Exploration of different methods for detection of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis, the cause of bacterial wilt and canker of tomato (*Lycopersicon esculentum* M.) and its management

Thesis

Submitted to the

G. B. Pant University of Agriculture & Technology, Pantnagar- 263 145 (U.S. Nagar), Uttarakhand, India

By

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M.Sc.Ag. (Mycology & Plant Pathology)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctorate in Philosophy

(PLANT PATHOLOGY)

JANUARY, 2018

ACKNOWLEDGEMENT

*It is by lavish love and blessing of the supreme **Lord Kashi Vishwanath Ji** that I have been able to complete my studies successfully hitherto and present the piece of work for which I am eternally indebted.*

*With immense pleasure and profound sense of gratitude, indeed, I take this opportunity to express my heartfelt and sincere thanks to my esteemed supervisor, **Dr. K. Vishunavat**, Professor and Head, Department of Plant Pathology, College of Agriculture, G B Pant University of Ag. and Tech., for her meticulous guidance, indelible inspiration, persistent encouragement, ingenious suggestions, mellifluous nature and indefatigable attitude. I will ever cherish the affection that she bestowed upon me throughout my tenure as a research scholar under her which helped me to cope with many difficult situations.*

*I humbly express my profound gratitude to **Dr. R. P. Singh**, t Professor, Department of Mycology & Plant Pathology, **Dr. Y. Singh,** Professor, Department of Plant Pathology and **Dr. S. N. Tiwari**, Professor Department of Entomology and **Dr. A. Kumar**, Professor Department of MBGE (the members of my advisory committee) for their valuable suggestion and liberal help rendered during the course of study and research work.*

*I extend my indebtedness to **Dr. Rashmi Tiwari**, Assistant Professor, Department of Plant Pathology, College of Agriculture , G B Pant University of Ag. and Tech. , for her immense encouragement and support, her discerning comments, valuable suggestions, co-operations and helpful attitude towards me during the course of investigation.*

*I feel paucity of words to express my gratefulness and warmest regard to my very esteemed teachers. **Dr. K.P. Singh**, **Dr. A.K. Tewari**, **Dr. Vishwanath**, and **Dr. B. Kumar** of the Department of Plant Pathology, College of Agriculture , G B Pant University of Ag. and Tech.., for their timely help and co-operation during the course of my study.*

With profound regard in a more personal sense, I owe deepest debts to my parents Shri J. C. Tripathi and Smt. Meena Tripathi who taught me the value of wisdom based on erudition but without enslaved by it and their persistent inspiration, selfless sacrifice, continuous encouragement and blessing gave untiring help and have enabled me to be so today.

Words are not enough to express my unbound gratitude to my younger sister and brother, Garima and Chetan who always motivated me through my hard times.

I am extremely thankful to my juniors Yogita Bohra, Anjna Kholiya, Sudha Nandini Sharma and Abhishek Gowda HR, for their keen interest, constant inspiration and assistance without which the work would not have been possible.

How can I forget the love, inspiration and motivation I constantly received from my best friends Sachin Pant and Neha Jayant Gautam. This thesis would not have been possible without both of you. I heartily thank you as your enthusiasm never failed me out of my blues.

I also want to give a special thanks to P. K. Agrawal Sir, Santosh Bhaiya, Aas Mohammad Bhaiya, Mahendra Bhaiya and Kaleshwar Bhaiya for their support during conducting my field trials. I would like to acknowledge the support from the non-teaching staff members of the Department of Plant Pathology, for their helpful assistance without showing any hesitation.

Finally I thank the many people whose names were not mentioned here, but who made this thesis possible with their biggest inspiration, encouragement and support. My apologies to those that I have inadvertently overlooked or forgot to mention in my acknowledgements but who equally deserve.

Above all, my humble and whole hearted prostration to ***Lord Baba Vishwanath, SankatMochan Baba*** *and* ***Goddess Sarswati*** *for their blessings.*

Great thanks to all

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Authoress

Date : January, 2018
Place: Pantnagar

CERTIFICATE-I

This is to certify that the thesis entitled **"Exploration of different methods for detection of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis, the cause of bacterial wilt and canker of tomato (*Lycopersicon esculentum* M.) and its management"** submitted in partial fulfilment of the requirements for the degree of **Doctorate in 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 **Miss Ruchi Tripathi, Id. No. 35685** 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 acknowledged.

Pantnagar
January, 2018

(K. Vishunavat)
Chairperson
Advisory Committee

CERTIFICATE-II

We, the undersigned, members of the Advisory Committee of **Miss Ruchi Tripathi, Id. No. 35685,** a candidate for the degree of **Doctorate in Philosophy** with major in **Plant Pathology** and minor in **Entomology** agree that the thesis entitled **"Exploration of different methods for detection of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis, the cause of bacterial wilt and canker of tomato (*Lycopersicon esculentum* M.) and its management"** may be submitted in partial fulfilment of the requirements for the degree.

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(Ex-officio member)
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Introduction

Chapter 1 INTRODUCTION

The tomato *(Lycopersicon esculentum* Mill.), belonging to family Solanaceae, is an economically important crop grown throughout the world and used as both fresh fruit and the processed food. It can be grown in a broad range of climatic conditions in the field and under protected cultivation **(Atherton and Rudich, 1986)**. Tomatoes are believed to have their origin in the coastal strip of western South America, from the equator to latitude of about 30^0 South. The species is native to South America, primarily in Peru and the Galapagos Islands. It plays a major role in human nutrition and is an excellent source of phosphorus, iron and vitamin A, B and C; **(Cobley and Steele, 1976, Varela *et al.,* 2003 and Naika *et al.,* 2005)**. There are about 7500 varieties of tomato grown all over the world. The total area under tomato cultivation across the world is 4.73 mha with a production of 163.96 mT. The major tomato growing countries in the world are China, USA, Italy, Turkey, India and Egypt **(FAOSTAT, 2016)**. In India the area under tomato cultivation is 0.791 mha, production is 17.398 mT and productivity is 22.00 T/ha (Anonymous, 2016). The major tomato growing states in the country are Andhra Pradesh, Karnataka, Madhya Pradesh, Maharashtra, Orissa, Bihar and Tamil Nadu The net contribution of tomato to the total vegetable grown is 8.04% and 9.67% in terms of area and production respectively. In Uttarakhand the area under tomato cultivation is 0.00946 mha, production is 0.1154 mT and the productivity is 12.2 T/ha. The major tomato growing districts are US Nagar, Uttarakashi, Nainital, Haridwar and Dehradun **(Anonymous, 2016)**.

The tomato processing industry is of high significance, however, the cultivation of the tomato crop is heavily affected by pest, fungi, bacteria and viruses which affect the fruit supply for its various industries. In the hilly region of northern India, the tomato crop is generally affected by different fungal, bacterial, viral and nematode diseases. Amongst bacterial diseases, the diseases that are often adding to the grower's losses are bacterial wilt (*Ralstonia solanacearum*) and bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) that have been reported earlier **(Gupta and Thind, 2006)**. A new tomato disease causing leaf necrosis, wilting and splitting of stem has been observed since last few years causing damage to the crop in the tomato growing areas

of northern hill region. The disease was found to be of a bacterial etiology and suspected to be the bacterial canker caused by *Clavibacter michiganensis* ssp. *michiganensis* (Smith) Davis. An extreme reduction in the qualitative and quantitative parameters is being done by the bacterium, leading to significant monetary loss both in protected and open field cropping conditions; which sometimes may even lead to complete yield loss **(Chang *et al.,* 1992; Gitaitis, 1991; Hadas *et al.,* 2005)**. Bacterial canker is a vascular and parenchymatal disease with a broad range of symptoms viz., leaf margin necrosis, and stunting, wilting, discoloration in vascular regions, leaf spots, fruit spots and eventual plant death. Small, raised, superficial necrotic spots are also induced by the pathogen on the fruit surrounded by white halos, referred to as "bird's eye lesions". The symptoms produced by this bacterium can be affected by the place of production (glasshouse or field), age of plant at the time of infection, cultural practices, cultivars, etc **(Gitaitis, 1991)**. The pathogen being seed-borne in nature, has been categorized by EPPO as an A2 category quarantine phytopathogen, occurring in almost all the tomato growing areas round the globe **(EPPO/CABI, 1998)**. Sowing seeds infected with the bacterium may lead to reduced germination, vigor and yield reduction. The pathogen also persists in plant debris in soil and on contaminated greenhouses. Contaminated seeds act as the source of long distance dispersal of bacterial inoculum **(De Leon *et al.,* 2009; Kawaguchi *et al.,* 2010)**. Contamination of previously disease-free areas is considered one of the most adverse effects of the seed borne bacteria, thereby acting as a source of primary inoculums for the disease spread. Even very low levels of seed-borne inoculum may lead to extremely high field incidence when the weather is congenial for disease development **(Neergaard, 1979)**. A high population of *Cmm* can also be present in completely asymptomatic tomato seedlings (Werner *et al*., 2002), eventually leading to infection of the other plants. The production of disease free tomato seed is of great economic importance for both growers and the seed industry. However the disease is generally erratic during growing season and it may be absent or of limited occurrence upto certain years and may cause epidemic in the next year **(EPPO/CABI, 2005)**.

The entry of *Cmm* generally occurs via infested seed source into a new area (Strider, 1969) or by the means of tomato transplants carrying dormant infection **(Chang *et al.,* 1991; Gitaitis *et al.,* 1991)**. The problem generally phased in the

management of the pathogen lies firstly in the unavailability of sufficient disease diagnostic tools due to an array of symptoms produced by the pathogen and the second reason can be accounted as the different inoculums sources, comparatively longer latency period of the bacterium and the lesser number of efficient chemicals for the management of the pathogen demands the need for the development of proper sanitation and preventive measures. The control of the disease becomes very difficult, once the disease initiates, as sufficient control measures are not yet available **(Ftayeh *et al.*, 2008)**. No resistant cultivar has yet been screened out and the chemicals may or may not be effective. The spread of the disease can be reduced to certain extent by the use of antimicrobial compounds. Till date, no chemicals can sucessfully control *Cmm* **(de Leon *et al.,* 2008, Hausbeck *et al.,* 2000)**. However, the use of copper compounds may at times lead to phyotoxicity **(Yang *et al.,* 2002)**. Plant defense system may also be activated by use of the chemicals or via priming against the ingressing pathogen, or through activating plant defense mechanism. Salicylic acid (SA), DL aminobutric acid (BABA), 2,6 di chloro isonicotinic acid (INA) and acibenzolar S methyl (ASM) are amongst some of the chemicals being described as defense inducers in plant against a range of phytopathogen **(Baysal *et al.,* 2005).**

However, bacterial canker of tomato has not been yet studied and documented from the outer himalayan belt of tomato growing areas and the two most significant problems associated with the disease are the absence of suitable detection and control measures, secondly due to being latent in nature the detection of disease may not be possible at right times and can also be confusing at times.

Thesis outline

Keeping in view the importance of the crop and heavy losses caused by this disease, this study aims on exploring the different detection tools for *Clavibacter michiganensis* subsp *michiganensis* (*Cmm*) and its management. The investigations have been proposed to be undertaken with following objectives:

- To record the incidence and severity of bacterial canker of tomato in Middle and Lesser Himalayan regions in Uttarakhand and Himachal Pradesh.
- To detect the isolated bacterial pathogen through morphological, physiological, biochemical, serological and molecular characterization.

- To evaluate available germplasm (cultivars, lines) against the bacterial pathogen in protected (glasshouse and polyhouse) and open field condition.
- Study of pathogenic variability existing among *Clavibacter michiganensis* subsp *michiganensis* isolates under glasshouse conditions through different inoculation techniques.
- Screening of test chemicals and biocontrol agents against the test pathogen under *in vitro* and *in vivo* condition and assessment of population dynamics of the pathogen.
- Study of defense inducers by explicating the biochemical basis of resistance in plant in *in vitro* conditions.

Review of Literature

Chapter 2 REVIEW OF LITERATURE

2.1 Historical background of the crop

Tomatoes (*Solanum lycopersicum* L. Syn. *Lycopersicon esculentum* Mill.) belonging to family Solanacae (nightshade family), genus. *Lycopersicon*, subfamily *Solonoaideae* and tribe *Solanae* **(Taylor, 1986)**, are amongst one of the most extensively cultivated and consumed 'vegetables' around the globe, for both the purposes of fresh fruit market as well as processed fruit industries **(Heuvelink, 2004)**. The plant is herbaceous and annual in nature with hairy stem and is differentiated either as indeterminate or determinate types based on the vigor or plant growth habit **(Papadopoulos, 1991)**. Andean region is considered to be the native place for all related wild species of tomato that includes part of Chile, Columbia, Equador and Peru **(Sims, 1980)**. Tomatoes were introduced by the Spanish into Europe during the early 16^{th} century **(Harvy *et al.,* 2002)** and it was introduced to China, South and Southeast Asia by European during the 17^{th} century **(Siemonsma and Piluek, 1993)**. Tomatoes are referred to be one of the most accepted vegetable in most of the parts of world, with respect to its high nutritive value, easy adaptability in new environment and varied uses. Even being such an important crop, it is prone to a number of fungal, bacterial and viral diseases leading to a devastating reduction in crop yield. The crop is being attacked by different bacterial pathogens amongst them, one of the bacterial disease i.e. bacterial canker and wilt of tomato caused by *Clavibacter michiginensis subsp michiganensis* is of utmost importance **(Chang *et al.,* 1992)**.

2.2 General introduction and nomenclature of the pathogen

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), a Gram positive bacterium causing wilt and canker, is aerobic, rod shaped, nonsporulating in nature. The bacterium belongs to genus *Clavibacter* that is currently is differentiated into five subsp. on the basis of host species viz., *C.m.* subsp. *michiganensis* infecting tomato **(Strider, 1969; Davis *et al.,* 1984**), *C. m.* subsp. *sepedonicus* infecting potato **(Manzer and Genereux, 1981)**, *C. m.* subsp. *nebraskensis* infecting maize **(Schuster, 1975)**, *C. m.* subsp. *insidiosus* infecting alfalfa **(McCulloch, 1925)** and *C. m. subsp. tessellarius* **(Carlson *et al.,* 1982)** infecting wheat. It is a seed borne pathogen and is of significant importance due to its destructive effect on crop yield. Due to the devastating impact of

the pathogen on the crop, it is considered as a quarantine pathogen in European Union (EU) and in other countries of the world **(Anonymous, 2000)**. Owing to the insufficient understanding of intricate behavior of *Cmm* as phytopathogen, unavailability of management tools and the lack of resistant genotypes have lowered the rate of successful prevention of the pathogen **(Chang *et al.,* 1991)**. The long route transportation of *Cmm* is facilitated via, seed trading, which itself is the reason for the presence of the pathogen in all most all the tomato growing areas of different countries **(EPPO, 2007)**. However, the survival of the pathogen is also observed in plant debris and also on volunteer plants or alternative hosts thereby acting as a local source of inoculum **(Gleason *et al.,* 1993)**.

2.3 Economic importance and geographical distribution of *Clavibacter michiganensis* subsp *michiganensis*:

Besides the fungal and viral, bacterial pathogens can also be considered of utmost importance, leading to significant losses in agriculture. Bacterial diseases can be some of the most serious and destructive diseases affecting field and greenhouse grown tomato crop both, thereby leading to a significant reduction in global tomato production. Different types of bacterial diseases affecting tomato are bacterial canker, bacterial speck, and bacterial spot. Amongst these diseases bacterial canker can be considered of significant importance worldwide causing considerable economic losses in open field as well as greenhouse field tomato production **(Chang *et al.,* 1992; EPPO/CABI, 2005)**. E. F. Smith first time reported the disease in 1910 from Grand Rapids, Michigan (USA) as Grand Rapids disease but later it was named as bacterial canker. Thereafter **McKeen (1973)** and **Chang *et al.* (1992)** reported bacterial canker in greenhouse and field grown tomatoes in other states of USA, like Ontario and Illinois.

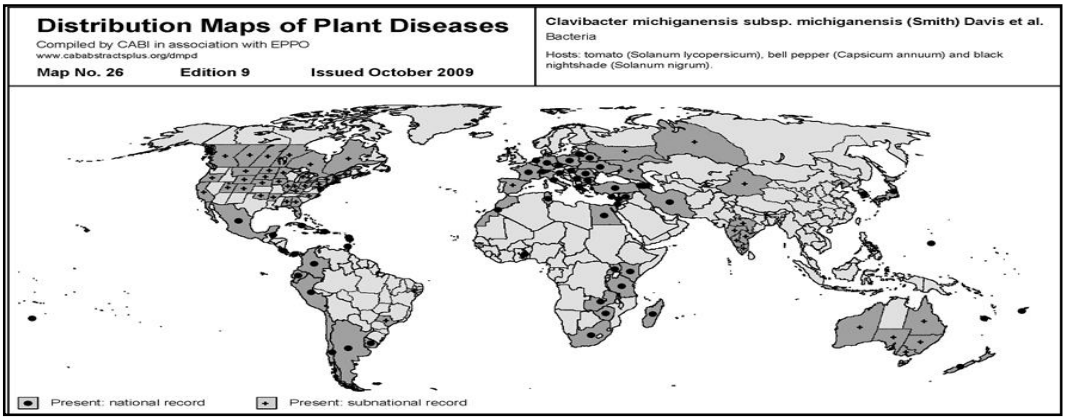


Figure 2.1 *Clavibacter michiganensis* subsp. *michiganensis,* distribution around the world as issued by CABI/ EPPO in 2009.

Thereafter the disease eventually started to spread in the other tomato growing areas of the world and was reported from Italy (Scortichini, 1997), Turkey (Basim *et al*., 2004), Serbia **(Milijasevic *et al.*, 2009a)**, Mexico (Holguin *et al.,* 2006), Cyprus **(Ioannou *et al.*, 2000)**, Iran **(Nazari *et al.*, 2007)**, Korea (Myung *et al.,* 2008), Indonesia **(Zainal *et al*., 2008)**, Syria **(Ftayeh *et al.,* 2010)**, Italy **(Lamichhane *et al.*, 2011)** etc. In India, the disease was first recorded during 1949 from Nagpur Maharashtra state but the pathogen was not confirmed **(Jain, 1951)**. The confirmed report of bacterial canker of tomato was from the state of Karnataka, India with an incidence of 25- 48 per cent by **Sarala and Shetty (2005)** and the disease was also reported from Karnataka by **Umesha, 2006** with an incidence up to 48 per cent. In other tomato growing countries where ever this disease occurs, causes huge crop losses **(Gitaitis, 1990; Chang *et al*., 1992; Boudyach *et al*., 2001**).

