similarly maximum plant height (247.93cm) was recorded with Th-14 followed by Th-2 (245.63cm), Th-R (241.63cm), Th-39 (239.66cm) and Psf-3 (237.66cm). Least plant height (230.70cm) was obtained with vermicompost. The maximum increase fresh biomass weight (560.0q/h) was observed with Th-2 followed by Th-14 (452.5q/h), Th-19 (407.8q/h), Th-R (372.5q/h) and Th-39 (365.0q/h). Maximum stem diameter (2.30cm) found with Th-14 followed by Th-2 (2.06cm). Least stem diameter (1.70cm) found with vermicompost.

Soil health and soil quality are fundamental to the sustained productivity and viability of agricultural systems worldwide. The utilization of organic wastes through earthworms is called vermicomposting. Although **Darwin (1881)** first drew attention to the great importance of earthworms in the decomposition of dead plants and the release of nutrients from them, it was necessary to wait more than a century until this was taken seriously as a field of scientific knowledge or even a real technology. Vermicomposting is a mesophilic bio-oxidative process in which detritivorous earthworms interact intensively with microorganisms and soil invertebrates within the

Table 4.19: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost and one foliar spray on stem diameter, plant height disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	1.90	238.80	22.53	30.76	26.64	36.11	402.5
Th-14	2.10	242.46	22.80	30.50	26.50	36.45	447.5
Th-19	1.80	231.80	23.50	34.10	28.80	30.93	395.0
Th-39	1.50	234.76	23.83	33.30	28.56	31.51	362.5
Th-R	1.70	234.66	23.20	33.40	28.30	32.13	370.0
Psf-3	1.60	229.70	25.50	36.20	30.85	26.01	345.0
Psf-7	1.73	231.60	24.20	36.40	30.30	27.33	335.0
Psf-12	1.50	228.43	24.20	35.10	29.65	28.89	335.0
Psf-18	1.42	227.80	26.80	37.70	32.25	22.66	327.5
Psf-24	1.60	235.90	24.60	36.53	30.56	26.71	362.5
Vermicompost	1.40	227.70	30.10	37.90	34.00	18.46	315.0
Control	1.06	215.90	33.80	49.60	41.70	-	222.5
CD at 5 %	0.47	3.37	4.12	6.73			4.70

Table 4.20: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates colonized vermicompost and two foliar spray on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	2.06	245.63	20.73	29.10	24.91	40.26	560.0
Th-14	2.30	247.93	21.80	30.70	26.25	37.05	452.5
Th-19	1.86	235.50	22.10	32.53	27.31	34.50	407.8
Th-39	1.80	239.66	22.80	31.16	27.31	34.50	365.0
Th-R	2.13	241.63	22.40	31.93	27.16	34.86	372.5
Psf-3	2.13	237.66	23.73	33.70	28.71	31.15	395.0
Psf-7	2.00	236.66	22.80	34.20	28.50	31.65	345.0
Psf-12	1.80	234.43	23.80	35.20	29.50	29.25	345.0
Psf-18	1.90	231.43	26.30	35.80	31.80	23.74	332.5
Psf-24	2.10	238.53	22.50	32.80	27.65	33.69	322.5
Vermicompost	1.70	230.70	24.80	35.40	30.10	27.81	320.0
Control	1.06	215.93	33.80	49.60	41.70	-	222.5
CD at 5 %	0.59	6.08	1.88	4.74			5.08

decomposer community, strongly affecting decomposition processes, accelerating the stabilization of organic matter, and greatly modifying its physical and biochemical properties **(Edwards and Bohlen, 1996; Dominguez, 2004 and Edwards *et al*., 2004)**. Vermicompost has been widely used in agriculture not only for its beneficial effects on soil structure and biota but also for its ability to inhibit plant pathogens, for example, **Asciutto *et al*. (2006)** reported that vermicompost at 25% controlled *Rhizoctonia solani* that causes damping off in Patience-plant (*Impatiens walleriana*). Several studies have reported the inhibition of plant pathogens by earthworm secretions. **Reiten and Salter (2002)** have reported strong inhibition for *X. campestris* pv. *carotovora* in culture plate method using compost tea. Their studies indicate that compost tea can be used to control *X. campestris pv. carotovora* both in the laboratory studies as well as in the field. **Hudson and Berman (1994)** have reported strong suppression of *Rhizactonia,* on application of compost to the soil. **Khalifa (1965)** has reported suppression and control of *Fusarium* using compost. Body wall and gut extracts have shown inhibitory effect on *F. oxysporum* in his studies. Apart from the soil borne plant pathogens, animal fungal pathogens *Candida albicans, Cryptococcus neoformans* and *Trycophytan metagrophyte* were also found to be inhibited by the body wall, gut and coelomic extracts **(Subhashini, 2005)**. **Reddy *et al*., (2012)** have found that soil application of vermicompost coupled with seed treatment with 10% aqueous extract of vermicomposted neem for one h can significantly reduce the severity of bacterial spot caused by *X. campestris* in tomato. **Hameeda *et al*., (2007)** showed significant improvement in shoot length (1-12%), leaf area (20-34%), plant biomass (9-27%) and root volume when vermicompost was applied in sorghum field. Several reports suggest that vermicompost extracts are effective antimicrobial agents against soil-borne pathogens **(Szczech *et al*., 1993; Orlikowski, 1999; Rodriguez *et al*., 2000; Szczech and Smolinska, 2001; Edwards and Arancon, 2004 and Zaller, 2006)** and do not produce any residual effects. Earthworms also modify microbial biomass and activity through stimulation, digeston, and dispersion in the casts and interact closely with other biological components of the vermicomposting system, thereby affecting the structure of the microflora and microfuna communities **(Dominguez *et al*., 2003 and Lores *et al*., 2006)**. Vermicompost can supply the full requirement of major nutrients for plant growth *i.e.* N, P, K; secondary nutrients *i.e.*

Ca, Mg, S and also micro nutrients *i.e.* Cu, Zn, Mn and Fe. The average nutrient content of vermicompost is much higher than in the other types of compost **(Singh, 1996).** On the other hand vermicompost is a rich source of vitamins and growth hormones like gibberellins which regulate plant growth. **Fragoso *et al*. (1996)** reported that single earthworm has to digest organic matter up to 5-30 times of its body weight per day in order to enhance the growth of beneficial soil bacteria which are the most divers and speediest agents for decomposing organic matter. Vermicompost is a nutritive 'organic fertilizer' rich in NKP (nitrogen 2-3%, potassium 1.85-2.25% and phosphorus 1.55-2.25%), micronutrients, beneficial soil microbes like 'nitrogen-fixing bacteria' and 'mycorrhizal fungi' and are scientifically proving as 'miracle growth promoters and protectors' **(Sinha *et al*., 2009)**. **Kale and Bano (1986)** reports as high as 7.37% nitrogen (N) and 19.58% phosphorus as P2O5 in vermicast. **Suhane (2007)** showed that exchangeable potassium (K) was over 95% higher in vermicompost. There are also good amount of calcium (Ca), magnesium (Mg), zinc (Zn) and manganese (Mn). Additionally, vermicompost contain enzymes like amylase, lipase, cellulase and chitinase, which continue to break down organic matter in the soil (to release the nutrients and make it available to the plant roots) even after they have been excreted. **(Chaoui *et al*., 2003; Lunt and Jacobson, 1994; and Tiwari *et al*., 1989)**. Annual application of adequate amount of vermicompost also lead to significant increase in soil enzyme activities such as 'urease', 'phosphomonoesterase', 'phosphodiesterase' and 'arylsulphatase'. The soil treated with vermicompost has significantly more electrical conductivity (EC) and near neutral pH. **(Tiwari *et al*., 1989)**. Vermicompost has very 'high porosity', 'aeration', 'drainage' and 'water holding capacity'. They have a vast surface area, providing strong absorbability and retention of nutrients. They appear to retain more nutrients for longer period of time. Study showed that soil amended with vermicompost had significantly greater 'soil bulk density' and hence porous and lighter and never compacted. Increase in porosity has been attributed to increased number of pores in the 30-50 μm and 50-500 size ranges and decrease in number of pores greater than 500 μm **(Lunt and Jacobson, 1994; Nighawan and Kanwar, 1952)**. It has been found to influence on all yield parameters such as-improved seed germination, enhanced rate of seedling growth, flowering and fruiting of major crops like wheat,