2.4 Cultural Morphology

Clavibacter michiganensis subsp. *michiganensis* causing bacterial wilt and canker of tomato, characterized as a gram positive, plant pathogenic actinomycetes **(Stakebrandt *et al.,* 1997)**, is an economically devastating pathogen that inflicts considerable damage throughout all major tomato-producing regions (Gleason *et al.* 1993).However the variant strains of the bacterium on nutrient-rich medium, show different colony characteristics including dry, sticky, mucoid and less mucoid, pink, red, yellow, orange, white, or colorless strains **(Davis and Vidaver, 2001**; **Kaneshiro *et al.* 2006)**.

2.5 Biology and Ecology

The phytopathogenic genus *Clavibacter* is amember of the group of Gram-positive actinomycetes, consisting of single species *Clavibacter michiganensis* subsp *michiganensis.* The pathogen starts its life cycle with its entry inside the plant **(Gartemann *et al.,* 2003)**.

Cmm is generally present in the xylem sap of the plant and injuries caused on the stem or leaves during the intercultural operations acts as the main source for the pathogen entry inside the plant (**Strider, 1969**). The tomato crop if planted in the soil infected by the pathogen through crop debris, then the roots could also serve as a medium for pathogen entry (**Eichenlaub *et al.,* 2006**). Infection of tomato leaves by the means of guttation droplets entering via hydathodes is also documented (**Carlton *et al.,* 1998**). The bacterium requires 72-96 hrs to develop colonies on agar plate generally with yellow pigmentation. *Cmm* may grow at a pH of 5 in the xylem vessels although the optimum pH for the growth of bacterium is between7 - 8 (**van den Bulk *et al.,* 1991**).

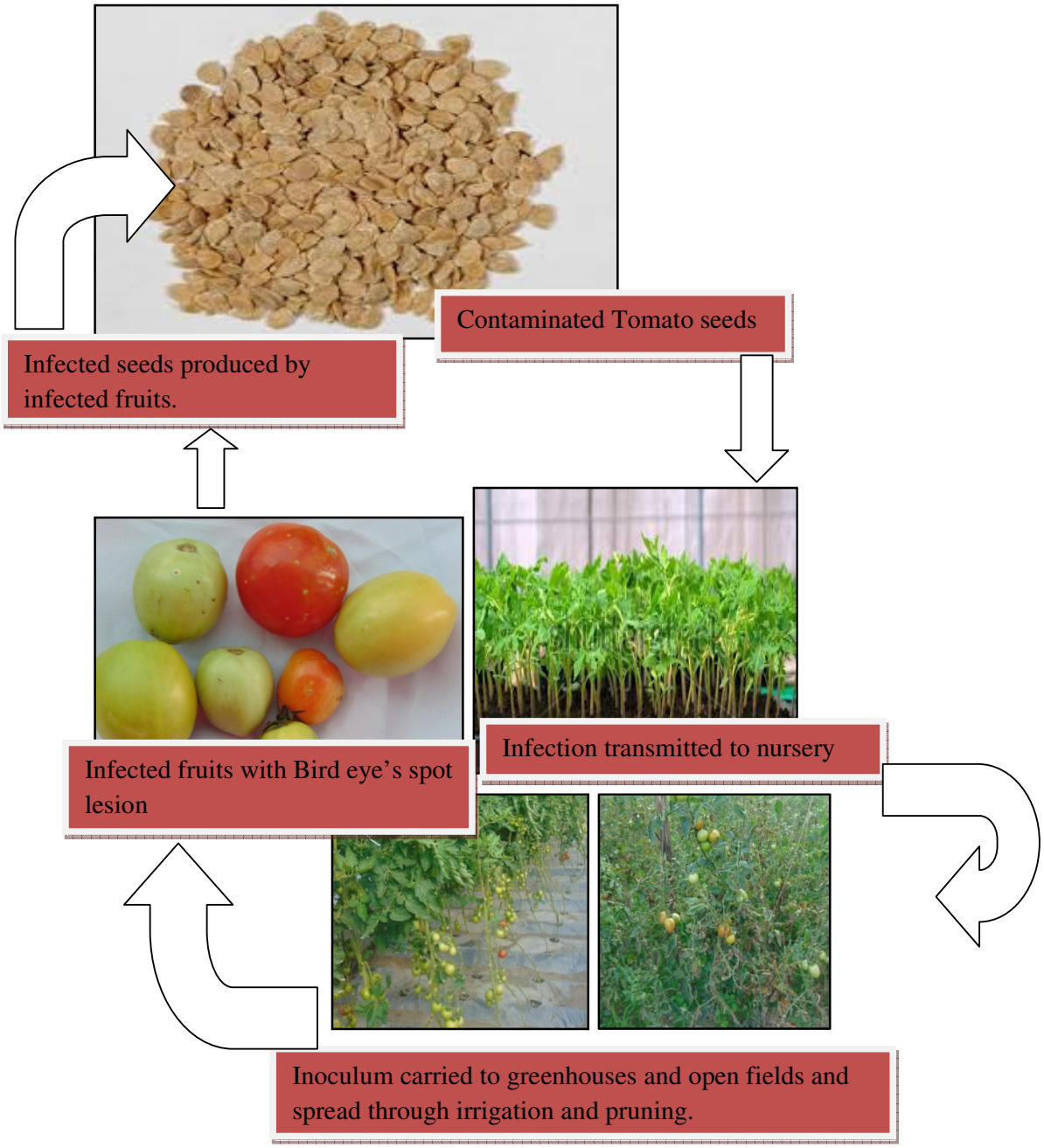


Figure 2.1: The ecology and spread of *Clavibacter michiganensis susbsp michiganensis* from seed to seed.

2.6 Symptomatology

The pathogen exhibits an array of symptoms, based on the infection character whether it is systemic or localized in nature (**Gleason *et al.,* 1993**). Lessening of the leaf photosynthetic area, plant wilting, early plant death and reduction in the fruit quality are some of the causes of infection. The virulent nature of the infecting pathogen, age of crop, growth state, infection site and inbuit nature of the crop leads to variance in the severity and symptom of the pathogen (**Chang *et al.,* 1992; Strider, 1969**).The entry of *Cmm* inside the tomato plant tissue occurs by stem or root injury, followed by bacterial multiplication and spread inside the plant leading to the eventual establishment of the bacterium in the xylem vessel region (**Gartemann *et al.,* 2003**). The initiation of the symptom takes place at the lower leaves of the plant and afterwards the wilting extends to all the leaves of the plant. Single sided and reversible wilting of leaves can be adjudged as the primary symptom succeeded by development of cankers on stem and consequently the plant dies. The symptoms on tomato fruit appears as dark brown spot surrounded by a distinct white halo and that is termed as bird's-eye spots (**Gitaitis, 1991**). Netted appearance of the fruits on symptomatic plants is another characteristic symptom associated with bacterial canker (**Seminis guide, 2006**).

2.7 Host Range

The host range of *Cmm* mainly includes solanaceous crops *viz.,* tomato, pepper, and eggplant. Variability in virulence is also observed in the strains of *Clavibacter* that are further characterized as being hypervirulent, hypovirulent, or nonvirulent. *Clavibacter* is currently classified as a member of the family *Microbacteriaceae* according to sequence analysis of 5S rRNA (**Park *et al.,* 1993**). *Clavibacter michiganensis* is the only species in the genus *Clavibacter* and it is divided into five subspecies according to host specificity. These subspecies include: subsp. *michiganensis,* which infects tomato*;* subsp. *sepedonicus,* which causes ring rot of potato; subsp. *tessellarius,* which induces leaf freckles and spots in wheat; and subsp. *insidiosus,* which is the pathogen causing wilting and stunting in alfalfa. These bacteria are all vascular pathogens and seed transmitted. Besides *Cmm*, *C. michiganensis* subsp. *sepedonicus* and subsp. *insidious* are also quarantine pathogens in many countries (**Jahr *et al.,* 1999; Eichenlaub *et al.,* 2006**). The pathogen can also infect pepper in nature or other solanaceous plants by artificial inoculation, but *Cmm* does not cause

severe systemic infection in hosts other than tomato. However, it is suspected that other solanaceous plants may serve as natural reservoirs of *Cmm* (**Thyr *et al.,* 1975; Ivey and Miller, 2000**).

2.8 Detection of *Clavibacter michiganensis subsp michiganensis*

Presence of receptive as well as consistent, quick and economical detection techniques are the essential strategies required for the management of *Cmm.* Detection also plays a significant role in tracing of the pathogen in case of a disease outbreak (**ISF, 2011**). The techniques recognized for the detection of *Cmm* includes serological and genetic methods, dilution plating and bioassays. For the purpose of detection of the pathogen in the contaminated seed, the seeds can be exposed to testing through incubation on nonselective medium like NBY medium and semiselective medium like SCM medium, (**Fatmi and Schaad, 1988**). Enzyme-linked immunosorbent assay (ELISA) (Wendy *et al.,* 2006; Nemeth and Van Vuurde, 2006,) and DNA based *Cmm* detection could be considered as one of the rapid and reliable methods. Specific primers for the detection of *C. michiganensis* subsp. *michiganensis* were designed by **Drier *et al.* (1995)** based on the plasmid-borne pathogenicity gene *pat*-1, **Pastrik and Rainey (1999)** designed oligonucleotide primers derived from the 16S-23S rRNA intergenic spacer region, that also act specifically for the detection of *Cmm.* Along with the progress of *Cmm* sequencing, primers were designed to amplify specific fragments by the polymerase chain reaction (PCR). **Santos *et al.,* 1997** successfully discriminated *Clavibacter michiganensis* (*Cm*) subspecies from other bacteria and saprophytes in tomato seeds. Comparing to the conventional microbiological detection of *Cmm* done by species determination and plant assays for virulence, immunological assays using monoclonal antibodies and ELISA are less time taking and précised in results. But the main drawbacks associated with this technique are the anticipation of false positives and false negatives when that can be observe while using either the immunodiagnostic tests as well as PCR assays (**Kaneshiro and Alvarez, 2001**).

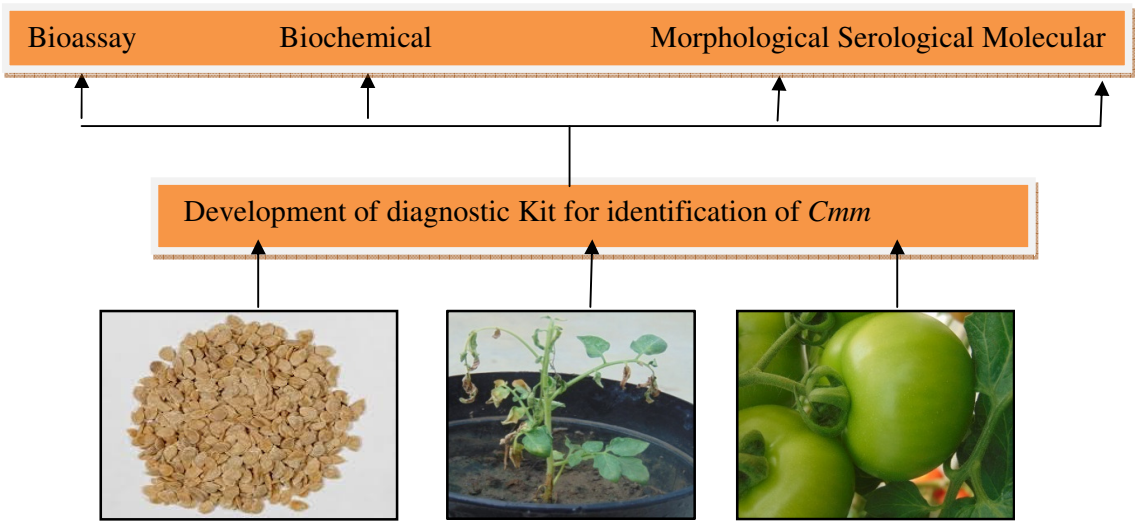


Figure 1.1: Methods to be explored for identification of *Cmm.*

2.9. Disease epidemiology

The temperature suitable for the growth of *Clavibacter* ranges from 20 to 30°C, however, the bacterium can survive upto 50°C, and grows optimally at 25°C. The optimum pH for growth of this bacterium is between 7 and 8, but *Clavibacter* can grow in plant xylem at pH 5 (**Eichenlaub *et al.,* 2006**). Infected seed and infested plant debris are the primary sources of inoculum for disease outbreaks (**Quesada *et al.,* 2004**), and seed is the primary vector over long distances. Seed to seedling pathogen transmission rates can vary from 0.25 to 85% (**Strider, 1969**), and population densities as low as five bacterial cells per seed can result in infected seedlings (**Lelis *et al.,* 2014**). It is known that *Cmm* is a good endophyte and moves into xylem after its entrance through wounds or nature openings (**Carlton *et al.,* 1998; Gartemann *et al.,* 2003**).

Mechanical wounds, which are easily made during clipping, harvesting, shipping and transplanting, promote the dissemination of *Cmm* directly into vascular tissue (**Chang *et al.,* 1991**). In addition, environmental conditions in seedling production greenhouses favor bacterial infection. Varying of temperatures (15-28°C) had no effect on canker development, but high relative humidity (87-97%) enhanced the symptoms in 2-3 week-old tomato seedlings. The bacterium is reported to spread by cultural practices and studies indicates *Cmm* movement in plants during latent infection and the influence of environment on *Cmm* growth as an endophyte in tomato transplants.(**Basu, 1966**).

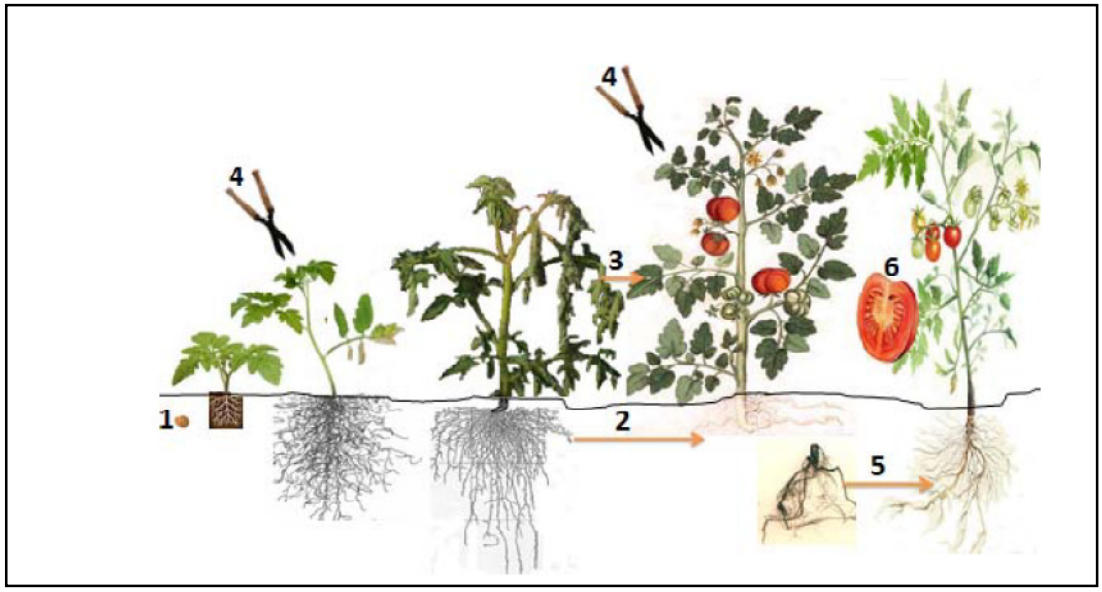


Figure 2.2: The *Cmm* entry points and infection route within the tomato plant. (1-5) (**Sen *et al.,* 2015**).

2.10 Taxonomy of the pathogen

Name: *Clavibacter michiganensis* subsp. *michiganensis* (**Smith, 1910; Davis *et al.,* 1984**).

Synonyms: *Corynebacterium michiganense* subsp. *michiganense* (**Smith, 1910; Carlson &** Vidaver, 1982), *Corynebacterium michiganense* pv. *michiganense* (**Smith, 1910; Dye & Kemp**, **1977**), *Corynebacterium michiganense* (**Smith, 1910; Jensen, 1934**).

Taxonomic position: Procaryotae Kingdom, Division II Firmicutes (**Gibbons & Murray 1978**), Class I Firmibacteria.

Clavibacter as a genus was established to include the plant pathogenic coryneform bacteria, the cell wall of these bacterium consists of peptidoglycan containing 2, 4-diaminobutyric acid as a dibasic amino acid. The pathogen is strictly aerobic in nature; Gram-positive rods which do not produce endospores (**Davis *et al.,* 1984**).

EPPO code: CORBMI.

Phytosanitary categorization: EPPO A2 List no. 50, EU

Annex designation II/A2 (EPPO/OEPP Bulletin, 2016).

2.11 Characterization of the test pathogen

2.11.1 Biochemical and physiological characterization of the pathogen

The bacterium may be identified for its phenotypic properties such as Gram reaction, metabolism of glucose, catalase activity, Kovac's oxidase test, levan formation, aesculin hydrolysis, starch hydrolysis, casein hydrolysis, H_2S production from peptone, acid production from mannose and mannitol and the use of sodium acetate as carbon source (**Lellott and Stead, 1987; Schaad *et al.,* 2001**).

2.11.2 Morphological characterization

Cells of *C. michiganensis* subsp. *michiganensis* are Gram-positive, non-motile, non-spore-forming, short rods (approx-imately 0.4–0.75 9 0.8–2.5 lm), which may be straight to slightly curved or wedge shaped. Coccoid forms may also be observed. They predominantly appear as single cells, but some V, Y and palisade arrangements are usually also present. Primary branching is uncommon in *Cmm* (**EPPO Bulletin, 2013**). Scanning electron microscopy served to be an important apparatus for better understanding of the morphology of *Cmm*.