paddy, corn, sugarcane, tomato, potato, brinjal, okra, spinach, grape and strawberry as well as of flowering plants like petunias, marigolds, sunflowers, chrysanthemums and poinsettias. In all growth trials the best growth responses were exhibited when the vermicompost constituted a relatively small proportion (10%-20%) of the total volume of the container medium. Suppression of soil borne diseases has been reported for several kinds of composts.

4.9.1.4 Pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates and chemicals

It is evident from data presented in Table 21 that maximum reduction in disease severity (17.07%) was recorded with blitox-50 followed by Tetracyclin (17.02%), Psf-24 (16.47%), Th-2 (16.30%), Th-R (14.79%), Th-14 (14.67 %), and Th-39 (14.50 %). Least reduction in disease severity (10.21%) was recorded with Psf-18. Maximum increase plant height was recorded with Th-14 followed by Th-R (230.70cm), Th-2 (229.76cm) and Th-39 (227.63cm). Least plant height (217.66cm) was obtained with Streptocyclin. Maximum fresh biomass weight (382.5q/h) was observed with Th-14 followed by Th-R (367.5q/h), Th-39 (340.0q/h) and Th-2 (337.5q/h). Psf-3 was found least effective increasing fresh biomass weight (255.0q/h). Maximum stem diameter (1.70cm) found with Th-14. Furthermore, for the effective biological control of soil-borne plant pathogens, a major consideration has been given to proliferation of the antagonist after introduction into the soil. Among the desirable attributes of a successful antagonist is its ability to produce inoculum in excess and to survive, grow, and proliferate in soil and the rhizosphere **(Baker and Cook, 1974)**. In particular, some antagonistic rhizobacteria such as *Burkholderia cepacia* **(Jee *et al.*, 1988)** and *Pseudomonas aeruginosa* **(Kim and Hwang, 1992)** have been very effective against Phytophthora blight in pepper plants under laboratory and greenhouse conditions. Similarly, application of *B. cepacia* granules into soil provided better suppression of Phytophthora blight on red-pepper seedlings, as compared to direct drenching with *Burkholderia cepacia* suspensions **(Park *et al.*, 1989)**. Soil drenches and dipping of seedling roots with the antagonist suspensions were found to be more effective in disease suppression than the coating and dipping pepper seeds **(Jee *et al.*, 1988)**.

Table 4.21: Effect of pre-plant soil drenching with *T. harzianum, P. fluorescens* isolates and chemicals on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Seed germination (%)	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
				45DAS	65DAS	Mean		
Th-2	79.36	1.60	229.76	28.90	40.90	34.90	16.30	337.5
Th-14	74.73	1.73	231.60	29.30	42.16	35.73	14.31	382.5
Th-19	77.66	1.40	224.76	30.76	43.80	37.28	10.59	310.0
Th-39	72.80	1.30	227.63	29.40	41.90	35.65	14.50	340.0
Th-R	79.66	1.50	230.70	28.86	42.20	35.53	14.79	367.5
Psf-3	77.46	1.40	221.76	29.70	41.46	35.58	14.67	255.0
Psf-7	70.50	1.60	225.70	29.90	42.90	36.40	12.70	280.0
Psf-12	76.30	1.43	222.13	29.50	43.09	36.29	12.97	320.0
Psf-18	70.66	1.50	220.70	30.80	44.09	37.44	10.21	277.5
Psf-24	78.86	1.26	226.70	28.90	40.76	34.83	16.47	285.0
Blitox-50	75.33	1.50	224.60	28.26	40.90	34.58	17.07	332.5
Tetracyclin	70.70	1.23	218.46	28.80	40.40	34.60	17.02	305.0
Bleaching Powder	71.50	1.40	221.40	29.30	42.20	35.75	14.26	310.0
Streptocyclin	69.80	1.23	217.66	30.30	43.60	36.95	11.39	292.5
Control	64.86	1.20	215.56	33.80	49.60	41.70	-	222.5
CD at 5 %	6.25	0.65	6.94	2.12	5.33			4.22

4.9.1.5 Pre-plant soil drenching with *T. harzianum*, *P. fluorescens*, chemicals and one foliar spray

It is evident from data presented in Table 22 that maximum reduction in disease severity (19.90 %) was recorded with Th-2 followed by Blitox-50 (19.30%), Th-14 (17.74%), Psf-24 (17.02%) and Th-39 (14.50 %). Least reduction in disease severity (13.30%) was recorded with Psf-18. Maximum plant height (233.00cm) was recorded with Th-R followed by Th-2 (231.00cm) and Th-39 (229.80cm). Least plant height (221.60cm) was obtained with Tetracyclin. Maximum fresh biomass weight (392.5q/h) was observed with Th-14 followed by Th-2 (365.0q/h), Psf-18 (352.5q/h), Psf-24 (345.0q/h), Th-39 (335.0q/h) Streptocyclin (332.5q/h) and Blitox-50 (332.0q/h). Similarly maximum stem diameter (1.83cm) was found with Th-14 followed by Psf-7 (1.56cm).

4.9.1.6 Pre-plant soil drenching with *T. harzianum*, *P. fluorescens*, chemicals and two foliar sprays

Data presented in Table 23 revealed that maximum reduction in disease severity (26.61%) was recorded with Th-2 followed by Th-14 (25.29 %), Blitox-50 (23.62 %) and Th-39 (16.54%). Least reduction disease severity (15.87%) was recorded with Psf-12. Plant height was found maximum (237.83cm) with Th-14 followed by Th-R (236.76cm), Psf-24 (235.70cm) and Blitox-50 (234.76cm). Maximum fresh biomass (green fodder) weight (395.0q/h) was observed with Th-14 and Blitox-50.

Stem diameter was found maximum (1.90cm) with Th-14 followed by Psf-7 (1.80cm). The use of microbial antagonists against *E. amylovora* is an alternative or complementary measure to the application of chemicals **(Pusey, 2002)**. Few species, mainly *Pseudomonas fluorescens* **(Cabrefiga *et al.*, 2007, Wilson and Lindow, 1993)**, *Pantoea agglomerans* (synonym *Erwinia herbicola*) **(Ishimaru *et al.*, 1988; Vanneste and Beer 1992; Wright and Beer 1996)** and *Bacillus subtilis* **(Broggini *et al.*, 2005)**, are active against *E. amylovora*, and only very few isolates have been developed for field application **(Broggini *et al.*, 2005; Highland and Walgenbach, 2006**, **Wilson, 1997)**.

Plate 25

Effect of chemicals on disease severity

A. Blitox-50, B. Streptocyclin, C. Bleaching Powder , D. Tetracyclin , E. Control

Table 4.22: Effect of pre-plant soil drenching with *T. harzianum*, *P. fluorescens* isolates, chemicals and one foliar spray on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	1.50	231.56	27.70	39.10	33.40	19.90	365.0
Th-14	1.83	233.00	28.40	40.20	34.30	17.74	392.5
Th-19	1.33	227.93	29.10	41.20	35.15	15.70	310.0
Th-39	1.40	229.80	29.30	40.30	34.80	16.54	335.0
Th-R	1.50	233.70	28.90	40.96	34.93	16.23	292.0
Psf-3	1.33	225.76	29.40	41.50	35.45	14.98	320.0
Psf-7	1.56	227.80	28.90	41.10	35.00	16.06	330.0
Psf-12	1.40	220.83	29.20	41.50	35.35	15.27	285.0
Psf-18	1.36	225.70	29.70	42.60	36.15	13.30	352.5
Psf-24	1.30	229.36	28.30	40.90	34.60	17.02	345.0
Blitox-50	1.43	226.76	27.40	39.90	33.65	19.30	332.0
Tetracyclin	1.30	221.60	28.70	40.60	34.65	16.90	315.0
Bleaching Powder	1.36	226.40	28.90	40.90	34.90	16.30	322.0
Streptocyclin	1.30	223.70	29.96	42.40	36.18	13.23	332.5
Control	1.20	215.23	33.80	49.60	41.70	-	222.5
CD at 5 %	0.25	4.80	4.35	7.82			5.39

Table 4.23: Effect of pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates and chemicals and two foliar sprays on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	1.60	234.66	25.90	35.30	30.60	26.61	382.5
Th-14	1.90	237.83	26.40	35.90	31.15	25.29	395.0
Th-19	1.63	231.26	27.30	37.20	32.25	22.66	352.5
Th-39	1.70	234.40	27.90	39.40	33.65	19.30	390.0
Th-R	1.40	236.76	27.80	39.90	33.85	18.82	347.5
Psf-3	1.50	227.60	28.50	40.40	34.45	17.38	392.5
Psf-7	1.80	229.80	28.10	40.80	34.45	17.38	322.5
Psf-12	1.50	224.76	28.60	41.56	35.08	15.87	335.0
Psf-18	1.73	228.80	29.10	39.90	34.50	17.26	320.0
Psf-24	1.40	235.70	27.90	40.10	34.00	18.46	280.0
Blitox-50	1.60	234.76	26.70	37.40	31.85	23.62	395.0
Tetracyclin	1.40	226.70	27.80	38.40	33.10	20.62	307.5
Bleaching Powder	1.50	231.56	27.30	37.90	32.60	21.82	315.0
Streptocyclin	1.40	226.66	27.53	38.10	32.81	21.31	330.0
Control	1.20	213.40	33.80	49.60	41.70	-	222.5
CD at 5 %	0.60	3.73	7.23	5.87			5.08

4.9.1.7 One foliar spray with bioagents and chemicals

It is evident from data presented in Table 24 that maximum reduction in disease severity (17.60 %) was recorded with Th-2 followed by Th-14 (16.06 %) and Blitox-50 (16.06 %). Maximum plant height (229.70cm) was recorded with Th-14 followed by Th-2 (225.63), Th-R (223.13cm), Blitox-50 (222.66cm) and Psf-12 (222.56cm). Maximum fresh biomass (green fodder) weight (360.0q/h) was observed with Blitox-50 followed by Psf-18 (347.5q/h) and Psf-12 (342.5q/h). Least fresh biomass weight (228.5q/h) was observed with Bleaching powder. Significantly higher stem diameter (1.66cm) was found with Th-14 over control.

4.9.1.8 Two foliar spray with bioagents and chemicals

It is evident from data presented in Table 25 that maximum reduction in disease severity (27.98 %) was recorded with Blitox-50 followed by Th-2 (20.38%), Th-14 (17.86 %) and Tetracyclin (15.10 %). Least reduction in disease severity (06.81%) was recorded with Psf-7. Maximum plant height (234.66cm) was recorded with Th-14 followed by Th-2 (231.30cm), Th-R (229.70 cm). Least plant height (221.46cm) was obtained with Psf-18. Maximum fresh biomass (green fodder) weight (390.0q/h) was observed with Psf-12 followed by Psf-3 (375.0q/h) and Psf-24 (370.0q/h).

Maximum stem diameter (1.80cm) found with Th-14 followed by Th-2 (1.66cm), Th-19 (1.53), Psf-12(150cm), Psf-18 (1.43) and Streptocyclin (140cm). Streptocyclin alone and with Blitox-50 WP has been found to be most effective against stalk rot of maize in glasshouse. Soil drenching with stable Bleaching power have been recommended in standing crop **(Lal and Saxena, 1979; Thind and Payak, 1984). Hepperly and Davila (1987)** tested 22 chemicals against Erwinia isolate of sorghum *in vitro* and *in vivo*, and reported that Tetercyclin, Gentamycin and Chloramphenical showed sensitivity, but chemicals of Penicillin group appeared insensitive. Seed, soil or foliage treatment with chemicals has not been found effective in controlling Erwinia soft rot diseases **(Christensen and Wilcoxan, 1966)**.

Table 4.24: Effect of one foliar spray with *T. harzianum, P. fluorescens* isolates and chemicals on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	1.46	225.63	28.30	40.43	34.36	17.60	310.0
Th-14	1.66	229.70	28.70	41.30	35.00	16.06	330.0
Th-19	1.40	221.40	29.26	42.40	35.83	14.07	350.0
Th-39	1.30	219.03	30.40	44.09	37.24	10.69	330.0
Th-R	1.13	223.13	29.90	43.30	36.60	12.23	285.0
Psf-3	1.26	220.80	30.90	44.70	37.80	09.35	305.0
Psf-7	1.20	219.53	30.40	43.90	37.15	10.91	320.0
Psf-12	1.40	222.56	29.60	41.40	35.50	14.86	342.5
Psf-18	1.30	218.13	31.50	44.09	37.79	09.37	347.5
Psf-24	0 .93	219.76	28.80	43.40	36.10	13.42	335.0
Blitox-50	1.20	222.66	28.50	41.50	35.00	16.06	360.0
Tetracyclin	0.90	217.70	30.20	43.80	37.00	11.27	335.0
Bleaching Powder	1.06	218.83	29.10	41.90	35.50	14.86	228.5
Streptocyclin	1.13	219.80	30.20	44.09	37.14	10.93	307.5
Control	1.20	214.36	33.80	49.60	41.70	-	222.5
CD at 5 %	0.42	6.60	6.23	7.40			6.01

Table 4.25: Effect of two foliar sprays with *T. harzianum, P. fluorescens* isolates and chemicals on stem diameter, plant height, disease severity and yield under field conditions

Treatment	Stem diameter (cm)	Plant height (cm)	Disease severity (%)			Decrease in disease severity (%)	Yield (Biomass) Q /hac
			45DAS	65DAS	Mean		
Th-2	1.66	231.30	27.20	39.20	33.20	20.38	342.5
Th-14	1.80	234.66	28.10	40.40	34.25	17.86	325.0
Th-19	1.53	229.03	28.90	42.80	35.85	14.02	350.0
Th-39	1.33	225.73	29.90	42.80	36.35	12.82	275.0
Th-R	1.13	229.70	30.40	43.90	37.15	10.91	357.5
Psf-3	1.30	225.56	30.10	43.20	36.65	12.11	375.0
Psf-7	1.20	226.80	33.93	43.80	38.86	06.81	300.0
Psf-12	1.50	229.56	29.10	43.09	36.09	13.45	390.0
Psf-18	1.43	221.46	30.70	43.80	37.25	10.67	350.0
Psf-24	1.20	228.16	29.40	41.83	35.61	14.60	370.0
Blitox-50	1.36	229.66	28.90	41.90	30.03	27.98	340.0
Tetracyclin	1.16	226.66	30.10	29.96	35.40	15.10	357.5
Bleaching Powder	1.13	224.76	29.20	42.30	35.75	14.26	307.5
Streptocyclin	1.40	223.70	29.90	43.30	36.60	12.23	327.8
Control	1.20	214.36	33.80	49.60	41.70	-	222.5
CD at 5 %	0.23	5.79	7.43	6.84			6.01