2.11.3 Serological characterization

Plant tissues infected by *Cms*, *Cmm*, and *Cmi* can be screened out by Enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody staining test (IFAS) (**De Boer 1982; De Boer and Wieczorek 1984; De Boer *et al.,* 1988; De Boer and McCann 1990; Slack *et al.,* 1978**).Even though the kits for diagnoses of some of the subspecies are available but the false positive or negative results and partial sensitivity of the technique remains the drawback for the serological detection of *Cm* (**Crowley and De Boer 1982; De Boer and Wieczorek 1984; Mills *et al.* 1997; Pastrik 2000**).

2.11.4 Molecular characterization

Numerous protocols based on DNA detection including Southern blot hybridization and the polymerase chain reaction (PCR)-amplified DNA products analysis are being used presently for the identification of *Cm*. Different set of specific primers have been developed to detect identify *Cmm* and *Cms* (**Thompson *et al.,* 1989;**

Rademaker and Janes 1994; Firrao and Locci 1994; Dreier *et al.,* 1997; Rademaker *et al.,* 1997; Rivas *et al.,* 2002; Arahal *et al.,* 2004; Hu *et al.,* 1995; Palacio-Bielsa *et al.,* 2009); unfortunately, the primers were insufficiently specific for screening environmental samples. During the past few years, PCR technique has shown the potential of an important diagnostic tool because of its high sensitivity and specificity (Palacio-Bielsa *et al.,* 2007). Regardless of the progresses that are being made in PCR technique, their execution for daily analyses has still not taken the pace, particularly in the sector of commercial seed testing p. As the methods based on PCR requires specific equipment along with the skilled and trained personnel, and may be for these reasons, conventional techniques are still being followed in some of the seed testing laboratories (**Gitatitis and Walcott, 2007; Munkvold, 2009**). The successful implication of PCR based techniques in the past years leaves no ground for not applying them in combined protocols, for the purpose of routine study. Numerous primer pairs have been generated specially for the detection of *Cmm*, and most of them are based on the 16S-23S rDNA intergenic region. EPPO protocol recommends the use of two primer pairs viz., CMM5/CMM6, designed by **Dreier *et al.,* (1995)**; and PSA-4/PSA-R, designed by Pastrik and Rainey (**Paistrik and Rainey, 1999**) for the supposed detection of *C. michiganensis* subsp. *michiganensis* (**EPPO, 2005**).

Other primer pairs, such as CM3/CM4 (**Santos *et al.,* 1997**) and Cmm1F/Cmm1R (**Kokoskova *et al.,* 2010**), have also been developed specifically for *C. michiganensis* subsp. *michiganensis*.

2.12 Management of *Clavibacter michiganensis* subsp *michiganensis*

One of the most difficult disease to manage in tomato is bacterial canker (**Zitter, 1985**), as identification of the diseased plant is problematic, due to an array of symptoms, the disease being highly infectious in nature, longer duration for symptom expression and unavailability of efficient chemicals highly recommends the need for the development of effective sanitation and preventive actions (**Luo *et al.,* 2007**).The methods employed for the biological control of pathogens have to be fast, sensitive and specific. Prevention and control can be considered as the two categories into which the disease management strategies can be classified. Management of

disease by controlling the pathogen infection involves the application of chemical and biological control measures. Antimicrobial copper compounds such as copper sulphate, copper hydroxide, streptomycin/ copper hydroxide are identified to lower the spread of pathogen and the disease incidence. No particular chemical is available presently that can completely control *Cmm*. (**de Leon *et al.,* 2008**). The use of copper compounds can result in phytotoxic effects (**Yang *et al.,* 2002**). Some antimicrobial substances can reduce bacterial spread; examples are lysozyme, endolysins of bacteriophages (**Wittman *et al.,* 2010**), and plant essential oils (**Daferera *et al.,* 2002**). As an alternative measure of disease management, defense induction by chemical inducers or by the way of priming can also be exercised to stimulate the plant defense system in a progressive way, leading to creation of obstruction in the pathogen ingress.Priming results into induction of defense mechanism in plant after pathogen recognition. Chemicals that have been illustrated for an effective disease resistance induction in plants are salicylic acid, jasmonic acid DL amino butyric acid (**Baysal *et al.,* 2005**). Efforts for the management of *Cmm* by biocontrol agents have been also attempted. A treatment with *B. subtilis* (**Uthkhede and Koch, 2004**) or a pre inoculation with avirulent *Cmm* (**Muller *et al.,* 2000**) reduces bacterial spread. However, the mechanism by which *Cmm* is controlled is not yet known. Growing practices such as lowering the pH of the growth solution can limit the growth of bacteria (**Huang and Tu, 2001**). Even though the rate of management through chemical treatments and biological treatments are observed to be significant, but this rate was not found to be efficient and sustainable to be used as a result these components were not used widely. Along with controlling the disease through chemical or biological technique, sanitary measures (clean propagation materials, clean irrigation water and clean humans) can also be applied for the disease prevention. Besides the preventive and control measures the disease management can also be assisted by the use of resistant cultivars that serves as the green and effective way of disease management. Inspite of the zero tolerance for *Cmm* in the commercial seed trade market, yet no cultivar is present that can be categorized as being resistant against the pathogen. In contrast to Gram-negative plant-pathogenic bacteria, an incompatible reaction between *Cmm* and a tomato cultivar has not yet been found and

all efforts to obtain resistant tomato cultivars by breeding so far have not been satisfactory. The only possibility to reduce agricultural losses by *Cmm*-infections is by removing and destroying infected plants, since survival of *Cmm* in the soil requires the association with plant material. Also, it is necessary to certify seed and cuttings of tomato to be free of *Cmm* (**Chang *et al.,* 1991; Mansfeld-Giese, 1997**). Restricted options are available with the growers for the management of Cmm in the open field conditions as a very few number of cultivars encompasses tolerance to *C. michiganensis* subsp. *michiganensis* (**Gleason *et al.,* 1993**). Applications of copper products, either alone or mixed with protectant fungicides, every 5 to 7 days in the field can reduce the less severe bacterial canker symptoms of foliar blight and fruit spotting in North Carolina (**Shoemaker., 1992**). Control of *B. cinerea* on strawberry by isolates of *Trichoderma* and *Gliocladium* spp. was reported (Peng and Sutton, 1990; Sutton *et al.,* 1997). Water extracts of compost have been effective in the control of apple scab and late blight of tomato (**Weltzein, 1991**). It was shown that yeast (*Rhodosporidium diobovatum*) has the potential to control *Botrytis* stem canker and increase the fruit yield of tomato under greenhouse conditions (**Utkhede *et al.,* 2001**). Strains of *Bacillus subtilis* can reduce disease and promoted growth in several crops (**Broadbent *et al.,* 1971; Merriman *et al.,* 1974; Chang and Kommedahl, 1988**). **Sule (1988)** observed locally induced resistance in tomato against *Cmm* by inoculation of wounded tomato petioles with suspension of various *Pseudomonas syringae* pathovars. According to **Boudyach *et al*. (2001)** 10 of the 178 bacterial strains, isolated from bulk soil, the rhizosphere and rhizoplane of tomato, reduced the *Cmm* infection totally when applied as a seed treatment followed by a root treatment before transplanting. **Girish and Umesha (2005)** have shown that some strains of *Bacillus subtilis* (Ehrenberg) Cohn and *B. amyloliquefaciens* Fukumoto protected the tomato plants from the bacterial canker. It was also shown that some biocontrol preparations based on *Bacillus subtilis, Trichoderma harzianum* Rifai or *Rhodosporidium diobovatum* applied preventively as foliar sprays, have the ability to prevent the incidence of bacterial canker of tomato under greenhouse conditions (**Utkhede & Koch 2004**).

2.12.1. Defense inducers as an alternative to chemical and biological plant disease and management

Plants possess an array of active defense responses that can be actively articulated during biotic stresses (pathogens and parasites) of different degrees (ranging from microscopic viruses to phytophagous insect). The period in which the defense response is expressed is of extreme importance as it reflects the efficiency of plant whether to succumb to or cop up with challenge of pathogens/parasites. The stimulus leading to triggering of the defense response prior to pathogen invasion, may lead to lowering of the disease incidence. The resistance activity exhibited by a suitably stimulated plant is known as induced resistance. The induced resistance can be characterized into two forms viz., Systemic acquired resistance (SAR) and induced systemic resistance (ISR) in which the earlier infection or treatment results into preconditioning of the plant defense apparatus against the challenging pathogen or parasite (**Choudhary *et al.,* 2007**). Treatment with different types of abiotic and biotic defense elicitors may lead to improved resistance against pathogen infection. Treatment with necrotizing pathogen or non pathogens belonging to same genus or cell wall fragments and plant-growth promoting rhizobacteria are included in biotic defense inducers. These resistance elicitors provide a broad spectrum and long lasting resistance; in many cases 20 - 85% of disease control can be observed The initiation of SAR occurs either locally or systemically as a defense response to infection by pathogen or by the treatment with the defense inducing chemical, this response is efficient against a broad range of phytopathogens and is interceded salicylic acid dependent process (**Walters *et al.,* 2005**). After the application of an inducer, the defense metabolism of the plant may be either stimulated directly or it may also be triggered following the pathogen infection (**Walters and Boyle, 2005**). Oxidative burst is the foremost defense response initiated in the plant which may lead to cell death (Heath, 1998), this response causes the trapping of the pathogen within the dead cells ; alteration in chemical composition of cell wall causing inhibition in the pathogen penetration; and antimicrobial compounds synthesis like phytoalexins (**Hammerschmidt, 1999**). (**Mucharromah and Kuc 1991**.). It was speculated that basic phosphates applied to plants could sequester apoplastic calcium, altering membrane integrity and influencing the activity of apoplastic enzymes such as

polygalacturonases, thereby releasing elicitor-active oligogalacturonides from plant cell walls (**Walters and Murray, 1992**). Indeed, subsequent work by **Orober *et al.* (2002)** showed that phosphate-mediated resistance induction in cucumber was associated with localized cell death, preceded by a rapid generation of superoxide and hydrogen peroxide. These workers also detected local and systemic increases in levels of free and conjugated SA following phosphate application. Recent work on barley showed that application of phosphate to first leaves reduced powdery mildew infection by 89% in second leaves. Application of phosphate to first leaves led to significant increases in activities of phenylalanine ammonia-lyase, peroxidase, and lipoxygenase in second leaves, and activities of these enzymes were increased further following pathogen challenge (**Mitchell and Walters, 2004**).

2.12.1.1 Role of Salicyclic acid in plant defense

An important role is played by Salicylic acid (SA), in defense induction in the plants against varied biotic and abiotic stress by alterations in the morphological, physiological and biochemical mechanisms of plants. The defense inducer is produced by plants as a response to challenge by a diverse range of phytopathogens and is the primary step required for the et up of local and systemic acquired resistance (SAR) within the plants. Treatment of SA in plants leads to induction of pathogenesis-related (PR) proteins (**Loeke and Grant, 2007**). The phenyl propanoid pathway interceded by SA has an important role against pathogens, insect pests and abiotic stresses. Application of SA on the plant surface alters a range of physiological, biochemical and molecular processes in plants including enzymatic activities of antioxidative (**Vicent and Placensia, 2011**). Moreover, SA regulates the components of its own signaling pathway besides getting involved in cross-talk with other pathways mediating plant resistance. It has been proposed that SA affects the plant growth under stress through nutrient uptake, water relations, stomatal regulation and photosynthesis (**Hayat *et al.,* 2009**). It regulates the activities of various enzymes such as, peroxidase (POD), polyphenol oxidase (PPO), Superoxide dismutase (SOD), Phenyl alanine ammonia lyase (PAL) etc., which are the major components of induced plant defense against biotic and abiotic stresses (**Zhao *et al.,* 2009**).

2.12.1.2 Role of Isonicotinic acid in plant defense

2, 6-Dichloroisonicotinic acid (INA) is an effective plant defense response inducer that includes PR protein synthesizes and improved disease resistance. 2, 6-Dichloroisonicotinic acid (INA) leads to induction of resistance in plants against various pathogens (**Kessman *et al.,* 1994**). The enzymatic activity of tobacco catalase is inhibited by INA both *invivo* and *invitro* by bounding to it in a manner similar to SA, this depicts that biological activity of both compounds is mediated through reduction in catalase enzymatic activity thereby resulting into enhanced ROS levels (**Conrath *et al.,* 1995**). The strong correlation between biological activity and ability to bind and inhibit catalase suggests that the physiological effects of INA, SA, and their active analogues are mediated by the same mechanism of action, namely, inhibition of catalase's ability to degrade H_2O_2. These results thus not only elucidate a likely mode of action of INA but also support the proposed role of ROS in the induction of plant defense responses (**Chen *et al.,* 1993**). *C. lagenarium* infection in cucumber was achieved by exogeneous application of INA by foliar spray, providing a high level of protection to the plants at a concentration of 104µM. The defense responses in these chemically treated plants were similar to those observed in plant's systemic tissue in which inoculation was done by TNV or *C. lagenarium* that lead to SAR response induction in upper leaves (**Kuc, 1982; Metraux *et al.,* 1991**). SAR induction in tobacco and Arabidopsis was also observed by INA application (**Ward *et al.,* 1991; Uknes *et al.,*1992**) against TMV, *Cercospora nicotianae, Peronospora tabacina, Phytophthora parasitica* var nicotianae, and *P. syringae* pv. tabaci (**Ward *et al.,* 1991**).

2.12.1.3 Role of Benzothiodiazole in plant defense

Benzothiodiazole as a SAR inducing chemical was firstly screened by Ciba-Geigy with a large number of benzo[1,2,3]thiadiazole-7-carboxylic acid derivatives that resulted in the identification of benzo(1,2,3)-thiadiazole- 7-carbothioic acid S-methyl-ester (BTH (**Schurter *et al.,* 1993; Kunz *et al.,* 1997; Oostendorp *et al.,* 2001**).Stimulation of defense response by BTH was observed in a range of plant species against varied pathogen, viz., TMV*, Cercospora nicotianae, Erwinia carotovora, Phytophthora parasitica* and *P. syringae* pv. tabaci (**Friedrich *et al.,*1996; Gorlach *et al.,*1996; Lawton *et al.,*1996; Kunz *et al.,* 1997**). BTH is regarded as antimicrobial by nature as it does not have any straight response on a number of phytopathogen

(Friedrich *et al.,* 1996). Similar to SA and INA, inhibition of APX and catalase function is also proposed to occur by BTH (**Du and Klessig, 1997; Wendehenne *et al.,* 1998**). BTH is observed to be a more efficient catalse inhibitor compared to SA and both of them differ in their mechanism. Catalase function in SA mediated response seems to be inhibited in an H_2O_2 and time-dependent manner while BTH inhibits this activity independently from time and H_2O_2 (**Wendehenne *et al.*, 1998**).INA activates an extensive spectrum of resistance under practical field conditions that includes fungal, bacterial and viral pathogens. Of particular relevance is the protection against the destructive blue mold as well as against bacterial and virus diseases (**Kuc, 1984**). BTH inhibits catalase and ascorbate peroxidase, two potential targets through which SA has been proposed to act. BTH was found to be a considerably better inhibitor of catalase than SA (**Wendehenne *et al.,* 1997**). Other dicots where ASM has shown activation of broad-spectrum disease resistance under field conditions are tomatoes, and some vegetable and fruit crops (**Tally *et al.,* 1999**).

2.12.1.4 Role of Lysozyme in plant defense

Lysozyme is a naturally occurring protein found in many organisms such as viruses, bacteria, plants, insects, birds, reptiles, and mammals. Bactercidal nature of lysozyme was observed against *C. michiganensis* subsp. *michiganensis* at as low concentration as 1 g/l. Foliar spray application of lysozyme at 10g/l and 100g/l were observed to slower down the ingress of *C. michiganensis* subsp. *michiganensis* subsp. *michiganensis*, which is a systemic pathogen (**Uthkhede *et al.,* 2004**).Lysozyme seems to have the potential to increase growth and prevent infection on tomatoes by *C. michiganensis* subsp. *michiganensis*. It is well known for its antibacterial protein activity against gram positive and negative bacteria. Its bacterial activity is hypothesized to reside in its murimidase activity, leading to degradation of murein layer, and reduction of the mechanical strength of bacterial cell wall. These actions eventually lead into killing of bacterial cell by lysis. There are also some report concerning its lytic activity against fungal pathogen such as *Phytophthora nicotinae* and *Fusarium oxysporium* (**During *et al.,* 1999**), *Pythium aphanidermatum* and *Botrytis cinerea* (**Uthkhede and Bogdenhoff, 2003**). The influence of hen egg white lysozyme (HEWL) on root rot caused by *Pythium aphanidermatum*, and of HEWL, yeast, azoxystrobin, and myclobutanil on stem canker caused by *Botrytis cinerea* and

gummy stem blight caused by *Didymella bryoniae* in cucumber grown under near-commercial greenhouse conditions was studied. HEWL treatments applied as a root drench at 5g/l every 3 weeks gave a significantly (55%) higher yield and reduced percent infection compared with the untreated control plants inoculated with *P. aphanidermatum* inoculation significantly reduced fresh weight and the height of cucumber seedlings. HEWL, yeast and azoxystrobin significantly reduced lesion lengths caused by *B. cinerea* and *D. bryoniae* on cucumber plants (**Uthkhede and Bogdanoff, 2003**).

2.12.2 Defense related compound

2.12.2.1 Hydrogen Peroxide

The role of reactive oxygen species, especially H_2O_2, in plant response to stresses has been the focus of much attention. Hydrogen peroxide has been postulated to play multiple functions in plant defense against pathogens. Intracellular accumulation of benzoic acid (BA) by H_2O_2 and then the conversion of BA to SA is catalyzed by benzoic acid 2-hydroxylase (BA2H), an inducible enzyme that is synthesized de novo in response to increased BA level (**Vidhyasekaran, 2014, Leon *et al.,* 1993**). Fivefold increase in the levels of benzoic acid was observed when H_2O_2 infiltrated into tobacco leaves. The defense stimulation by elicitors provokes the oxidative burst, leading to H_2O_2 production. Accumulation of BA2H protein in the cell is observed by higher BA level, thereby providing more substrate for the enzymatic activity (**Leon *et al.,* 1995**).