4.10 Population dynamics analysis of earthworms

For population dynamics analysis of earthworms, following parameters were taken into consideration these includes, population no. of young earthworms per plant rhizosphere, no. of adult earthworms per plant rhizosphere (15cm×10cm), total no. of earthworms (Young + Adults)/ plant rhizosphere (15cm×10cm), total no. of (young +adult) earthworms per hectare (10,000m^2), average size (length) of earthworms, no. of earthworm's heap of cast per plant rhizosphere. In analysis of earthworm's population dynamics (the data presented in Table 4.26 and Plate 26, 27) an interesting co-relation among the treatment was found. Maximum no. (4.60) of young earthworms per plant rhizosphere was obtained with Th-2 followed by vermicompost (1.36) and Psf-24 (1.33). Minimum no. (0.10) of young earthworms per plant rhizosphere was obtained with Bleaching Powder. None the young earthworms per plant rhizosphere was obtained with Psf-18, Blitox-50 and control. Maximum no. (2.80) of adult earthworms per plant rhizosphere was obtained with Th-2 followed by Th-14 (2.66) and Psf-18 (2.40). Number of adult earthworms per plant rhizosphere was obtained equal (1.70) in three treatment viz. Th-39, Bleaching Powder and Streptocyclin. Similarly, in the analysis of total no. of (young + adult) earthworms per hectare (10,000m^2) per plant rhizosphere; maximum no. (49.33×10^4) of earthworms was obtained with Th-2 followed by Th-19 (21.99×10^4), and Th-14 (21.73×10^4). Minimum no. (11.99×10^4) of (adult+ young) earthworms per hectare (10,000m^2) per plant rhizosphere was obtained with Bleaching Powder. Maximum length (15.9cm) of earthworm measured which was obtained from the treatment Th-2. Minimum length (2.8cm) of earthworm measured which was obtained from the treatment Psf-7. Another parameter considered was no. of earthworm's heap of cast per plant rhizosphere. Maximum no. (17.80) of earthworm's heap of cast per plant rhizosphere was obtained plant treated with Th-2 followed by Psf-19 (11.55), Psf-12 (10.54) and Psf-18 (9.68). Minimum no. (3.25) of earthworm's heap of cast per plant rhizosphere was obtained plant treated with Streptocyclin . There was no heaps of earthworms casting per plant rhizosphere was also obtained plant treated with Streptocyclin. It have been demonstrated that it reduces bacterial disease and anthracnose on fruit and increased yield in organically-produced tomatoes produced in soil amended with compost. More importantly, research conducted in Australia has

Plate 26

A. Earthworms with rhizospheric soil adhered on sorghum plant
B. Earthworm casting on the top of the rhizospheric soil
C. Structure of earthworm castings with an opening

Plate.27

A, B, C. Earthworm of varying size

D. Young and adult earthworms in mass

Table 4.26: Effect of soil drenched vermicompost on population of earthworms in rhizospheric soil of sorghum plants 45 DAS

Sample taken from Treatment	No. of Earthworms in sorghum rhizospheric soil			Total no. of earthworms (Young +Adult) per hectare (10,000m^2) per plant rhizosphere	Average length of earthworms (Young+Adult) (cm)	No. of heaps of earthworms casting per plant rhizosphere
	No. of young earthworms per plant rhizosphere (15cm×10cm)	No. of adult earthworms per plant rhizosphere (15cm×10cm)	Total No. of earthworms (Young + Adults)per plant rhizosphere (15cm×10cm)			
Th-2	4.60	2.80	7.40	49.33×10^4	3.5-15.9	17.80
Th-14	0.60	2.66	3.26	21.73×10^4	4.2-11.5	4.55
Th-19	0.40	2.20	3.30	21.99×10^4	4.8-12.6	11.55
Th-39	1.10	1.70	2.80	18.66×10^4	6.0-8.5	6.45
Th-R	0.90	1.90	2.80	18.66×10^4	3.5-14.0	5.56
Psf -3	0.26	2.20	2.46	16.39×10^4	5.9-13.9	4.67
Psf-7	0.30	1.80	2.10	13.99×10^4	2.8-11.9	8.85
Psf-12	0.80	1.80	2.60	17.33×10^4	7.8-14.2	10.54
Psf-18	0.00	2.40	2.40	15.99×10^4	5.8-7.5	9.68
Psf-24	1.33	1.90	3.23	21.53×10^4	4.5-9.2	7.54
Blitox-50	0.00	2.00	2.00	13.33×10^4	6.5-6.8	5.55
Bleaching Powder	0.10	1.70	1.80	11.99×10^4	7.5-8.0	3.75
Streptocyclin	0.53	1.70	2.23	14.86×10^4	6.0-15.0	3.25
Tetracyclin	0.20	1.90	2.11	14.06×10^4	5.5-9.8	5.65
Vermicompost	1.36	1.30	2.66	17.33×10^4	5.5-12.5	8.75
Control	0.00	1.70	1.70	11.33×10^4	6.6-7.5	0.00
CD at 5%	0.46	1.17				

∗Mean value of 5 random samples taken from each treatment

shown that earthworms were associated with decreased severity of field diseases of clover, grains, and grapes incited by *Rhizoctonia* spp. **(Stephens and Davoren, 1997)** and *Gaeumannomyces* spp. **(Clapperton *et al*., 2001; Davoren, 1994)**. **Edwards and Arancon (2004)** have shown that vermicomposts, an end-product of the breakdown of organic matter by earthworms, are disease suppressive. Earthworm castings are rich in nutrients and support a diverse microbial community **(Lunt and Jacobson, 1944; Parle, 1963)**. Castings are also rich in calcium humate, a binding agent **(Edwards and Arancon, 2004)** that reduces desiccation of individual castings and favors the incubation and proliferation of beneficial organisms, such as *Trichoderma* spp. **(Tiunov and Scheu, 2000)**, *Pseudomonas* spp. **(Schmidt *et al.*, 1997)**, and mycorrhizal spores **(Doube *et al.*, 1995, Gange, 1993)**. **Clapperton *et al.* (2001)** showed that earthworms increase communities of gram negative bacteria and concluded that any disease suppression was mediated by enhancing beneficial microbes. Although other microbial communities have been associated with disease suppression, such as filamentous actinomycetes **(Postma *et al.*, 2005)** and Mn-reducing microbes **(Elmer, 1995**; **2003)**, the effect of earthworms on these communities has not been studied. Several reports that vermicompost extracts are effective antimicrobial agents against soil-borne pathogens **(Szczech *et al*., 1993; Orlikowski, 1999; Rodriguez *et al.*, 2000; Szczech and Smolinska, 2001; Edwards and Arancon, 2004; Zaller, 2006)** and do not produce any residual effects.