2.12.2.2 Lignin

A variety of secondary metabolites having a phenol or hydroxyl active moiety called as phenol are produced by plants, acting as an important line of defense for plants against pests and diseases (**Wuyts *et al*., 2006**). A highly branched polymer of phenyl-propanoid groups, formed from three different alcohols viz., coniferyl, coumaryl and sinapyl which oxidized to free radicals (ROS) by a ubiquitous plant enzyme-peroxidase, reacts simultaneously and randomly to form lignin The physical toughness rendered by lignin to plants deters the ingress of pathogen and pest, as well as its chemical composition and durability makes it comparatively unsuitable for insects pathogens (**Mader and Amberg-Fisher, 1982**). On being exposed to different

stress condition, there may be a change in lignin content in plants. In many cases, particularly for plant-microbe interactions, this has been suggested as defense responses of plants to the stress (**Moura *et al.,* 2010)**. An increase in lignification is often observed in response to attack by pathogen. This defense reaction is supposed to depict one of the varied mechanisms designed to block parasite invasion, thus reducing the susceptibility of the host, since lignin is a non-degradable mechanical barrier for most microorganisms (**Lauvergeat *et al.,* 2001**). Noteworthy enhancement in the lignin content was observed in the inoculated plants at 1st, 2nd and 3rd day after inoculation (**Zhang *et al.,* 2007**). Ferulic acid production is induced by *Agrobacterium* occurs in wheat. Ferulic acid is a precursor in lignin biosynthesis, suggesting the existence of a defense response preventing infection by the bacteria in these plants (**Parrott *et al.,* 2002**). The deposition of lignin in necrophylatic periderm in the early stages of infection by *Mycosphaerella* explains the greater resistance of *E. nitens* as compared with *E. globulus*, as this response may prevent the spread of toxins and fungal enzymes to the host, thereby preventing the displacement of water and nutrients from the host cell to the fungus (**Smith *et al.,* 2007**). Lignifications block the growth of pathogens and are a frequent response to infection or wounding (**Gould, 1983**).

2.12.3. Enzymatic antioxidants

2.12.3.1. Peroxidases

PODs constitute an important group of defense enzymes that defend plants against various stresses.

Among the proteins induced during plant defense and playing a key role in several metabolic responses, class III plant peroxidases (EC 1.11.1.7) are well known (**Passardi *et al.,* 2004a**).

Peroxidases can create a physical barrier to limit pathogen invasion in host tissues by catalysing the cross-linking of cell wall components in response to different stimuli such as wounding and pathogen interactions and thus, cell wall rigidification is, in most cases, the result of the Prxs-mediated H_2O_2 dependent cross-linking of cell wall components (**Ros Barcelo, 1997**). Enhanced peroxidases levels have been correlated with resistance in a number of plant species that consist of tobacco, rice, barley, cotton, wheat and cucurbits. Lignin, suberin or protein polymerization into the plant cell wall

occurs as a result of these enzymatic activity (**Ride, 1983; Mohan and Kolattukudy, 1990; Bradley *et al.,* 1992**), thereby forming an obstruction in pathogen movement and cell wall penetration. In resistant or incompatible interactions between rice and the bacterial blight pathogen, *Xanthomonas oyzae pv oryzae*, increases in the activities of three extracellular peroxidases (two anionic [PO-A1 and PO-A2] and one cationic [PO-Cl]) have been correlated with the accumulation of lignin-like compounds, a reduction in bacterial multiplication in the leaves, and the onset of the HR (**Reimers and Leach, 1991; Reimers *et al.,* 1992; Guo *et al.,* 1993**). Activity of the cationic peroxidase PO-C1 increased more dramatically with the onset of resistance when compared with the two anionic peroxidases (**Reimers *et al.,* 1992**).

2.12.3.2. Polyphenol oxidase

Polyphenol oxidase (E.C. 1.10.3.2) has been purified and characterized from a wide range of plant species and a variety of tissues (**Constabel *et al.,* 1996; Mayer 2006**). Polyphenol oxidases (PPOs) are ubiquitous copper-containing enzymes which use molecular oxygen to oxidize common *ortho*-diphenolic compounds such as caffeic acid and catechol to their respective quinines (**Constabel and Barbehenn, 2008**), playing a pivotal role in plant defense (**Bhonwong *et al.,* 2009**). Polyphenol oxidase (PPO) catalyses the oxidation of monophenols and/or *o*-diphenols to *o*-quinones with the concomitant reduction of oxygen to water which results in protein complexing and the formation of brown melanin pigments. The most frequently suggested role for PPO in plants has been in defense against herbivores and pathogens, based on the physical separation of the chloroplast-localized enzyme from the vacuole-localized substrates. The *o*-quinone–protein complexes, formed as a consequence of cell damage, may reduce the nutritional value of the tissue and thereby reduce predation but can also participate in the formation of structural barriers against invading pathogens (**Boeckx *et al.,* 2015**).

2.12.3.4 Phenyl ammonia lyase

Phenylalanine ammonia lyase (EC 4.1.3.5) catalyzes the first step of the general phenylpropanoid pathway, a step that is common to the production of many metabolites including flavonoids, coumarins, and phytoalexins and lignin. The suppression of PAL expression in tobacco results into a plethora of mechanism comprising growth

reduction, altered leaf shape, reduced pollen viability (**Elkind *et al*., 1990**), and increased susceptibility to the fungal pathogen *Cercospora nicotianae* (**Maher *et al*., 1994**). Thinner cell walls in the secondary xylem and reduced lignin content have been observed in the plants with low PAL activity (**Bate *et al*., 1994; Elkind *et al*., 1990**). Phenylpropanoid compounds have been proposed to play crucial roles in plant defense to microbial pathogens based on the correlation between rates of phenylpropanoid accumulation and expression of resistance *in vivo* (**Dixon and Paiva, 1995; La Camera *et al.*, 2004**). Some phenylpropanoid compounds accumulate to high levels in plants that are resistant to an invading pathogen (**Dixon, 2001; La Camera *et al.*, 2004**). These pathogen-induced phenylpropanoids, such as pterocarpans, isoflavans, stilbenes, and coumarines, act as phytoalexins, which have antimicrobial activity against plant-pathogenic fungi and bacteria (**Dixon and Paiva, 1995; Barber *et al.*, 2000; La Camera *et al.*, 2004**).

2.12.3.5. PR-2 protein

PR proteins are a heterogeneous group of low molecular weight proteins with selective solubility at low pH, resistant to proteolytic degradation and predominantly accumulated in intercellular leaf spaces (**Van-Loon, 1985; Bol *et al.,* 1990; Schroder *et al*., 1992**). They accumulate rapidly at the intra or extracellular level under various biotic and abiotic stimuli including fungal, elicitor and physical or chemical treatments (**Heller and Gessler, 1986; Van- Kan *et al*., 1992; Van-Loon and Van-Strien, 1999; Graham *et al*., 2003**). Plant Glucanases (PR-2) family comprises of large and highly complex gene families involved in pathogen defense (**Hong and Meng, 2004; Saikia *et al*., 2005**). β-1,3-glucanases usually expressed at low concentration in plants, but when plants are infected by fungal, bacterial, or viral pathogens, β-1,3-glucanases enzyme concentration increases dramatically, **Van Kan *et al.,* 1992** showed that mRNA for a tomato acidic β-1, 3-glucanase accumulated to a higher level in leaves infected by the fungal pathogen *Cladosporium fulvum.* Several studies showed that the expression levels of these enzymes increased after infected with pathogens, such as barley infected by powdery mildew (**Ignatius and Chopra, 1994**), maize infected with *Aspergillus flavus* (**Lozovaya *et al.,* 1998**), pepper infected with *Xanthomonas campestris* pv. *vesicatoria* and *Phytophthora capsici*, soybean infected with *Pseudomonas syringae* (**Jung and Hwang, 2000**), wheat infected with *Fusarium*

graminearum (**Li *et al.,* 2001**), chickpea infected with *Ascochyta rabiei* (Pass.) Labr. (**Hanselle and Barz, 2001**) and peach infected with *Monilinia fructicola* (**Zemanek *et al.,* 2002**) the host in response to host signals.

2.12.3.6. Total Phenol Content

Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. Phenolics are one of the most ubiquitous groups of secondary metabolites found throughout the plant kingdom (**Boudet, 2007; Harborne, 1980**).They encompass a very large and diverse group of aromatic compounds characterized by a benzene ring (C6) and one or more hydroxyl groups. Generally, the classification of phenolics is based on the number of carbon atoms present in the molecule (**Harborne and Simmonds, 1964**). Phenolics are often produced and accumulated in the subepidermal layers of plant tissues exposed to stress and pathogen attack (**Cle *et al.,* 2008; Schmitz-Hoerner and Weissenbock, 2003**). The concentration of a particular phenolic compound within a plant tissue is dependent on season and may also vary at different stages of growth and development (**Lynn and Chang, 1990**; **Ozyigit *et al.,* 2007; Thomas and Ravindra, 1999**). Several internal and external factors, including trauma, wounding, drought and pathogen attack, affect the synthesis and accumulation of phenolics (**Kefeli *et al.,* 2003; Zapprometov, 1989**). Phenolics serve a dual function of both repelling and attracting different organisms in the plant's surroundings. They act as protective agents, inhibitors, natural animal toxicants and pesticides against invading organisms, i.e. herbivores, nematodes, phytophagous insects, and fungal and bacterial pathogens (**Dakora and Phillips, 1996; Lattanzio *et al.,* 2006; Ravin *et al.,* 1989**).

Materials and Methods

Chapter 3 MATERIALS AND METHODS

The experiments of the present investigation were conducted in the laboratories of Dept. of Plant Pathology, Dept. of Genetics and Plant Breeding, College of Veterinary and Animal Sciences and under controlled conditions in the glass house, Department of Plant Pathology, College of Agriculture. Field trials were conducted at Vegetable Research Center, (VRC) Pantnagar for the years 2015-16, 2016-17 in order to assess the loss incurred due to *Clavibacter michiganensis* subsp *michiganensis.*

3.1.1 Disease Survey and Sample Collection

For recording the occurrence and spread of bacterial canker, periodic surveys of tomato growing regions of Uttarakhand and Himachal Pradesh were undertaken from during cropping seasons of 2014-15 and 2015-16. The incidence and severity of bacterial canker were observed at different locations in the field. Around three tomato fields per location were visited in different regions of Himachal Pradesh and Uttarakhand. Disease incidence was calculated with the help of following formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

Data on disease severity at each location was recorded following 0- 5 scale developed by **Bogo and Takatsa (1997) and Shukla and Gupta (2005)**. The per cent disease severity was calculated as per the method of **McKinney (1923)**.

RATING/ GRADE	SYMPTOMS
0	Apparently healthy foliage
1	Very few necrotic areas on leaves while fruits completely free from disease symptoms
2	Up to 10% leaf area necrotic and fruits showing 1 or 2 spots
3	Necrosis covering up to 25% of leaf area and 10 % of fruit area covered with spots. One bud showing necrosis/wilting.
4	Pronounced necrosis covering up to 50% of leaf area and 25 % fruit areas covered with spots. Few elongated cankerous lesions on stem and branches. One or two buds showing necrosis /wilting
5	More than 50% of leaf showing necrosis and 50% fruit area covered under spots. Lesions on stem, branches and petioles, wilting of branches started to occur.

$$\text{Disease severity (\%)} = \frac{\text{Sum of all disease ratings}}{\text{Total number of ratings} \times \text{Maximum disease grade}} \times 100$$

Table 3.1. List of *Cmm* isolates used in the present study

S. No.	Isolate	Designation
1	Nauni	*Cmm* 1
2	Khaltu	*Cmm* 2
3	Kalaghat	*Cmm* 3
4	Kotla Panjola	*Cmm* 4
5	Gaulapar	*Cmm* 5
6	Garganoo	*Cmm* 6
7	Narag	*Cmm* 7
8	Kyar	*Cmm* 8
9	Nainatikkar	*Cmm* 9
10	Deothal	*Cmm* 10

3.2. Laboratory experiment

3.2.1 Sterilization of Glassware and Media preparation

Petriplates (Borosil) (90 mm diameter), test tubes (Borosil) (10 ml and 20 ml), flasks (Tarsons) (50, 100, 250, 500 and 1000 ml), Buchner funnel (10 cm diameter) measuring cylinder (Tarsons) (100, 250, 500 and 1000 ml), pipettes (0.1, 1.1, 2.5 and 10.0 ml) and beaker (Tarsons) (100, 250 and 500 ml) were used during the course of investigation. Digital electronic balance (Sartorius) was used to take the weight of media constituents; autoclave (NSW) used for the sterilization of media and glassware and BOD incubator (REMI) used for incubation. Orbital shaker (REMI) was used for the proper shaking of inoculated broth; vertical laminar air flow chamber (Klendiaz) was used for maintaining the proper sterilization conditions; Spectrophotometer (Thermo Scientific) was used for measurement of optical density of broth cultures of isolates; digital pH meter was used for getting the accurate value of pH; for maintaining temperature, multi chamber incubator (REMI) was used.

3.2.2. Isolation of Pathogen

Isolation of the pathogen was done as per the method described by **Janse (2005)**. The bacterium was isolated from the seeds, from infected seedlings, infected plant tissue of tomto plant stem, leaves and fruits. Such infected tissues were surface disinfected by alcohol (70%) and were then placed in a test tube having sterilized water. Tissue was left for 30 minutes in water and was crushed gently so that the bacterium can be released out of the tissue into water. Subsequently 100µl of suspension was poured into Nutrient Agar Glucose Yeast medium (NGY). The seeds collected from infected fruits exhibiting the disease symptom were placed on the NGY medium and inoculated in the growth chamber at 28±1ºC. The seeds were examined 72hrs after for the recovery of the bacterium.

3.2.3. Purification and preservation of the bacterium

The purification of the bacterium was done on D_2ANX medium, that shows the characteristic feature of the bacterium by streaking freshly growing single colonies from NGY medium and incubated at 28ºC for five days. *Cmm* isolates were stored in NGY slants at 4ºC and in glycerol stocks at -20ºC for long term storage.

3.3. *In vitro* Evaluation

3.3.1. Biochemical and Physiological Characterization of *Cmm* isolates

3.3.1.1 Morphological characterization of *Cmm* isolates

3.3.1.1.1 Colony Characters on Culture Medium

3.3.1.1.1.1 SCM medium

For the assessment of growth efficiency of *Cmm* on SCM medium the bacterial cell suspension was made in 0.01M phosphate buffer saline (PBS) (pH: 7.0), the inoculums concentration in the suspension was adjusted spectrophotometerically to an OD value of 0.06 at 660nm that corresponds to 10^8 cfu ml^{-1}, and afterwards diluted to 10^4 cfu ml^{-1}. Lastly, 100 µl of bacterial cell suspension from each strain was streaked on the surface of the growth medium by a "L" shaped glass spatula. Replication of the experiment was done thrice. The observations were recorded in the form of growth area of *Cmm* 4_{th} and 10th day after plating.

Area of growth in the medium = cfu count x area of colony (πr^2)

3.3.1.1.1.2 D_2ANX medium

For the assessment of growth efficiency of *Cmm* on D2ANX medium the bacterial cell suspension was made in 0.01M phosphate buffer saline (PBS) (pH: 7.0), the inoculums concentration in the suspension was adjusted spectrophotometerically to an OD value of 0.06 at 660nm that corresponds to 10^8 cfu ml^{-1}, and afterwards diluted to 10^4 cfu ml^{-1}. Lastly, 100 µl of bacterial cell suspension from each strain was streaked on the surface of the growth medium by a "L" shaped glass spatula. Replication of the experiment was done thrice. The observations were recorded in the form of growth area of *Cmm* 4_{th} and 10th day after plating.

Area of growth in the medium = cfu count x area of colony (πr^2)

3.3.1.1.1.3 YDCA medium

For the assessment of growth efficiency of *Cmm* on YDCA medium the bacterial cell suspension was made in 0.01M phosphate buffer saline (PBS) (pH: 7.0), the inoculums concentration in the suspension was adjusted spectrophotometerically to an OD value of 0.06 at 660nm that corresponds to 10^8 cfu ml^{-1}, and afterwards diluted to 10^4 cfu ml^{-1}. Lastly, 100 µl of bacterial cell suspension from each strain was streaked on the surface of the growth medium by an "L" shaped glass spatula. Replication of the experiment was done thrice. The observations were recorded in the form of growth area of *Cmm* 4_{th} and 10th day after plating.

Area of growth in the medium = cfu count x area of colony (πr^2)

3.3.1.1.1.4 SPY medium

For the assessment of growth efficiency of *Cmm* on SPY medium the bacterial cell suspension was made in 0.01M phosphate buffer saline (PBS) (pH: 7.0), the inoculums concentration in the suspension was adjusted spectrophotometerically to an OD value of 0.06 at 660nm that corresponds to 10^8 cfu ml^{-1}, and afterwards diluted to 10^4 cfu ml^{-1}. Lastly, 100 µl of bacterial cell suspension from each strain was streaked on the surface of the growth medium by a "L" shaped glass spatula. Replication of the experiment was done thrice. The observations were recorded in the form of growth area of *Cmm* 4_{th} and 10th day after plating.

Area of growth in the medium = cfu count x area of colony (πr^2)

3.3.1.1.1.5 YPGA medium

For the assessment of growth efficiency of *Cmm* on SCM medium the bacterial cell suspension was made in 0.01M phosphate buffer saline (PBS) (pH: 7.0), the inoculums concentration in the suspension was adjusted spectrophotometerically to an OD value of 0.06 at 660nm that corresponds to 10^8 cfu ml^{-1}, and afterwards diluted to 10^4 cfu ml^{-1}. Lastly, 100 µl of bacterial cell suspension from each strain was streaked on the surface of the growth medium by a "L" shaped glass spatula. Replication of the experiment was done thrice. The observations were recorded in the form of growth area of *Cmm* 4_{th} and 10th day after plating.