Summary and Conclusion

Chapter 5 SUMMARY AND CONCLUSION

Stalk rot caused by *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock is one of the most destructive diseases of Sorghum crop in India. The disease mainly affect sorghum stem showing water-soaked symptoms that later turn reddish dark brown color. The infected stem pith disintegrates and show slimy soft-rot symptoms and eventually the whole plant is wilted. The experiments were conducted on this pathogen to study the characterization of test bacterium, screening of inoculation methods for creating artificial epidemic under glass house conditions, pathogenic variability (the degree of virulence and aggressiveness) existing among *Erwinia chrysanthemi* isolates under glasshouse conditions by using leaf whorl and stem inject inoculation methods, genomic variability existing among *E. chrysanthemi* isolates, efficacy of biocontrol agents against test pathogen under *in vitro* and *in vivo* conditions, efficacy of vermicompost colonized with biological control agents against test pathogen by pre-plant soil drenching method under glasshouse and field conditions and screening of chemicals against test pathogen under *in vitro* and *in vivo* conditions.

The salient findings of the study are summarized as below:

1. The disease mainly affected sorghum stem showing water-soaked symptoms that later turned reddish dark brown color. The infected stem pith disintegrated and showed slimy soft-rot symptoms and eventually the whole plant wilted. Losses are due to (1) premature plant death (2) harvest losses associated with stalk breakage or lodging. Affected stalks suddenly collapse and are usually twisted. The tip of the uppermost leaves often wilt, followed by a slimy soft rot at the base of the whorl. The decay spreads rapidly downward until the affected plants collapse. Lodged plants usually have a foul odor.
2. The pathogenicity of the test bacterium was established and it proved to be a potential pathogen.
3. Twenty one isolates of *Erwinia chrysanthemi* were established by biochemical and physiological testing.

4. The antagonistic potential 21 isolates of *T. harzianum* were evaluated against the *E. chrysanthemi* by dual culture assay. Th-39 performed best which gave 90.40 % inhibition of radial growth followed by Th-R (89.6 %), Th-19 (87.7%), Th-43 (79.6 %) and Th-31(76.6 %) whereas least inhibition was obtained with Th-75 (30.7 %)

5. Using dual culture method, antagonistic potential of 12 isolates of *P. fluorescens* was evaluated against the pathogen *E. chrysanthemi.* The most effective isolates in inhibiting the bacterial colony of *E. chrysanthemi* were Psf-3(91.8 %) and Psf-2 (90.7%).

6. In sensitivity test of *Ech* to fungicide Blitox-50, Bleaching powder and 4 chemicals s, Tetracyclin was found most effected followed by Streptocyclin at all concentrations. Maximum zone of inhibition was observed with Tetracyclin (3.80cm) followed by Streptocyclin (2.84cm), Streptomycin (2.14cm) at 400 ppm concentrations.

7. rep- PCR were used to analyze the haplotypic distribution of isolates from different location of district U.S. Nagar. All the 21 isolates differentiated into 21 distinct banding patterns (haplotypes) by the rep-PCR technique used. UPGMA Cluster analysis of the fingerprints generated by BOX primer revealed the presence of four groups at 58% similarity level. Cluster A consisting of 17 haplotypes was found the biggest. Cluster C and D were represented by only single haplotype. Cluster B was represented by two haplotypes, showed 43% genetic similarity with each other. Only a2 A was found to be robust with a bootstrap P value of 90.6 to 100 % (Subcluster). Cluster analysis of ERIC patterns generated binary data showed two clusters at 64% similarity level. Cluster B was the biggest and composed of six haplotypes. Cluster A was consisted of two haplotypes. Cluster B was further subdivided into six sub clusters, b1, b2 b3 b4 b5 and b6, which were related to each other at approximately 80% similarity level. Cluster B was found only robust with a bootstrap P value of 92%. Cluster analysis of REP, grouped the isolates into three clusters (A, B and C) at the demarcation of 67% genetic similarity level. The major cluster B comprised of 15 haplotypes. The members of this cluster

showed approximately 82 to 100% genetic similarities with each other. Cluster A and C was further subdivided into two minor clusters, showing approximately 65% similarity to each other and was represented by three haplotypes in each. Cluster B consisted of three haplotypes and showed a genetic similarity of approximately 32% with a single representative of cluster A. Clusters A and B was found to be robust with a bootstrap value of 100%.

8. A separate phenogram for combined rep-PCR was also prepared and the reliability of the groups was measured by INTYSIS program. Phenograms generated by the PCR analysis and combined cluster analysis detected the same type of grouping. With combined data three clusters were formed at 65% similarity level. Cluster A consisted of three haplotype, whereas, cluster B and C contained four and two haplotypes, respectively. The clusters B was found highly robust with a bootstrap P value of 100% and for cluster A and C were 55, 60% respectively in combined rep-PCR analysis. Combined dendogram from the data obtained from the rep-PCR analysis detected the same kind of grouping, which was found to be highly reliable.

9. Evaluation of inoculation techniques was done for creating artificial Erwinia stalk rot epidemic in sorghum under glasshouse conditions. Highest stalk rot severity (92.40 %) with typical rotting, wilting and uniform type disease development was found in case of root tip cut methods followed by stem injection (85.96%), leaf-whorl (80.60%), and tooth-pick (74.76%) method of inoculation. Partial rotting was encountered with midrib injection (29.22) and cotton wool method (22.57%) over control.

10. In testing for aggressiveness and virulence variability analysis by measurements of disease severity under glasshouse conditions revealed existing variability in virulence and aggressiveness for each *E. chrysanthemi* isolates. Most of the *Ech* isolates were found least virulent in producing disease except *Ech*-12 (53.37 % disease severity) which was found most virulent. Four isolates (*Ech*-3, 8, 10 and 11) were found moderately virulent. In aggressiveness analysis, only five isolates (Ech-1, 17, 18, 20 and 21) were found most aggressive, rest other isolates were found moderately aggressive.

Similarly, some isolates were least virulent (percept disease severity) but found most aggressive (host tissue colonization) viz. *Ech*-1, 18, 19, 20 and 21.

11. In pre-plant soil drenching with vermicompost colonized bioagents experiment under glasshouse conditions, maximum seed germination was recorded with Th-2 (88.53%) followed by Th-R (84.83%), Th-19 (82.70%) and Psf-24 (82.50%). Among chemicals maximum seed germination was observed with Blitox-50 (79.46%) followed by Tetracyclin (77.13%) and Bleaching powder 73.10%). Least seed germination was recorded with vermicompost (68.46%) while it was found statistically at par with treatment Psf-31(70.43%) and Chloramphanicol (70.58%).

12. Application of the *T. harzianum* and *P. fluorescens* isolates colonized vermicompost and two foliar sprays under glasshouse conditions, significantly reduced the disease severity of the stalk rot compared with that of the control. Maximum reduction in disease severity (40.03%) was observed with the isolate Th-2 followed by Th-14(38.49 %), Th-19(34.35%), Th-39 (33.38%) and Th-R (32.65%) as compared to control.

13. In pre-plant soil drenching with *T. harzianum* and *P. fluorescens* isolates and two foliar spray experiment under glasshouse conditions, maximum reduction in disease severity was observed with Th-2 (32.04%) followed by Th-14 (29.70%), Th-R (29.48%), Psf-7 (27.30), Blitox-50 (27.17%), Th-19 (24.85%) and Th-39 (23.20%). Least reduction in disease severity was observed with treatment Th-31 (17.12%).

14. Two foliar sprays with *T. harzianum, P. fluorescens* isolates and chemicals experiment under glasshouse conditions, maximum reduction in disease severity was observed with Th-2 (32.04%) followed by Th-14 (29.70%), Th-R (29.48%), Psf-7 (27.30), Blitox-50 (27.17%), Th-19 (24.85%) and Th-39 (23.20%).

15. Under field conditions in artificial inoculation experiment, maximum disease severity (41.65%) was recorded with leaf whorl inoculation methods followed by stem injection (38.85%) and tooth pick methods (34.35%).

16. In pre-plant soil drenching with vermicompost colonized bioagents experiment under field conditions, maximum seed germination (88.66%) was recorded with Th-2 followed by Th-19 (87.46%) Th-R (87.26%) and Psf-7 (83.50%). Maximum reduction (32.37%) disease severity was recorded with Th-2 followed by Th-14 (29.97%), Th-R (29.25%) and Psf-3 (26.25%). Least reduction in disease severity (19.54%) was recorded with vermicompost.

17. Application of *T. harzianum* and *P. fluorescens* isolates colonized vermicompost and two foliar sprays under field conditions; Maximum reduction in disease severity (40.26%) was recorded with Th-2 followed by Th-14 (37.05%) and Th-R (34.86 %). Least reduction in disease severity (23.74%) was recorded with Psf-18. Similarly maximum plant height (247.93cm) was recorded with Th-14 followed by Th-2 (245.63cm), Th-R (241.63cm), Th-39 (239.66cm) and Psf-3 (237.66cm).

18. In field experiment, an application of two foliar sprays with *T. harzianum* and *P. fluorescens* isolates and chemicals, maximum reduction in disease severity (27.98 %) was recorded with Blitox-50 followed by Th-2 (20.38%), Th-14 (17.86 %) and Tetracyclin (15.10 %). Least reduction in disease severity (06.81%) was recorded with Psf-7. Maximum plant height (234.66cm) was recorded with Th-14 followed by Th-2 (231.30cm), Th-R (229.70 cm). Least plant height (221.46cm) was obtained with Psf-18. Maximum fresh biomass (green fodder) weight (390.0q/h) was observed with Psf-12 followed by Psf-3 (375.0q/h) and Psf-24 (370.0q/h).

19. In population dynamics experiment of earthworms, in the analysis of total no. of (young + adult) earthworms per hectare (10,000m^2) per plant rhizosphere; maximum no. (49.33×10^4) of earthworms was obtained with Th-2 followed by Th-19 (21.99×10^4), and Th-14 (21.73×10^4). Minimum no. (11.99×10^4) of (adult+ young) earthworms per hectare (10,000m^2) per plant rhizosphere was obtained with Bleaching Powder.

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Appendix

APPENDIX I

Medium used in studies: Composition and preparation

All the media used in this study were sterilized by autoclaving at 121 °C for 15 min. The pH of the medium was adjusted using NaOH or HCl before autoclaving. After autoclaving the medium was allow to cool down about 45-50 °C.

Logan's medium (Fahy and Hayward, 1983)

Composition	**Amount**
Nutrient agar	28.0 g
Yeast extract	5.0 g
Glucose	5.0 g
Dist. water	1000.0 ml

After autoclaving and cooling to about 45-50 °C the medium is poured into plates.

NGM medium

Nutrient agar	23 g
MnCl2d. 4H2O (2 mM)	0.4g
Glycerol (1% v/v)	10 ml
Distilled water	1000ml

The pH of NGM medium was adjusted to 6.5 and media was autoclaved at 15 pounds psi (temperature 121^0C) for 20 minutes.

Nutrient Agar (NA) (Lelliott and Stead, 1987)

Composition	**Quantity**
Beef extract	1.0 g
Peptone	5.0 g
Yeast extract	2.0 g
Agar	15.0 g
NaCl	5.0 g
Dist. water	1000.0 ml

King's medium B (King *et al.*, 1954)

Composition	**Quantity**
Proteose peptone	20.0 g
K_2HPO4	1.5 g
$MgSO_4$.7H2O	1.5 g
Glycerol	10.0 ml
Agar	15.0 g
NaCl	5.0 g
Dist. water	1000.0 ml
pH	7.2

Yeast- dextrose- chalk agar (YDC) (Lelliott and Stead, 1987)

Composition	**Quantity**
Yeast extract	10.0 g
$CaCO_3$	20.0 g
D-Glucose	20.0 g
Agar	15.0 g
Dist. water	1000.0 ml

Dissolved ingredients, except dextrose, in 900 ml water and sterilize at 120 ºC for 1 h. Cool to 50 ºC and aseptically add 100 ml of a 20 % filter sterilized (0.45 µm filter) dextrose solution. Mix well to keep the calcium salt in suspension and pour in Petri dishes. Inoculate YDC medium with test bacterium and incubate at 27 ºC for 48 h.

Simmon's citrate agar

Composition	**Quantity**
Ammonium dihydrogen phosphate	1.0g
Dipotassium phosphate	1.0g
Sodium chloride (NaCl)	5.0g
Sodium citrate	2.0g
Magnesium sulphate	0.2g
Agar	15.0g
Bromothymol blue	0.8g
Distilled water	1000.0ml

CVP medium **(Cuppels and Kelman. 1974)**

Composition	**Amount**
1 N NaOH	4.5 ml
10% CaCl2-2H2O	3.0 ml
NaNO3	1.0 g
Agar	1.5 g
Sodium Polypectate	10 g
10% SDS	0.5 ml
0.075% crystal violet	1.0 ml

APPENDIX II

rep-PCR FINGERPRINTING

DNA extraction and rep-PCR amplification was done for all pathogenic isolates. For each isolates rep-PCR is carried out by ERIC, REP and BOX primer. The primer sequences are given below:

Primer sequences of rep-PCR

5' ——————→ 3'

1. REP-PCR

R IIIICGICGICATCIGGC

F ICGICTTATTATCIGGCCAC

2. ERIC-PCR

R ATGTAAGCTCCTGGGGATTCAC

F AAGTAAGTGACTGGGGTGAGCG

3. BOX-PCR

CTACGGCAAGGCGACGCTGACG

PCR conditions

Rep-PCR was performed in the thermocycler (BIORAD, C1000^{TM} Thermal cycler), under the following conditions as initial incubation of 95^0C for 2 min, followed by 30 to 35 cycles of 94^0C for 3 sec, 92^0C for 30 sec, 40^0C for 1 min for ERIC-PCR, and BOX-PCR and 65^0C for 8 min. Lastly, final extension cycle at 65^0C for 8 min and a final waiting temperature of 4^0C.

PCR-PROTOCOLS

Table 1: PCR reaction master mixture and Thermal profile for ERIC, REP and BOX-PCR

A. Master mix preparation for ERIC-PCR (50 µL reaction)

Reagents	Volume/reaction (µL)	Required for no. of sample	Total amount (µL)
5X PCR-buffer	10	23	230
25.0mM $MgCl_2$	4	23	92
25.0mM dNTPs	2.5	23	57.5
DMSO	5	23	115
BSA	0.4	23	9.2
10mM ERIC R Primer	2	23	46
10mM ERIC F Primer	2	23	46
5U/ µL Taq Pol	0.66	23	15.18
PCR grade water	20.44	23	470.12
100ng Template (DNA)	3		
Total Volume	50		

a. Thermal profile for ERIC-PCR

PCR Steps	Temperature	Time
Initial denaturation	95 °C	7 Min
Denaturation	94°C	1 Min
Annealing	46°C	1 Min
Extension	65°C	8 Min
Final extension	65°C	15Min
Forever	4°C	