Area of growth in the medium = cfu count x area of colony (πr^2)

3.3.1.1.1.5 Cell shape and size study by Scanning Electron Microscopy (Chalupowicz *et al.,* 2011)

1. Bacterial Culture grown for 24-48 hour in Luria Bertani broth is taken.
2. Centrifugation of the bacterial broth is done at 5000 rpm for five minutes.
3. The pellet is collected and the broth is completely discharged without disturbing the pellet.
4. Add 2.5 % glutaraldehyde (Prepared in Na-Phosphate buffer, pH: 7.0) and place the pellet at 4ºC for four hours.
5. Centrifuge again for five minutes at 5000 rpm and discard the glutaraldehyde solution.
6. Wash the pellet with Na-Phosphate buffer (pH: 7.0) three times.
7. Wash the pellet with M.B. grade water three times.
8. Quick spin the bacterial suspension.
9. Prepare the SEM stub by taking 2 µl of bacterial suspension and spread it on the circular cover slips.
10. Place the cover slips below the incandescent lamps for proper drying.
11. Visualize the sample. Capture the images with SEM (JSM 840A; Jeol Ltd., Tokyo)

3.3.2 Biochemical and physiological testing

3.3.2.1 Catalase test (Reiner, 2010)

For the purpose of routine testing of aerobes, 3% hydrogen peroxide was used. The catalase enzyme to neutralizes the bactericidal effects of hydrogen peroxide. Catalase accelerates the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen ($2H_2O_2$ + Catalase $\rightarrow$ $2H_2O + O_2$). Rapid formation of bubbles evident the positive reaction.

3.3.2.2 H_2S production (Clarke, 1953)

A strip of lead acetate paper was held in the mouth of the tube by a cotton-wool plug. The tubes were incubated at 37ºC either in an incubator and observation was taken after one week. Blackening of the strips confirms the positivity of the reaction.

3.3.2.3. Gelatin liquification (Lelliott and Stead, 1987)

12 per cent gelatin was added into nutrient broth (NB) medium and pH was adjusted to 7.0. The medium was autoclaved for 15 minutes and poured into petriplates. The plates were inoculated by streaking a loopfull of inoculum into the center of the medium, and then incubated the plates at 20-25^{0}C. The agar surface was flooded by 0.2% mercuric chloride solution in 20%HCL and leaved for few minutes. The plates were then observed for rapid or slow liquefaction by formation of clear zone around the growth.

3.3.2.4 Starch Hydrolysis (Lelliott and Stead, 1987)

Bacteria were grown on NA medium containing soluble starch (0.2%). The plates were incubated at 25ºC for 2. 4. 7 and 14 days and were flooded with Lugol' iodine to observe the starch hydrolysis. Formation of the reddish color or clear zone on the blue background indicates a positive reaction.

3.3.2.5 3% KOH test (Suslow *et al.,* 1982)

A drop of 3% (w\v) KOH was placed on a clean slide. The bacterial cells(24h growth) were aseptically transferred from agar plate to the drop of KOH with a sterile toothpick. The cells were mixed properly with the toothpick. The appearance of watery suspension with no string production indicates a gram positive bacterium.

3.3.2.6. Levan production (Lelliott and Stead, 1987)

Bacteria were streaked onto sterilized NA medium supplemented with sucrose (5%). Absence of large, white and mucoid colonies indicates a negative reaction.

3.3.2.7. Casein hydrolysis (Vidaver and Davis, 1988)

The solution of 2% skimmed milk was poured into NA medium after autoclaving and was poured into Petri plates. Bacteria were streaked onto plates and incubated for 24 – 48 hours. The absence of clear zone around the bacterial colonies indicates absence of hydrolysis.

3.3.2.8. Aesulin hydrolysis (Swan, 1954)

NA medium, containing 0.1% aesculin was prepared and autoclaved. After autoclaving, media was poured into Petri plates. Bacteria were streaked onto plates and incubated for 72hrs at 25ºC. The brown color of medium confirms the positive test.

3.3.2.9 Gram Reaction (Strider, 1969)

A thin bacterial film spread on a clean slide, and the bacterial smear was prepared by heat fixing the cells gently at the flames for a few seconds The smear was then flooded with crystal violet solution for 1 minute followed by washing under tap water for few seconds. The smear was then flooded with iodine solution and covered for 1 minute. The slide was rinsed in running water blot dried, decolorized with ethyl alcohol (90%) drop wise till the dye run off the smear.. Rinsed in tap water for about 2 seconds and blot dry. Observe under oil immersion lens. The gram positive cell will be stained purple.

3.3.2.10. Differentiation of *Cmm* at different temperatures

NGY broth in 150 ml 'Erlenmeyer' flasks, each containing 50 ml of sterilized nutrient broth (pH 7.0) were autoclaved at 15 lbs. /Inc^2 for 20 minutes to avoid contamination of the media. The broth was inoculated with a 48h culture using straight inoculation needle. Then broths were inoculated at a series of temperature ranging from 10ºC, 20ºC, 30ºC and 40ºC. Each treatment was replicated thrice. The OD value at 660nm by using Spectrophotometer was observed for each replication.

3.3.2.11. Oxidase test (Jurtshuk and Mcquitty, 1976)

A loopful of bacteria grown on NGY medium was transferred on to a disc impregnated with tetramethyl- p-phenylenediamine dihydrochloride (Hi-media). Presence of blue color indicates positive reaction and no color change depicts a negative reaction.

3.4. Studies on Variability of different isolates of *Clavibacter michiganensis subsp michiganensis*

3.4.1 Cultural variability of different isolates of *Cmm*

'Erlenmeyer flasks' containing NGY medium was sterilized and autoclaved at 15 lbs. /inch^2 for 20 minutes. Each of the isolate was plated in the medium with three replication and incubated at 28±1ºC temperature for the growth of bacterium in BOD incubator 48 - 72 hours. After 72hrs of incubation the bacterial growth was observed in the media and the variability in the cultural morphology was study on 96hrs after incubation.

3.4.2. Pathogenic variability of different isolates of *Cmm*

All the isolates of *Cmm* were evaluated for the symptom of wilt and canker under glass house conditions using 1-5 rating scale Shukla and Gupta (2005) and finally transformed into % disease severity.

3.4.2.1 Raising of tomato plants

Seeds of tomato cv. "Arka Vikas" were sown in pots (25cm in dia.) filled with sterilized soil comprising of soil + sand + vermicompost (2: 1:1 w/w/w) and kept in glass house conditions in the glass house at College of Agriculture. One seedling was maintained per pot. The plants were inoculated after 25 to 30 days.

3.4.2.2 Preparation of inoculum

One loopfull culture of 48hr old bacterial colonies raised in the petriplates was inoculated into 50mL of autoclaved nutrient broth in 150 ml "Erlenmayer flask", for the preparation of bacterial cell suspension. The suspension was adjusted to an OD value of 0.06nm at 660nm that corresponds to 10^8 cfu ml^{-1} by adding required quantity of sterilized distilled water. Pathogenicity tests were conducted on young tomato seedlings (5th week) as described below.

3.4.2.3 Syringe inoculation of stem

In the stem of tomato plants (5 Week Stage), 0.2 ml of standardized bacterial suspension (1.0x 10^8 cfu/ml) was injected with the help of a sterilized hypodermic syringe. Plants were kept under identical conditions separately and observed periodically for the appearance of the symptoms.

3.4.2.4 Observations

Data on disease development was recorded on the basis of % wilting and canker formation on the stem. Evaluation of disease appearance and development was determined using a 0-/5 arbitrary scale (**Soyulu *et al.,* 2003**). Ratings were as follows:

0 no leaves showing wilting;

1 slight marginal wilting, 1-/10% of leaves with wilt;

2 11-/25% of leaves with wilt;

3 sectored wilting, and canker formation, 26-/49% of leaves showing wilting associated with chlorosis;

4 pronounced leaf collapse, 50-/74% of leaves showing wilting;

5 whole leaf wilted

3.5. Statistical analysis

The data was analyzed using simple ANNOVA on Completely Randomized Design (CRD) and Randomized Block Design (RBD). Data recorded in percentage were further transformed to arcsine (angular) values before analysis. Critical difference was calculated at probability level of 0.05 to identify significant effects of treatment means.

$$CD = {}^{t}\sqrt{\frac{2EMS}{r}}$$

Where,

CD = critical difference

t = t value at error degree of freedom

EMS = Error Mean Square

r = number of replications

3.6. Evaluation of biocontrol agents

3.6.2 Evaluation of isolates of *Trichoderma* Spp. against *Cmm* by using dual culture method

Nine isolates of *Trichoderma* spp., were screened for their antagonistic potential against the pathogen following the dual culture technique (Morton and Stroube, 1955). 20 mL sterilized and melted Nutrient Agar (NA) was aseptically poured in sterilized 90mm diameter petri plates and allowed to solidify. Spot inoculation of bacterial suspension (conc. 10^4 cfu/ml) of 48 h old culture of test bacterium of *Cmm* and biocontrol agents cut with the help of sterilized cork borer from the edge of four days old culture, were placed in solidified NA in such a manner that they lie just opposite to each other. Inoculated petri plates were incubated at 28± 1º C. Na petri plates inoculated only with bacterial suspension served as control. The process was replicated thrice for four days. Experiments were conducted in Completely Randomized Design (CRD) with three replications.

3.6.2 Evaluation of *P. florescens* isolate against *Cmm* by using dual culture method

Two isolates *P. fluroscens* of, one alone and one in consortium with *T. harzianum* were screened for their antagonistic potential against the pathogen following the dual culture technique (Morton and Stroube, 1955). 20 mL sterilized and melted Nutrient Agar (NA) was aseptically poured in sterilized 90mm diameter petri plates and allowed to solidify. Spot inoculation of bacterial suspension (conc. 10^4 cfu/ml) of 48 h old culture of test bacterium of *Cmm* and biocontrol agents, were placed in solidified NA in such a manner that they lie just opposite to each other. Inoculated petri plates were incubated at 28± 1º C. NA petri plates inoculated only with bacterial suspension served as control. This process was replicated thrice for four days. Experiments were conducted in Completely Randomized Design (CRD) with three replications.

3.7. Evaluation of Chemicals

3.7.1. Efficacy of chemicals against *Cmm* by using the disk diffusion method

Kirby –Bauer and Stoke´s method (**Hedges, 1999**) was used for antibiotic susceptibility test. Whatman filter paper no. 1 was used for preparing disc of approximately 6mm in diameter, which were sterilized in hot air oven at 80ºC for 30 min. 100 µl of bacterium was poured into the media under aseptic conditions. Then the

disc of different antibiotic concentration was dispensed at the centre of the petri plate. Each disc was pressed down to ensure complete diffusion. Control disc was dipped into water. After 48 hrs of incubation at 28 ± 1ºC each plate was examined for the resultant zone of inhibition.

3.8. Evaluation of defense induers

3.8.1 Sample collection and biochemical analysis:

The plants of three cultivar viz., P.Ruby, US2853 and Rohini were sprayed with four de fense inducers at three different concentrations (200µM, 500µM and 800µM) at woe different durations. The first spray was given as prophylactic before the inoculation of the pathogen and second spray was given to another set of plants of all the three cultivar after the inoculation of pathogen. The samples were taken for biochemical analysis after 48hrs in both the set of experiment to assess the change in enzymatic activity and % disease severity. The experiments were replicated thrice. In the similar manner plants were also taken out carefully from the pots in where only water and pathogen were inoculated as control and the sample was further taken for biochemical analysis.

3.8.2 Biochemical Analysis

3.8.2.1 Polyphenol Oxidase EC 1.14.18.1 (PPO) assay:

Leaf samples (0.1 g) were homogenized in 2 ml of ice cold phosphate buffer (0.1 M/l), at pH 6.5. The homogenate was centrifuged at 16 000 rpm for 30 min at 4ºC, and the supernatant, thus, obtained was used directly in the enzyme assay. The reaction mixture consists of 0.4 ml catechol (1 mM/l) in 3 ml of (0.05 M/l) sodium phosphate buffer pH 6.5 and 0.4 ml enzyme extract. Only substrate containing reaction mixture served as control. The substrate for PPO estmation was catechol, and the change in absorbance was recorded at 405 nm **(Gauillard *et al.,* 1993)**. The PPO enzyme activity was expressed as change in OD min/mg/ FW.

3.8.2.2 Phenylalanine Ammonia Lyase EC 4.1.3.5 (PAL) assays:

Leaf samples (0.1 g) were homogenized in 2 ml of (0.1 M/l), sodium borate buffer (pH 7.0; 4ºC) containing 1.4 mM/l) 2-mercaptoethanol and centrifuged at 16,000 g at 4ºC for 15 min. The supernatant was used as enzyme source. To the reaction

mixture containing 0.2 ml of enzyme extract, 0.5 ml of 0.2 M/l) borate buffer pH 8.7 and 1.3 ml of water was added. The reaction was initiated by the addition of 1 ml, 0.1 M/l of l-phenylalanine pH 8.7 followed by an incubation at 32ºC for half an hour. The termination of the ongoing reaction was done by pouring 0.5 ml of trichloroacetic acid (TCA, 1 M/l) into the reaction mixture. The measurement of PAL activity was done by estimating trans-cinnamic acid formation at 290 nm according to **Brueske (1980)** and was expressed as µmol/ min/g fresh weight (FW) TCA.

3.8.2.3 Peroxidase EC 1.11.1.7 (POD) assay:

Leaf samples (0.1 g) were homogenized in 2 ml of ice cold phosphate buffer (0.1 M/l), (pH 7.0), at 4ºC, centrifuged at 16,000 g at 4ºC for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml pyrogallol (0.05 M/l), 0.05 ml enzyme extract and 0.5 ml H_2O_2 (1% v/v). Reaction mixture without enzyme served as control. The changes in the absorbance at 420 nm were recorded after 30 s intervals for 3 min. The enzyme activity was expressed as change in the U /min / g FW according to **Hammerschmidt *et al.,* (1982)**.

3.8.2.4 Total Phenol content estimation:

Total phenol content estimation was done in accordance to the procedure described by **Zheng and Shetty (2000)**. Leaf tissues (0.1 g) were placed in 5 ml ethanol (95%) and were placed at 0^oC for 48 h. Individual samples were homogenized followed by centrifugation at 10,000 rpm for 10 min. 1 ml of 95% ethanol, 5 ml of autoclaved distilled water and 0.5 ml of 50% Folin–Ciocalteau reagent was added to 1 ml of the supernatant, and the reaction mixture was shaken vigorously for proper mixing of the constituents. 1 ml of 5% sodium carbonate was added after 5 min, the reaction mixture was incubated at room temperature for an hour and the absorbance of the color developed was recorded at 725 nm. Standard curves were prepared for each assay using different gallic acid concentrations in 95% ethanol. Absorbance values were converted to mg GA equivalents (GAE) per g FW.

3.8.2.5 Hydrogen Peroxide Production:

Histochemical detection of H_2O_2 was done by 3, 3-diaminobenzidine (DAB) staining as described by Thordal-Christensen *et al.,* 1997. H_2O_2 reacts with DAB to form a reddish-brown stain. Leaf disks were incubated in 1 mg ml^{-1} DAB solution pH

7.5. After incubation in the dark at room temperature for 20 h, leaf tissues were boiled in a solution containing alcohol and lactophenol (2:1) for 5 min and rinsed twice with 50% ethanol. For H_2O_2 quantification, a 0.1 g leaf sample from each of the treatments was homogenized in an ice bath with 2.0 ml of 0.1 % (w/v) of trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min and 0.5 ml of the supernatant was mixed with 10 mM potassium phosphate buffer pH 7.0 and 1 ml of 1 M potassium iodide solution and incubated for 5 min. The oxidation product formed was measured at 390 nm (**Velikova 2000**). The amount of H_2O_2 formed was determined from the standard curve made with known concentrations of H_2O_2 and expressed as mM H_2O_2/g fresh weight (FW). For qualitative estimation of H_2O_2 leaf discs were incubated in 1mg/ml dab solution pH 7.5. After incubation in dark at room temperature for 20 hours the leaf disc were boiled in 15 ml solution containing absolute ethanol and lactophenol (2 :1) for 5min and then rinse twice with 1 ml ethanol (50%). The leaf discs were then viewed under microscope in order to view H_2O_2 production in leaf tissues.

3.8.2.6 Histochemical staining (Lignification):

Examination of transverse stem sections was performed by taking stem of the plant from each treatment which were fixed in 95% (v/v) ethanol and mounted on a slide in a solution of saturated aqueous phloroglucinol in 20% HCl and observed with light microscope (Leica) with positive lignin staining indicated by red-violet color (**Jensen 1962**).

3.8.2.7. PR2 Protein Assay:

β- 1,3- glucanase activity was assayed by the laminarin-dinirosalicyclic acid method (**Pan *et al.,* 1991**). The reaction mixture consisted of 62.5µL of 4% laminarin and 62.5µL of enzyme extract. The reaction mixture was carried out at 40ºC for 10 min. The reaction was stopped by adding 375µL of dinirosalicyclic acid and was heated for 5 min in boiling water, vortexed and the absorbance was measured at 500nm. The enzyme activity was measured as µg glucose released min^{-1} mg^{-1} protein.

3.9. Biofilm formation by *Cmm* on different media

Crystal violet assay for bacterial attachment. The assay for bacterial attachment was carried out following the procedure described by Davey and O'Toole (2000). Culture of *Cmm* was grown in LB broth for 49 h at 28°C and then transferred to the same medium at 1:1000 dilution. After reaching an OD595 = 0.5, the bacterial suspension was centrifuged and the pellet was washed once and resuspended in 3 ml of sterile water. The

suspension (50 µl) was added to individual wells of 24-well multidishes containing 150 µl of XS, M9, or LB medium. The multidishes were incubated for 72 h at 28°C without agitation. After incubation, the culture was gently removed using a pipette, and wells were washed twice with 150 µl of sterile water. Following fixation at 60°C for 20 min, surface-attached bacteria were stained with 0.1% crystal violet solution for 1 h at room temperature. The multidishes were then washed gently three times with 150 µl of sterile water and air dried for 1 h for visual qualitative analysis. For quantitative analysis, crystal violet in each well was solubilized by adding 100 µl of 95% ethanol and absorption of the solution was then measured at 595 nm using a microtiter plate reader.