Total time approximately 6hrs

B. Master mix preparation for BOX-PCR (25µL reaction)

Reagents	Volume/reaction (µL)	Required for no. of sample	Total amount (µL)
5X PCR-Buffer	5	23	115
25 mM $MgCl_2$	2	23	46
25mM dNTPs	1.25	23	28.75
DMSO	2.5	23	57.5
BSA	0.2	23	4.6
10mM BOX A1R-Primer	1	23	23
5U/ µL Taq Pol	0.33	23	7.59
PCR grade water	10.72	23	246.5
100ng Template (DNA)	2		

b. Thermal profile for BOX-PCR

PCR Steps	Temperature	Time
Initial denaturation	95 °C	7 Min
Denaturation	94°C	1 Min
Annealing	46°C	1 Min
Extension	65°C	8 Min
Final extension	65°C	15 Min
Forever	8°C	
30 Cycles		

Total time approximately 6hrs

C. Master mix preparation for REP-PCR (25 µL reaction)

Reagents	Volume/reaction (µL)	Required for no. of sample	Total amount (µL)
5X PCR-Buffer	5	23	115
25 mM $MgCl_2$	2	23	46
25 mM dNTPs	1.25	23	28.75
DMSO	2.5	23	57.5
BSA	0.2	23	4.6
10mM REP R Primer	1	23	23
10mM REP F Primer	1	23	23
5U/ µL Taq Pol	0.5	23	11.5
PCR grade water	9.55	23	219.65
100 ng Template (DNA)	3		

c. Thermal profile for REP-PCR

PCR Steps	Temperature	Time
Initial denaturation	95 °C	7 Min
Denaturation	94°C	1 Min
Annealing	46°C	1 Min
Extension	65°C	8 Min
Final extension	65°C	15 Min
Forever	4°C	
30 Cycles		

Total time approximately 6hrs

Gel-Electrophoresis

Preparation of 0.8% Agrose gel for observing DNA 0.8 gm agrose +100ml of distilled water + 2ml 50XTAE Buffer+4µl of EtBr.

4.3.6 Gel Electrophoresis of PCR products

Preparation of 1.5% gel for 100 ml:

Composition	Quantity
Agarose (1.5%)	1.5g
TAE buffer	2ml
Distilled water	98ml
Ethidium bromide	4µl

Procedure:

1.) Taken 98 ml distilled water.

2.) Added 2 ml of 50X TAE Buffer.

3.) Added 1.5 mg Agarose, mix and keep it in oven for approx 2 min.

4.) Allowed to cool.

5.) Mixed 4µl of Ethidium bromide (EtBr).

6.) Placed it on electrophoresis tray with comb.

7.) Allowed to solidify.

APPENDIX III

GENETIC DIVERSITY

Genomic DNA Extraction

Preparation of Solutions:

1. 0.5 M EDTA (pH-8)

Prepare 1000 ml using:

Sodium EDTA	186.1 g
NaOH	20 g
Deionized water	800 g

2. 1X TE Buffer

Prepare 1000 ml using

100X TE Tris, pH 8.0	10ml
Distilled Water	990 ml

3. Resuspension Buffer (0.15M NaCl, 0.01M EDTA, pH-8)

Prepare 1000 ml using

NaCl	8.77g
0.5 M EDTA, pH 8.0	20 ml

4. 7.5 Ammonium Acetate

Prepare 1000 ml using:

Ammonium acetate	578.1g

Dissolve in 1000 ml of distilled water.

5. Chloroform/Isoamylalcohol (24:1)

Prepare 1000 ml using

Chemical	**Quantity**
Isoamyl-alcohol	40 ml
Chloroform	960 ml

(Note: Chloroform is highly toxic wear suitable protective clothing and work under a fume hood depending on number of samples for extraction is to be done.

6. **Isopropanol**
7. **70% Ethanol**
8. **RNase solution (100 µl/ml)**

(Note: all materials need to be sterilized by autoclaving except solution number 5, 8, and 9.)

4.3.3 DILUTION OF PRIMER

1. Add given amount of water into the primer
2. Mix it with help of centrifuge at approx. 2500-3000 rpm for 2 min.
3. Keep it on ice.
4. For 100 ml need 10 ml of standard primer.
5. Add 90 ml water
6. All work should done on ice then keep it at -20^0C

Quantification of genomic DNA

Genomic DNA was quantified by using NANODROP SPECTROPHOTOMETER. 1µl of extracted genomic DNA was taken to quantify the concentrations (ng/µl) and purity (OD). The optical density was taken at 260 nm and 280nm (wavelength).

Isolate no.	Concentration of genomic DNA(ng/ µl)	Optical density
1	852.6	1.89
2	1317.7	1.90
3	996.1	1.97
4	192.6	1.98
5	5709	1.89
6	119.8	1.70
7	2531.1	1.99
8	156.8	1.68
9	338.9	1.88
10	225.6	2.10
11	484.4	1.76
12	171.5	1.88
13	325.5	1.78
14	1166	2.0
15	189.7	1.90
16	227.8	1.69
17	902	1.89
18	1448.0	1.92
19	335.4	1.85
20	898.8	2.03
21	443	1.69

VITA

The author was born on 4th august 1985 at Majra Farm (Khatima). He earned his M.Sc. (Ag.) Plant Pathology from Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, in 2009. Then he joined the Ph.D. degree programme with major in Plant Pathology minor in Entomology at Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, in August 2009. The Author was recipient of University Fellowship during his degree programme. He has been qualified for ICAR-SRF (2009-10) and ICAR-NET (2010) examination conducted by N.A.A.R.M. and A.S.R.B. respectively. His research interests centered on Diagnosis of Plant Diseases, Microbial Taxonomy and Molecular Plant Pathology.

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ABSTRACT

Sorghum bicolor (L.) Moench is one of the prime food and fodder crops of the world. The yield potential of this crop is affected by a number of diseases. Stalk rot caused by *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock is one of the most destructive diseases of sorghum crop in India. Considering the destructive nature of this disease and availability of only little information on chemical, biological control and existing diversity among the isolates of *E. chrysanthemi*, present investigations were carried out with the following objectives to study the; (1) Characterization of *E. chrysanthemi* isolates, (2) Pathogenicity and inoculation methods for creating artificial epidemic under glass house conditions, (3) Pathogenic variability existing among *E. chrysanthemi* isolates under glasshouse conditions, (4) Genomic variability existing among *E. chrysanthemi* isolates (5) Antagonistic potential of biocontrol agents against test pathogen under *in vitro* and *in vivo* conditions, (6) Glasshouse and field evaluation of vermicompost colonized with biocontrol agents against test pathogen by pre-plant soil drenching method, (7) Screening of chemicals against the test pathogen under *in vitro* and *in vivo* conditions. The antagonistic potential of 21 isolates of *Trichoderma harzianum* was evaluated against the *E. chrysanthemi* by dual culture assay. Th-39 performed best which gave 90.40 % inhibition of radial growth followed by Th-R (89.6 %), Th-19 (87.7 %), Th-43 (79.6 %) and Th-31 (76.6 %) whereas least inhibition was obtained with Th-75 (30.7 %). Most effective isolates in inhibiting the bacterial colony of *E. chrysanthemi* were found, Psf-3(91.8 %) and Psf-2 (90.7%). In the sensitivity test of *E. chrysanthemi* to chemicals, maximum zone of inhibition was observed with Tetracyclin (3.80cm) followed by Streptocyclin (2.84cm), Streptomycin (2.14cm) at 400 ppm concentrations. rep-PCR were used to analyze the haplotypic distribution of isolates *E. chrysanthemi* from different location of district U.S. Nagar. All the 21 isolates differentiated into 21 distinct banding patterns (haplotypes) by the rep-PCR technique used. With combined data of rep-PCR (BOX, ERIC and REP) three clusters were formed at 65% similarity level. Cluster A consisted of three haplotype, whereas, cluster B and C contained four and two haplotypes, respectively. The clusters B was found highly robust with a bootstrap P value of 100% and for cluster A and C were 55, 60% respectively in combined rep-PCR analysis. Combined dendogram obtained from the rep-PCR data analysis detected the same kind of grouping, which was found to be highly reliable. Highest stalk rot severity (92.40 %) with typical rotting, wilting and uniform type disease development was found in case of root tip cut methods followed by stem injection (85.96%), leaf-whorl (80.60%), and tooth-pick (74.76%) method of inoculation under glasshouse conditions. In aggressiveness analysis, only five isolates (*Ech*-1, 17, 18, 20 and 21) were found most aggressive, rest other isolates were found moderately aggressive. Similarly, some isolates were least virulent (in producing amount of disease) but found most aggressive (host tissue colonization) viz. *Ech*-1, 18, 19, 20 and 21. Application of the vermicompost colonized bioagents (*T. harzianum* and *P. fluorescens* isolates) + two foliar sprays under field conditions, maximum reduction (40.26%) in disease severity was recorded with Th-2 followed by Th-14 (37.05%) and Th-R (34.86 %). In population dynamics experiment of earthworms, the analysis of total no. of (young + adult) earthworms per plant rhizosphere per hectare (10,000m^2) was done. Maximum no. (49.33×10^4) of earthworms was obtained with vermicompost followed by Th-19 (21.99×10^4), Th-14(21.73×10^4) and control (21.53×10^4).