3.10. Effect of Different Inoculation Methods on Incubation Period of *Clavibacter michiganensis subsp. michiganensis* on Tomato Seedlings

For the purpose of finding out the best inoculation method for the expression of the bacterial canker symptoms in tomato plants different inoculation methods were performed on young tomato seedlings as follows:

3.10.1.1 Raising of tomato plants

The raising of tomato plants was done as per **3.4.2.1**

3.10.1.2Preparation of inoculum

The preparation of inoculums was done as per **3.4.2.2**

3.10.1.3 Inoculation methods used:

3.10.1.3 1 Seed inoculation

Seeds (50 g) of the tomato cultivar "Arka Vikas" were packed in a muslincloth bag and placed in a 5oomL flask (Borosil) containing 200 ml of the bacterial suspension ($1.0x10^8$ cfu/ml). The seeds were left in the bacterial cell suspension for about half an hour and the bacterial suspension was removed by placing the muslin cloth inside the vaccum suction cups and suction pressure was applied for the removal of suspension bacterial cell suspension. Then the seeds were kept for drying on a sterilized blotter paper sheet under the laminar air flow cabinet.

3.10.1.3.2 Foliar spray inoculation

Bacterial suspension ($1.0x10^8$ cfu/ml) was sprayed on the leaves of tomato plants with the help of atomizer. Inoculated plants were covered with polythene cover for 48 h to maintain high relative humidity by frequently spraying distilled water and were observed periodically for the appearance of the symptoms.

3.10.1.3.3 Syringe inoculation of stem

In the stem of tomato plants, 0.2 ml of standardized bacterial suspension ($1.0x10^8$ cfu/ml) was injected with the help of a sterilized hypodermic syringe. The plants were observed periodically for the appearance of the symptoms.

3.10.1.3.4 Wound inoculation of stem with toothpick

Stem of tomato plants were inoculated with bacterial suspension ($1.0x10^8$ cfu/ml) with the help of toothpick. The plants were observed periodically for the appearance of the symptoms.

3.11. Serological Characterization

The indirect ELISA technique of **Benedict *et al.,* (1989)** was used on pure cultures of the test strains. The harvested bacterial cells were washed three times in phosphate buffer saline, (PBS, pH: 7.0).and were re suspended into 0.05 M carbonate-bicarbonate buffer (pH 9.6) and adjusted to an absorbance of 0.1 D at A_{600}nm in spectrophotometer. Polyvinyl chloride 96 well plates were coated with 100µl of the cell suspensions by drying in 37ºC circulating air incubator. The plates were blocked with BLOTTO (**Johnson *et al.,* 1984**) in PBS and washed once with 0.16 M borate buffer (pH 8.3). 100µl of each of the reagents, each diluted in 1:3 dilutions of 5% BLOTTO in borate buffer was added sequentially, and each was incubated 1 hr at room temperature followed by three washes with borate buffer : mAb, 1:1000 rabbit anti mouse globulin, 1:1000 protein - A- horseradish peroxidase. Finally substrate consisting of 0.05% 5-amino-salicyclic acid, 0.06% H_2O_2in phosphate-EDTA buffer (Alvarez and Lou, 1985), and after 1 hour absorbance was measured at 450nm by multiskan plate reader.

3.12. Molecular Characterization

3.12.1 DNA isolation (Ausubel *et al.,* 1995)

Bacterial cell harvested from 48 hrs bacterial broth suspension were resuspended in 567 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) by repeated pipetting, and adjusted to a final volume of 700 µl with 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K. The mix was subsequently incubated for 1 h at 37 °C. Selective precipitation of proteins and polysaccharides was performed using 80 µl of CTAB/NaCl (10% CTAB, 0.7 M NaCl) in presence of 100 µl of 5 M NaCl. The samples were gently mixed by inversion and incubated for 10 min at 65 °C. Nucleic

acids were thereafter isolated by a phenol: chloroform: isoamyl alcohol (25:24:1, Sigma-Aldrich) separation, followed by one chloroform: isoamyl alcohol (24:1, Sigma-Aldrich) separation. DNA was finally recovered by precipitation using 0.6 volumes of isopropanol and centrifugation (5 min, 4 °C, and 15,000 rpm). The DNA pellets were washed with 1 ml of cold 70% ethanol. Finally, the tubes were centrifuged one last time 5 min (4 °C, 15,000 rpm), the supernatant was discarded, and each pellet dried before being resuspended in 100 µl TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

3.12.2 Amplification of Genomic DNA

Amplification of genomic DNA was performed by polymerase chain reaction (PCR) in Applied Biosystem Thermal Cycler. PCR was carried out with one universal primer pair 27F&1492R **(Lane *et al.,* 1985)** and two specific primer pairs CMR 16F1, CMR 16R1 designed from the 16S rDNA region (Lee *et al*. 1997) and primers CMM-5, CMM-6 (Drier et al., 1995) designed based on the pat-1 gene residing in pCM -2 plasmid region.

Primer	**Sequence (5′–3′)**	**Reference**
CMR16F1 CMR16R1	GTG ATG TCA GAG CTT GCT CTG GCG GAT C GTA CGG CTA CCT TGT TAC GAC TTA GT	Lee *et al.* (1997)
CMM 5 CMM 6	GCG AAT AAG CCC ATA TCA A CGT CAG GAG GTC GCT AAT A	Drier *et al.,* (1995)
27f 1492r	AGA GTT TGA TCM TGG CTC AG CGG TTA CCT TGT TAC GAC TT	Lane *et al.,* (1985)

Table 3.2. List of primer pairs used in the study

Polymerase chain reaction components:

Components	**Volume/Reaction(µl)**
Hi-Chrom PCR master mix	12.5
Primer 1 forward	1.0(0.5µM)
Primer 2 reverse	1.0(0.5µM)
Genomic DNA	4.0(40ng)
Molecular biology grade water	6.5
Total	25.0

The required concentration of master mix was prepared in a 200 µl eppendorf tube and 21 µl of this mixture was distributed to each PCR tubes. Four µl of template DNA(40ng) was added to each PCR tube. The tubes were then placed on thermocycler for cyclic amplification. Conditions for amplifications were programmed as follows:

PCR cycle set up for Primers by Lee *et al.,* 1997

Steps	**Temperature (^{0}C)**	**Duration (min)**
Initial Denaturation	94	5:00
Denaturation	94	1:00
Annealing	62	2:00
Extension	72	3:00
Final Extension	72	10:00

The PCR was carried out with a total of 35 cycles.

PCR cycle set up for Primers by Drier *et al.,* 1995

Steps	**Temperature (^{0}C)**	**Duration (min)**
Initial Denaturation	96	2:00
Denaturation	96	1:00
Annealing	55	1.5:00
Extension	72	1:00
Final Extension	72	10:00

The PCR was carried out with a total of 30 cycles.

PCR cycle set up for Primers by Lane et al., 1985

Steps	**Temperature (^{0}C)**	**Duration (min)**
Initial Denaturation	94ºC	3min
Denaturation	94ºC	30sec
Annealing	50ºC	30sec
Extension	72ºC	90/60sec
Final Extension	72ºC	7min

The PCR was carried out with a total of 35 cycles.

3.12.3. Electrophoresis in Agarose Gel:

The solutions for the agarose gel electrophoresis were prepared as under:

i) **Ethidium bromide:** Ethidium bromide was added to sterile water (10 mg/ml) and stirred on a magnetic stirrer until the dye was completely dissolved. The container was wrapped in aluminium foil or the solution was transferred to a dark bottle and stored at 4^{o}C temperature.

ii) **6X Loading Dye:** Dissolved 0.25% bromophenol blue, 0.25% xylene cynol FF and 30% glycerol in H_2O**.** The dye was kept at 4^{0}C till further use.

iii) **50X TAE/TBE (Tris Acetate/Tris Borate):** 270 g of tris base and 137.5 g of boric acid were taken in a container. 100 ml of 0.5 M EDTA and 200 ml of distilled H2O were added and stirred on a magnetic stirrer until a clear solution was formed. The pH of the volume was adjusted to 11 and sterilized by autoclaving.

iv) **Agarose Gel (1 %):** 1 g of agarose was poured in 200 ml of 1X TAE buffer and boiled for 3-5 minutes. 10 µl ethidium bromide solution was added when temperature reached to 50-600C. This solution was poured into the casting tray for gelling.

3.12.4. Running Conditions:

Electrophoresis of amplified DNA was done in 0.8 % agarose gel under submerged conditions, 1X TAE buffer was used as gel and tray buffer.

1X TAE/TBE buffer	: 100 ml
Agarose	: 1.0 gm
Ethidium bromide dye	: 5 µl

Before loading the sample in the wells, 1 µl of 6X loading dye was added to each sample. DNA ladder 100 bp plus, obtained from Hi media was loaded in one well for size comparison. The sample was loaded into wells and amplified DNA was fractioned at 50 volts at constant supply.

3.12.5. Viewing of Gel and Photography:

The amplified DNA products in the gel were viewed under and the gel was photographed by Gel Documentation System (Alpha Innotech Corporation).

3.13 . Germplasm Screening

Screening of seeds of different cultivars (25) from different private and public sector enterprises was done to check the presence of bacterium followed by screening of the cultivars in glasshouse, polyhouse and open field condition and in two different cropping seasons was also done in order to assess the nature of cultivars against the bacterium. All the 25 tomato lines/cultivars were screened against the disease after artificial inoculation conditions in pots and field during 2016. The seedlings of different cvs. / lines were raised in pots for 30 days as well as in nursery for the open field condition, and plants inoculated with the stem inoculation by syringe (3.9.9.3). The relation of temperature with the spread of bacterium was also assed in the three above location in two different growing season viz., rabi and kharif. The evaluation of disease appearance and development was determined using a 0-/5 arbitrary scale (**Soyulu *et al.,* 2003**).

3.14 Host range studies

For the purpose of determining the alternate host of the pathogen, the pathogen was syringe inoculated to different crop plant belonging to the solanaceous family *viz.,* pepper, bellpepper (*Capsicum annum* group), brinjal (*Solanum melongena*), potato (*Solanum tuberosum*) and weed host makoi (Solanum nigrum), bathua (*Chenopodium album*). Evaluation of disease appearance and development was determined using a 0-/5 arbitrary scale (**Soyulu *et al.,* 2003**). Ratings were as follows:

0 no leaves showing wilting;

1 slight marginal wilting, 1-/10% of leaves with wilt;

2 11-/25% of leaves with wilt;

3 sectored wilting, 26-/49% of leaves showing wilting associated with chlorosis;

4 pronounced leaf collapse, 50-/74% of leaves showing wilting;

5 whole leaf wilted.

3.15. Field Experiment

Screening of 3 tomato cultivars (P. Ruby, Rohini, US2853) against *Clavibacter michiganenis subsp michiganensis* was conducted in the growing season 2015-16 and 2016-17. The field experiment was laid out in Randomized Block Design using 3 cultivar and replications. Seedlings of 4 week old were transplanted into the field with 60cm inter and 50cm intra row spacing in plots measuring 2.5 × 2.0 m.

3.15.1 Land Preparation:

Field was prepared 15 days prior to sowing. In order to get well pulverized soil, the field was ploughed thoroughly with disc harrow and finally leveled with tractor operator leveler.

3.15.2. Nursery preparation and transplanting

Tomato seed were first sown in the nursery beds to raise seedlings and transplanted in the main field when attained a height of 10-15 cm and 4-6 true leaves. The seeds were sown at the depth of 1-15 cm in properly prepared raised seed bed.

3.15.3. Fertilizer Application

Fertilizers were applied at the rate 100 Kg N_2, 60 Kg P_2O_5 and 60 Kg K_2O per hectare. Half dose of nitrogen and full dose of phosphorus and potassium were applied to the soil at the time of transplanting and remaining quantity was applied as top dressing four weeks after transplanting.

3.15.4. Intercultural practices

Gap filling was done replacing the plants showing mortality. Weeding was done time to time as and when required. Mulching, staking and intercropping with marigold were applied as integrated cultural management practices.

3.15.5. Crop protection from insect damage

In order to keep the crop free from insect pest and other viral vectors, two sprays of Acetamiprid at the rate of 0.3% at vegetative and flowering stage was done.

3.15. Effect of different treatment on the pathogen through intra plant Population Dynamics

3.15.1 Inoculum preparation

The test bacterium *Cmm* isolated and purified from infected parts of tomato plant was used throughout the investigation. For preparation of inoculums LBB medium was used. Single typical colony of *Cmm* was inoculated in each flask aseptically and then the flasks were incubated at 28 ± 1ºC for 48 hrs, during incubation, the flasks were regularly shaken for uniform bacterial growth the OD value was measured10^8 cfu/ml (OD of 0.06 at 660 nm and the bacterial cell suspension of 1×10^8 cfu was inoculated into the plant at first true leaf region.

3.15.2 Artificial inoculation in field experiments

The inoculation of the plants was done as per **3.9.9.3**

3.15.3 Prophylactic treatment of pathogen and estimation of population densities

The plants were given prophylactic spray of the chemicals and the biocontrol agent before pathogen inoculation. The pathogen was inoculated into the tomato plants two days after the spray with the best effective concentration of the chemical compound screened from the invitro assay and with the biocontrol agents suspension was adjusted to an OD value of 0.1 at 600nm corresponding to $1*10^8$cfu. The pathogen population was allowed to build up within the plants. After three weeks the region of the plant tissue 1cm above the point of inoculation one gram of plant material from each sample (stems and leaves) was macerated in 2 ml 0.05 M phosphate buffer. Each homogenate was subjected to a 10-fold serial dilution and the diluted samples spread on NBY medium. Population densities were estimated based on the number of bacterial colonies that grew from the serial dilutions on NBY medium. The number of bacterial colonies per gram of plant material (cfu/g) was calculated, the mean values were log-transformed and separated in ANOVA by t-test ($P<0.05$).

3.15.4. Fruit yield data/ treatment

Fruit yield was recorded for the three cultivars (P.Ruby, US2853 and Rohini) for the thirteen different treatments and percent increase in yield due to treatment was calculated by using the following formula:

$$\text{Fruit yield} = \frac{\text{Yield in treated plot- Yield in control}}{\text{Yield in control}} \times 100$$

Results and Discussion

Chapter 4 RESULTS AND DISCUSSION

The results obtained during the course of present investigation are being presented here under:

4.1 DISEASE SURVEY

To assess the magnitude of bacterial canker of tomato, systematic surveys of tomato growing areas of Uttarakhand and Himachal Pradesh was conducted during the crop season of 2015-16 and 2016-17 at fruiting stage of the crop growth. The data on disease incidence and disease severity were recorded and presented in table 4.1.

Table 4.1 Incidence and Severity of bacterial canker of tomato in tomato growing areas of Uttarakhand and Himachal Pradesh

Location (Uttarakhand)	Disease incidence (%)	Disease severity (%)
Haldwani	0.00	0.00
Gaulapar	27.78	22.60
Ramnagar	0.00	0.00
Kotabagh	0.00	0.00
Lohaghat	0.00	0.00
Mean	5.55	4.52
Himachal Pradesh		
Nauni	27.88	23.64
Khaltu	32.69	28.48
Kalaghat	18.01	12.31
Kotla Panjola	16.24	14.12
Deothal	64.23	58.39
Garganoo	36.22	32.35
Narag	31.82	26.70
Kyar	38.94	31.22
Nainatikkar	26.36	22.26
Mean	33.59	27.71
Overall mean	19.57	16.11

It is apparent from the data in Table 4.1 that the overall incidence and severity of bacterial canker of tomato was observed to be 19.57 per cent and 16.11 per cent, respectively. The data provides the evidence that the disease was earlier not reported from Uttarakhand has shown its presence in one of the tomato growing region Golapar area of Haldwani, District Nainital. However, the disease incidence was found to be 27.78 % and disease severity was found to be 11.60%. During the survey of Himachal Pradesh the disease was found to be widely spread in most of the tomato growing region of the state with varying percentage of disease incidence and severity. In HP, the disease incidence and diseases severity was found to be 33.59 % and 27.71 %, respectively. The maximum disease incidence and severity was 64.23% and 58.39%, respectively at Deothal region followed in Kyar where the diseases incidence and severity has been 38.94% and 31.22, and in Garganoo region of Himanchal Pradesh where the disease incidence was 36.22% and 32.35%, respectively. The lowest disease incidence of 16.24% and severity of 14.12% was observed at Kotla Panjola in HP.