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शोध शीर्षक	: ''ज्वार के तना विगलन कारक *इरविनिया क्राईजेन्थिमाइ* का लक्षण वर्णन, जीनोमिक फिंगरप्रिंटिंग एवं प्रबन्धन पर अध्ययन''		
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सारांश

सोर्घम वाइकलर (एल.) मोएंक विश्व की प्रमुख खाद्य एवं चारे की फसल है। इस फसल की उत्पादन क्षमता को बहुत से रोग प्रभावित करते हैं। *इरविनिया क्राईजेन्थिमाइ* वरखोल्डर, मैकफोडेन एवं डाइमॉक द्वारा जनित ज्वार फसल का तना विगलन रोग भारत में बहुत ही विनाशकारी है। इस रोग की विनाशकारी प्रकृति रासायनिक, जैविक नियंत्रण एवं *इ. क्राईजेन्थिमाइ* के विलगों में विविधता की अल्प जानकारी उपलब्ध होने के कारण वर्तमान अध्ययन किये गये, 1. *इ. क्राईजेन्थिमाइ* के विलगों का लक्षण वर्णन, 2. कॉचघर परिस्थियों में रोजगनक क्षमता एवं कृत्रिम महामारी उत्पन्न करने की विधियां, 3. कॉचघर परिस्थितियों में *इ. क्राईजेन्थिमाइ* के विलगों में उपस्थित रोग जनक क्षमता की विविधता, 4. *इ. क्राईजेन्थिमाइ* के विलगों में उपस्थित जीनोमिक विविधता, 5. पात्रें एवं जीवे परीस्थितियों में जैव नियत्रकों की रोग जनक के विरूद्ध एन्टागोनिस्टिक क्षमता, 6. पादप–पूर्व मृदा मिलान विधि द्वारा केचूये की खाद में पाले गये जैवनियत्रकों का रोगजनक के विरूद्ध कॉचघर व खेत में परीक्षण, 7. पात्रे एवं जीवे में रसायनों का रोगजनक के विरूद्ध परीक्षण। *ट्राइकोडर्मा हरजियनम* के विलगों का *इ. क्राईजेन्थिमाइ* के विरूद्ध में डयूलकल्चर विधि द्वारा परीक्षण किया गया, सर्वाधिक त्रिज्यक वृद्धि को टीएच–39 ने घटाया (90.40%) तत्पश्चात् टीएच–आर (89.60%), टीएच–19 (87.7%), टीएच–43 (79.6%) एवं टीएच–31 (76.6%) जबकि न्यूनतम 30.7% टीएच–75 ने घटाया। *इ. क्राईजेन्थिमाइ* की कॉलोनी को अवरूध करने में पीएसएफ–2 (91.8%), पीएसएफ (90.7%) सर्वाधिक प्रभावी पाये गये। *इ. क्राईजेन्थिमाइ* की रसायनों के प्रति संवेदनशीलता परीक्षण में 400 पीपीएम सान्द्रता पर सर्वाधिक अवरोध क्षेत्र 3.8 सेमी. टेट्रासाइक्लिन, स्ट्रेप्टोसाइक्लिन (2.84 सेमी.) तथा स्ट्रेप्टोमाइसिन (2.14 सेमी.) पाया गया। उत्तराखण्ड के उ0सि0 नगर जिले के विभिन्न क्षेत्रों से *इ. क्राईजेन्थिमाई* के विलगों के हेप्लोटाईपिक वितरण का रैप–पीसीआर द्वारा विश्लेषण किया गया। रैप–पीसीआर द्वारा सभी 21 विलगों को स्पष्ट 21 बैन्डिग प्रारूपों (हेप्लोटाईपस) में विभक्त पाया गया। रैप–पीसीआर (बॉक्स, इरीक एवं रैप) के आकड़ों को मिश्रित करने पर, 65% समानता स्तर पर, तीन समुह बने। समुह ए, बी एवं सी क्रमशः तीन, चार व दो हेप्लोटाईपों से बने। समुह बी 100% तथा समुह ए व सी क्रमशः 55% एवं 60% बूट स्ट्रेप मान पी के साथ रोबस्ट पाये गये। रैप–पी.सी.आर. द्वारा प्राप्त मिश्रित डेन्डोग्रामों में समान समूहीकरण प्राप्त हुआ जोकि बहुत ही विश्वनीय पाये गये। कॉचघर कृत्रिम टीकाकरण परीक्षण में आर्दशभूत विगलन, म्लानि एवं समान रोग विकास के साथ रूट टिप विधि में सर्वाधिक तना विगलन उग्रता (92. 4%) ततपश्चात् तना इन्जेक्शन (85.96%), पर्ण समुह छिड़काव (80.6%) एवं टूथपिक (74.76%) विधि द्वारा पाया गया। रोगजनक की आक्रामकता विश्लेषण में, इसीएच के केवल पांच विलगों 1, 7, 18, 20 एवं 21 सर्वाधिक आक्रमक पाये गये, कुछ विलग सबसे कम उग्र (प्रतिशत रोग उग्रता में) जबकि सर्वाधिक आक्रामक (होस्ट टिस्सु कोलोनाइजेशन में) पाये गये जो इस प्रकार है इसीएच–1, इसीएच–18, इसीएच–19, इसीएच–20 एवं इसीएच–21। खेत की परीस्थितयों में, बायोऐजेन्ट (*टी. हरजियनम* एवं *पी. फ्लोरीसेन्स* विलगों) कोलोनाइज्ड वर्मीकम्पोस्ट विलगों एवं दो पर्णीय छिड़काव के प्रयोग से सर्वाधिक रोग उग्रता न्यूनीकरण (40.26%) टीएच–2 ततपश्चात् टीएच–14 (37.05%) एवं टीएच–आर के साथ दर्ज की गयी। ज्वार के प्रति पौध के जड़ मण्डल प्रति हेक्टेयर में केचूओं के जनसंख्या गतिविद्या का विशलेषण किया गया। केचूओं की सर्वाधिक संख्या टीएच–2 (49. $33x10^4$) ततपश्चात् टीएच–19 ($21.95x10^4$) एवं टीएच–14 ($21.73x10^4$) के साथ पायी गयी।

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