Survey has not been conducted earlier in the Outer Himalayan regions to record the disease incidence and severity of bacterial canker of tomato in Uttarkhand. Hence, the present investigation represented the first report of bacterial canker in Gaulapar region of Haldwani, District Nainital in Uttarakhand State of India. However, **Sarala and Shetty (2005)** and **Umesha (2006)** have reported the occurrence of bacterial canker in tomato fields in the state Karnataka, India with an average incidence of 48 per cent. **Singh *et al.,* 2017**, however, have reported the disease in Himachal Pradesh. The nature of pathogen *Cmm* is described to be seed borne and seed act as a main source of long distant transmission (**Tsiantos, 1987**). Therefore it can be said that the bacterium *Cmm* might have appeared in the tomato growing areas of Uttarakhand and Himachal Pradesh through contaminated seed. Due to which it is necessary to certify seeds of tomato to be free of *Cmm* **(Chang *et al.,* 1991; Mansfeld-Giese, 1997)**. Although less than 1 % of *Cmm* transmission through infected seed has been reported **(Grogan and Kendrick, 1953)**, even though 0.01 to 0.05% of infested seed are sufficient to cause an epidemic in suitable environmental conditions for multiplication and dissemination of the bacterium (**Chang *et al.*, 1991**). Transmission of soil borne inoculums of *Cmm* is reported to be less significant (**Ftayeh, 2004; Ftayeh *et al*., 2004; Strider, 1969)** hence, tomato seed indexing for efficient disease management is of utmost importance **(Biggerstaff *et al.*, 2000).**

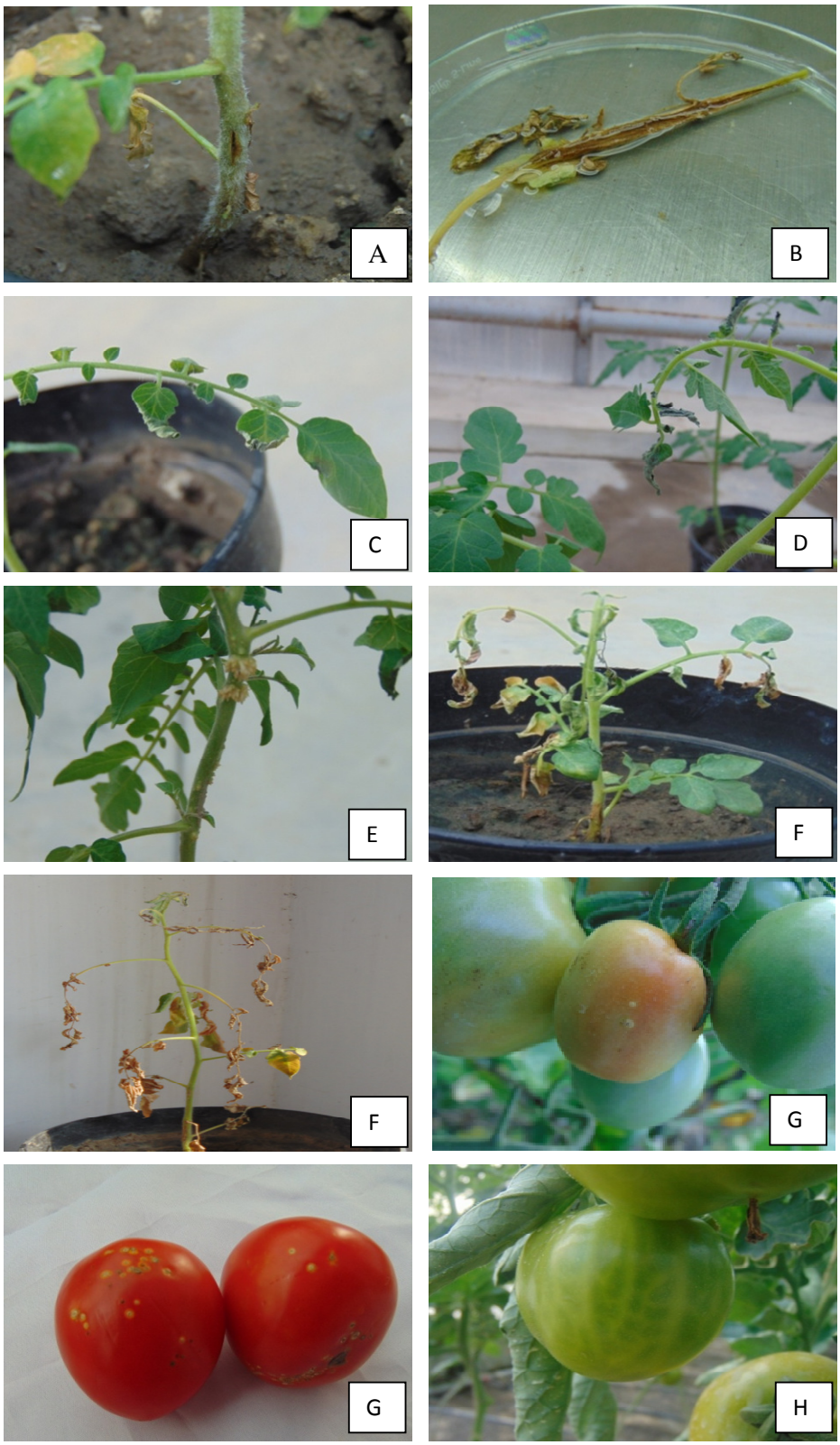


Plate 1: Varied types of symptoms formed by *Cmm* in different plant parts

Wilting symptom observed in tomato plants in open field and polyhouse conditions

4.2. Symptomatology

The bacterial canker and wilt disease caused by *Clavibacter michiganensis* subsp *michiganesis* (Smith) Davis is one of the most destructive diseases of tomato causing an array of symptoms. Local infection by the pathogen leads to formation of symptom like spots on the surface of leaves, peduncles and fruits. A wide range of symptoms are produced by the pathogen on the basis of plant stages, time of infection, intercultural operations, location of production (glasshouse or field), cultivar, etc **(EPPO Bulletin, 2016)**. The disease affects all the plant part showing marginal necrosis in the leaves and canker on stem and fruits followed by wilting of the entire plant leading to the plant death. The disease is generally observed during the months of May- August, where high temperature is accompanied by rainfall. The rain splashes act as the resource for the spread of the bacterium. Economic losses are generally caused by the reduction in the marketable quality of the fruit due to appearance of the symptom on the fruit.

The symptom of the disease becomes visible on the stem region as cankerous lesion, which increases in size chronically (A), infection on the stem often also girdles the stem and may cause premature plant death. Splitting the stem longitudinally, a mealy appearance and brown colored discoloration of the internal tissues can be observed (B). The symptom on the leaves appears in the form of unilateral wilting in plant, that appears starting from the top most part and follows downside (C), marginal necrosis in leaves is also observed accompanied with downward turning of one or a few of the leaves occurs as the infection progresses (D), adventitious root formation at the nodal region of the plant takes place (E), whole plant wilting (F) is observed. Symptoms commonly observed on the leaf surface can be seen in form of dark brown spot surrounded by yellow –orange halo, observed usually at the edge of the leaf; resulting due to hydathodal infection. Curling of the oldest infected leaves followed by marginal chlorosis followed by necrosis is also observed in plants by **Carlton *et al.,* (1998).**

Symptom on the fruit appears in the form of bird´s eye spot formation (G), infected fruit also exhibit netted symptom on unripe green fruit (H) (Plate1). The netted or marbled appearance on infected fruit in greenhouse has also been reported **(ASTA, 2010).**

Symptom on the fruit appears in the form of bird´s eye spot formation (G), infected fruit also exhibit netted symptom on unripe green fruit (H) (Plate1). Fruits of systemically infected plants may fail to develop, ripen unevenly or fall. Bird's-eye lesions are observed on both unripe and ripe fruit **(Bryan, 1930**; **Gleason *et al.,* 1993)**. Infected fruit in greenhouse may be symptomless or appear netted or marbled **(ASTA, 2010).**

4.3. Isolation and Colony Characteristics of the *Cmm*

Bacterial pathogen was isolated on nutrient agar glucose yeast (NGY) medium from different parts of the plant infected with bacterial canker. Ten different isolates of bacterial pathogen were thus isolated which were named as *Cmm*1, *Cmm*2, *Cmm*3, *Cmm4, Cmm*5*, Cmm*6*. Cmm*7*, Cmm*8*, Cmm9 and Cmm10.* The colony characteristics of these isolates were recorded and presented in Table - 4.3 (Plate 5).

4.4. *In – vitro* Evaluation

4.4.1. Biochemical and Physiological Characterization of *Cmm* Isolates

The identification of the bacteria was done by determining various physiological and biochemical properties on the basis of "**Bergey's manual of determinative bacteriology**", 1994. The results thus obtained are presented in Table-4.2 (Plate-4).

On the basis of the biochemical and physiological tests to differentiate the isolated bacterium; the subjective bacterium was screened for characterization by the set of tests (Plate2, Table 4.2). On the basis of these results, these 10 bacterial isolates were considered to be belonging to the genus *Clavibacter*, as also been established by **Milijasevic *et al.* (2006).**

These set of biochemical and physiological testing has been recommended by several investigators: Gram staining (**Strider, 1969**), string formation test in 3% KOH (**Suslow *et al.,* 1982**), oxidase test (**Jurtshuk and Mcquitty, 1976**), aesulin hydrolysis (**Swan, 1954**), casein hydrolysis (**Vidaver and Davis, 1988**), levan production, gelatin hydrolysis, starch Hydrolysis **(Lelliott and Stead, 1987** and test for differentiation of *Cmm* (**Singh *et al.,* 2017**).

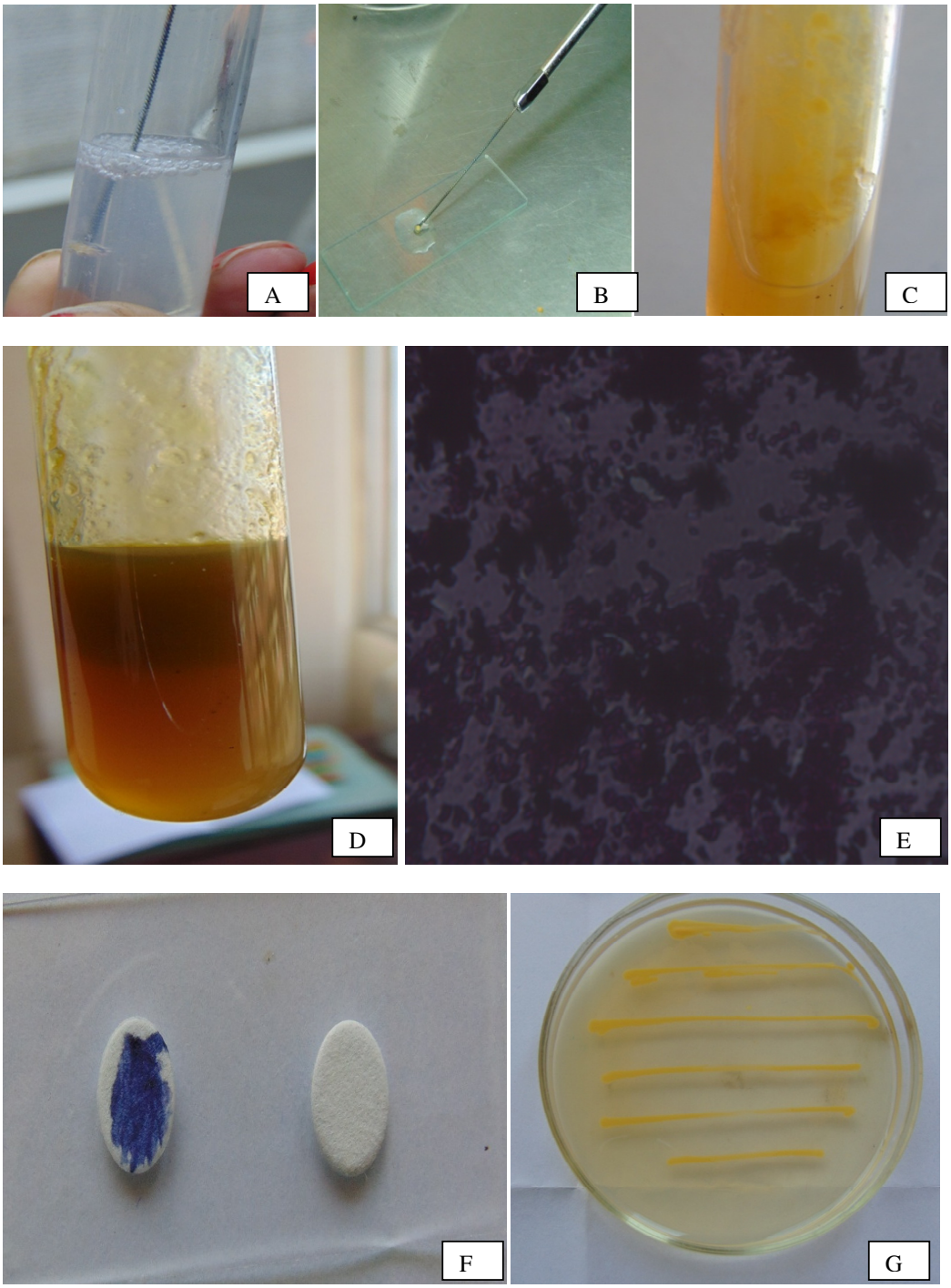


Plate 2: Biochemical testing:

A. Catalase test
B. KOH Test
C. Aesculin hydrolysis test
D. Mannose oxidation test
E. Gram staining
F. Oxidase test
G. Gelatin liquification test

Table 4.2 Biochemical characterization of the bacterial isolates

Tests	Character acc. to Bergey's Manual	ISOLATES									
		*Cmm*1	*Cmm*2	*Cmm*3	*Cmm*4	*Cmm*5	*Cmm*6	*Cmm*7	*Cmm*8	*Cmm*9	*Cmm10*
Gram stain	+	+	+	+	+	+	+	+	+	+	+
KOH test	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	-	-	-	-	+	+	-	-
Aesulin hydrolysis	+	+	+	+	+w	+	+	+	+w	+	+
Mannose oxidation	+	+	+w	+	+	+w	+	+	+	+w	+
Starch	+w	+	+	+	+	+	+	+w	+	+	+
Gelatin	+w	+	+	+w	+	+w	+w	+	+	+	+w
Casein hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Levan Production	-	-		-		-	-	-	-	-	-
H_2S production	+	+	+	+	+	+	+	+	+	+	+
Pathogenecity	+	+	+	+	+	+	+	+	+	+	+

+ = strains shows positive reaction
- = strains shows negative reaction
+w = strains showed weakly positive reaction

4.4.2. Morphological characterization

The study of the morphology of bacterial cell and its size was done through Scanning electron microscopy (Plate 3). The bacterial cells were rod shaped to pleomorphic, ranging from 0.6-0.8×0.7-1.3µm. Similar morphology of the *Cmm* bacterial cell were reported by **Chalupowicz *et al.* (2011); Choi *et al.* (2014).**

4.4.3 Colony Characters and Evaluation of selectivity and growth areas on different media

The colony characteristics and growth areas of bacterial isolates were studied on seven different media and observations have been recorded on 4th day and 10th day after plating and compared with the growth on NGY medium at the same period. Fast growth of the bacterium was obtained in the non selective medium followed by a slower growth in the semi-selective medium. Relatively fast growth was recorded on NGY, followed on NA, YPGA, YDCA and SPY media. On semi-selective media viz., D2ANX and SCM, a slower growth of the bacterium was observed in comparison with the growth on non selective media. On comparing the two semi-selective media, the growth of the bacterium was relatively faster in D2ANX medium, and only the beginning of the growth was observed in SCM medium at 4th day of plating (Fig.1).

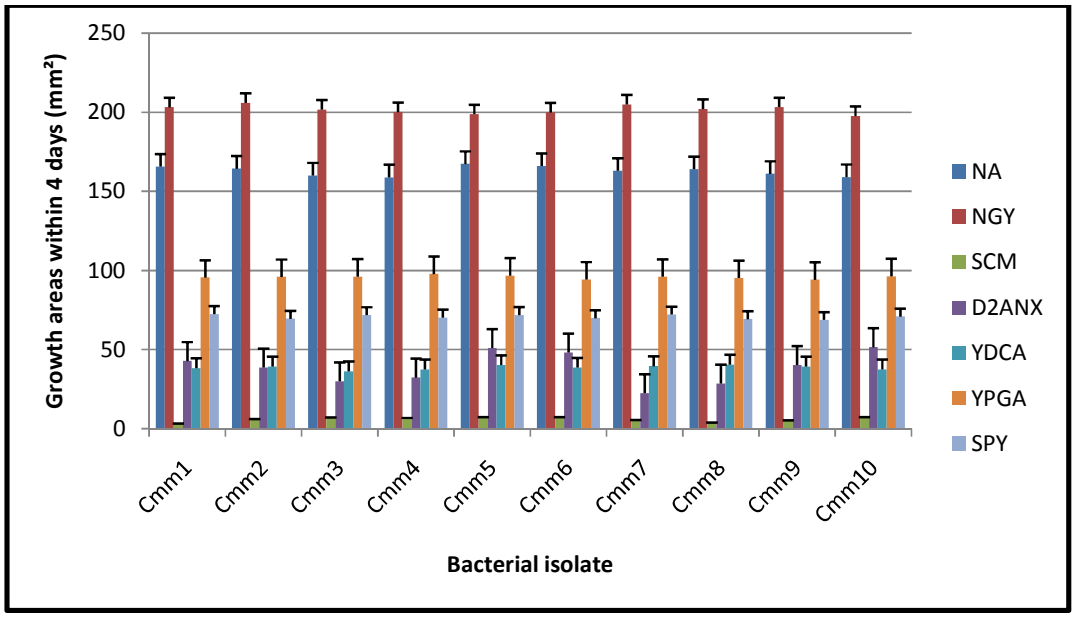


Fig. 1: Growth areas in mm^2 of 10 strains (as the mean of three replicates for each strain) on different media (without addition of antibiotics) at the 4th day after plating following incubation at 28°C. Growth area = number of CFU × π r^2 (r: average radius of colonies in mm). Bars represent standard error.

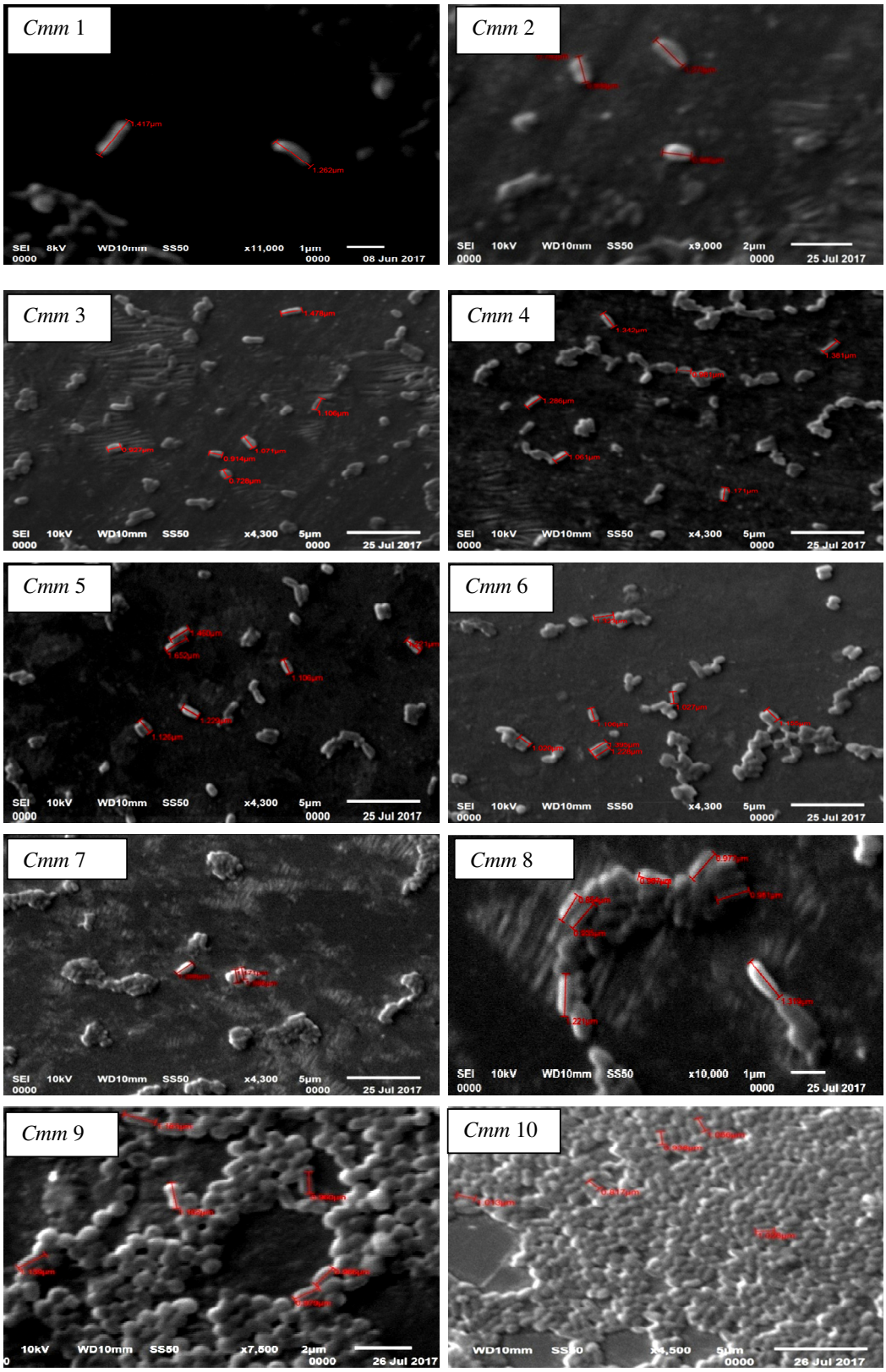


Plate 3: Morphological characterization of bacterial isolates on the basis of SEM *Cmm* 1 – *Cmm* 10.

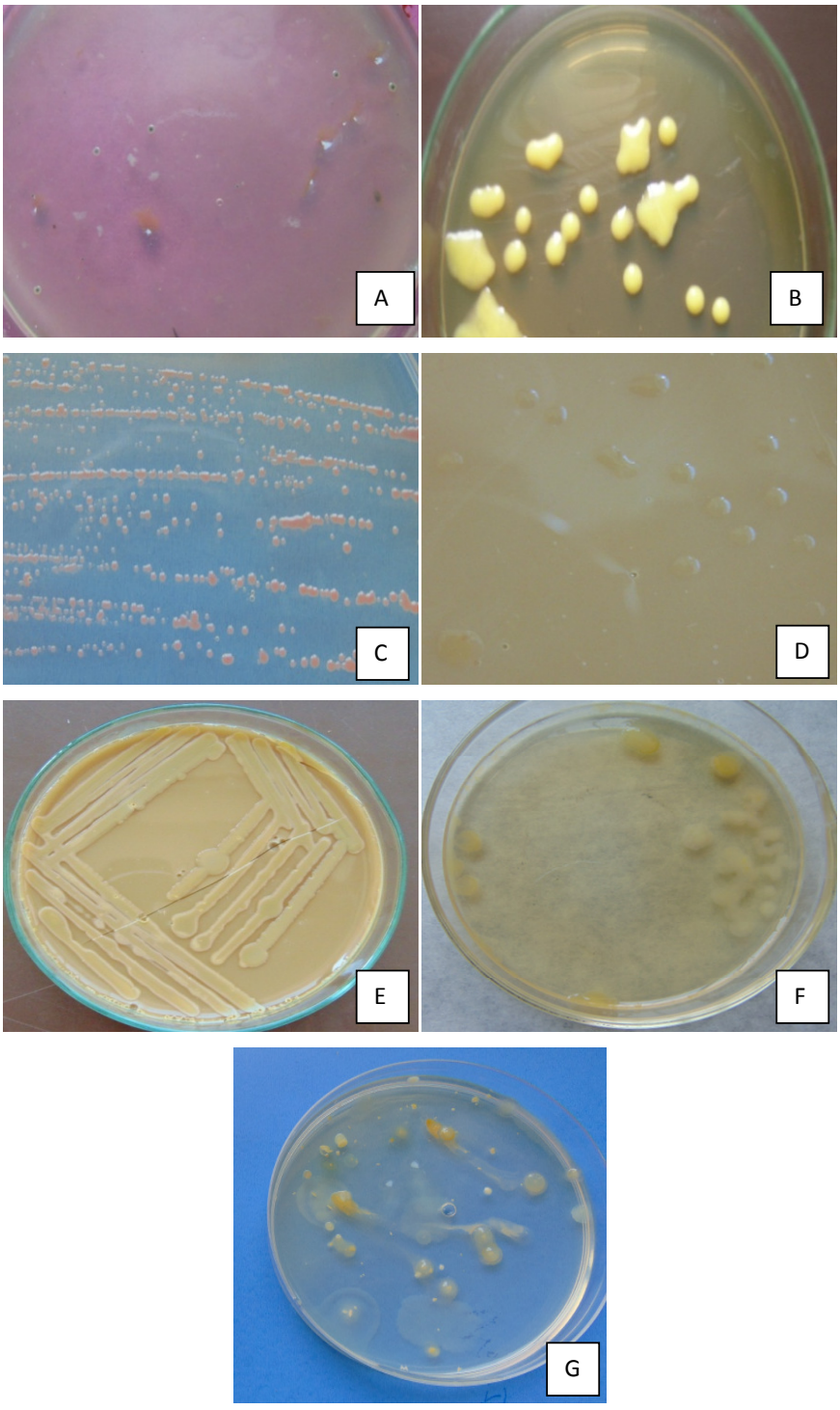


Plate4: Growth of bacterium isolate *Cmm*10in different medium

A. SCM, B. D2ANX, C. NGY, D. YDCA, E. YPGA, F. SPY, G. NA

In comparison with the NGY medium, a significant difference in growth areas was observed on the other medium used. After ten days of plating on the semiselective media, the maximum mean number was recorded on D2ANX, followed by SCM, and amongst the nonselective medium the maximum mean number was recorded on NGY, followed by NA, YPGA, YDCA and SPY.

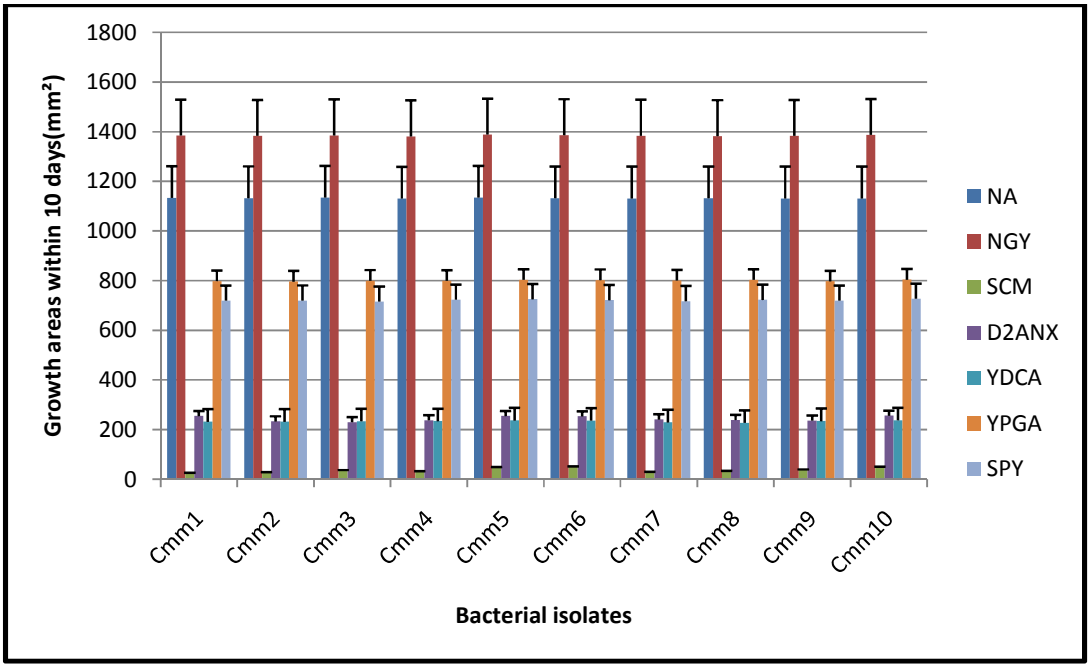


Fig. 2: Growth areas in mm^2 of 10 strains (as the mean of three replicates for each strain) on different media (without addition of antibiotics) at the 10th day after plating following incubation at 28°C. Growth area = number of CFU × π r^2 (r: average radius of colonies in mm).Bars represent standard error.

On most of the nonselective media, the maximum bacterial growth was recorded within the first four days, while in D2ANX medium the growth in some strains started to grow between two and four, and in other strains between four and six days, this may be due to variable growth potential of isolates. On SCM medium the bacterial colonies appeared to grow very slowly with growth observed fourth day after plating and *Cmm*1 and *Cmm* 8 showed poor growths on the medium. The colony characteristics of the bacterial isolates in different medium are shown on Plate4.

Roy and Sasser (1990) also mentioned that semi-selective media are valuable and essential tools in phytobacteriology for disease diagnosis, indexing and epidemiological studies**.** Direct isolation and plating onto semi-selective media remain

the most widely used detection methods for detecting the specific bacterial pathogen. Semi-selective media are based on knowledge of the nutritional requirements and physiological tolerances of the target bacterium. Plating onto sem-iselective media is easier and less expensive and results in recovery of viable bacterial cultures that can be used to determine pathogenicity **(Schaad, 1982; Schaad *et al.*, 1997).** This includes choosing suitable carbon and nitrogen sources that allow growth of the target organism. Other methods which could increase selectivity of semiselective media include incubation temperatures **(Gitaitis *et al.*, 1997)** that allows growth of the target bacterium but inhibit growth of the background microflora.

The bacterial colonies (*Cmm10*) on NGY medium were light yellow, creamish and orange, round and semifluidal. On SCM medium, the colonies appear to be translucent green–grey, mucoid, with a variable grey to black centre. The results obtained are in the agreement with **Fatmi and Schaad (1988) and Umesha (2006).** Further on YPGA medium, the colonies appeared to be light yellow, flat and semi-fluidal, round or irregular becoming deeper yellow, opaque and glistening with the increase in the incubation period. On YDCA medium, the bacterial colonies were yellow, mucoid but often orange in colour which is supported by the observations made in the **EPPO Bulletin (2016).** On the D2ANX medium, the colonies were yellow, concave and become glistening with the time. Similar growth pattern of bacterial colonies of *Cmm* was observed by **Chun (1982).** However, in the studies carried out by **Hadas *et al.* (2005)**, one of the *Cmm*-strains did not grow on D2ANX medium and other *Cmm* strains grew with very low plating efficiency. Target and non-target bacteria were detected using both SCM and D2ANX media and most of the colonies on both the selective media were indeed C. *michiganensis* subsp. *michiganensis*. Similar results have been obtained by **Ftayeh (2010).**

4.5. Studies on variability of different isolates of *Clavibacter michiganensis* subsp *michiganensis*

4.5.1 Cultural variability of different isolates of *Cmm*

On NGY medium the bacterial colonies of most of the isolates were small, 1-4 mm in diameter and developed within 72-96 hr from the day of inoculation. The bacterial colonies were light yellow, orange, round and semifluidal (Plate- 5). Colonies become deeper yellow and glistening with longer period of incubation (Table- 4.3). The

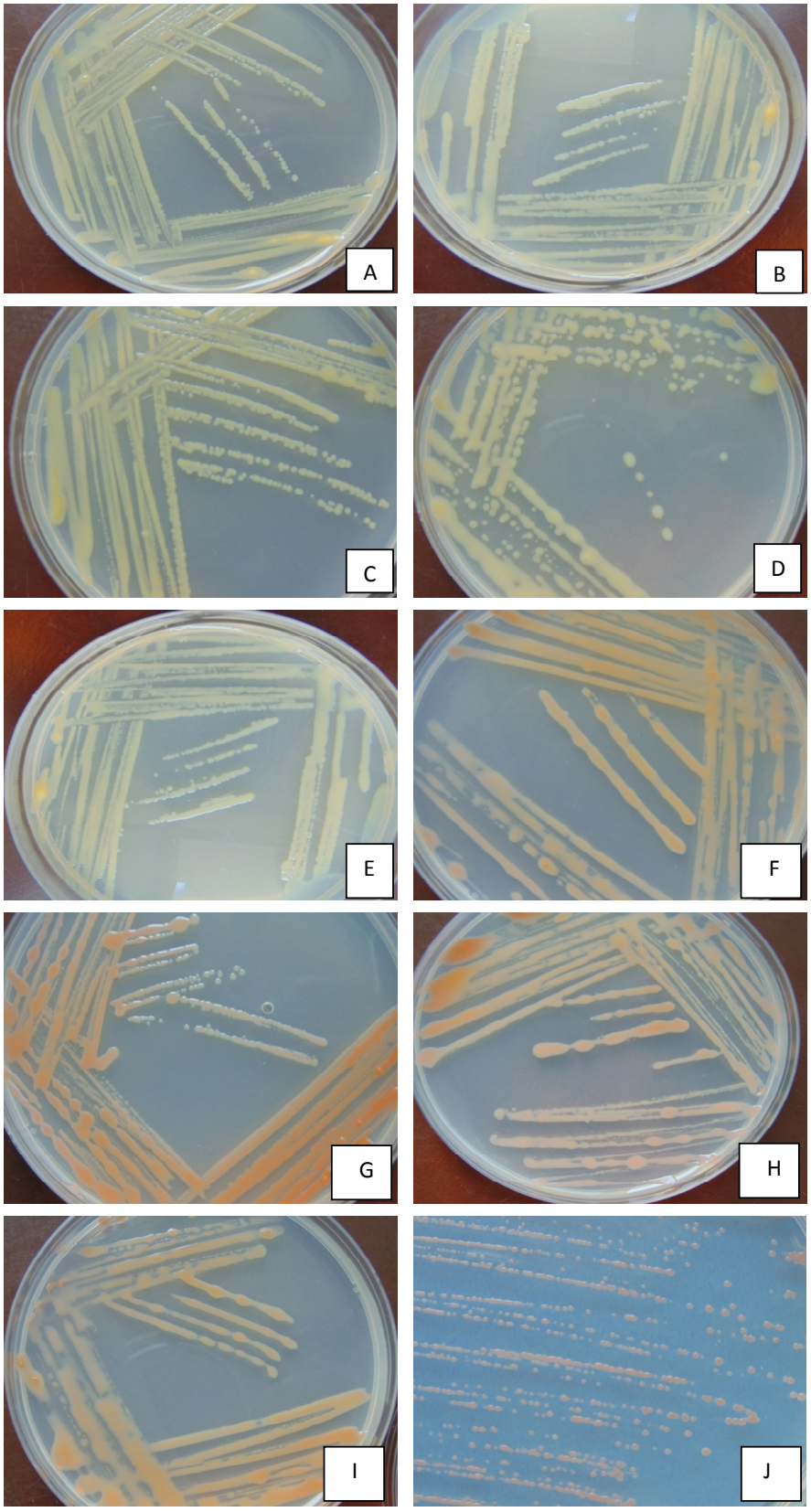


Plate 5: Cultural variability of different isolates (*Cmm*1-*Cmm*10) of the bacterium

colony color of the bacterial isolates *Cmm*1, 3, 4, 6 and 8 was yellow and that of isolate *Cmm*2 was creamish white to yellow while it was orange in isolate *Cmm5,* 7, 9 and 10.The shape of colonies was concave to dome shaped in case of all the isolates.

Table 4.3 Colony characteristics of different isolates of bacterial canker pathogen on nutrient agar glucose yeast medium.

S.No.	Isolates	Colony characteristics		
		Color	Shape	Size (mm)
A	*Cmm1*	Yellow	Round, mucoid	1-2
B	*Cmm2*	Creamish white to yellow	Round	1-3
C	*Cmm3*	Yellow	Circular, fluidal	1-3
D	*Cmm4*	Yellow	Circular	2-4
E	*Cmm5*	Yellow	Round, mucoid	2-3
F	*Cmm6*	Orange	Round	1-3
G	*Cmm7*	Orange	Round	1-3
H	*Cmm8*	Orange	Circular, fluidal	2-4
I	*Cmm9*	Orange	Circular	2-3
J	*Cmm10*	Orange	Round, mucoid	1-3

4.5.2 Pathogenic variability of different isolates of *Cmm*

The ten of the bacterial isolates were inoculated into the cultivar "A. Vikas" by the method of syringe inoculation at the nodal point of true leaf emergence and the observation for disease rating was taken as described below:

Evaluation of disease appearance and development was determined using a 0- 5 arbitrary scale (**Soyulu *et al.,* 2003**). Ratings were as follows:

0 no leaves showing wilting;

1 slight marginal wilting, 1- 10% of leaves with wilt;

2 11- 25% of leaves with wilt;

3 sectored wilting, 26- 49% of leaves showing wilting associated with chlorosis;

4 pronounced leaf collapse, 50- 74% of leaves showing wilting;

5 whole leaf wilted.

Table 4.4: Pathogenicity test of different isolates of the bacterial canker pathogen on tomato seedlings

Isolates	Method of inoculation	Disease scoring (Days after inoculation)							
		0	**6**	**12**	**18**	**24**	**30**	**36**	**42**
Cmm1	Stem inoculation	0	0	1	2	3	3	3	4
Cmm2	Stem inoculation	0	0	1	2	3	4	4	4
Cmm3	Stem inoculation	0	0	1	2	3	3	4	4
Cmm4	Stem inoculation	0	0	0	1	2	3	3	3
Cmm5	Stem inoculation	0	1	2	3	3	4	4	4
Cmm6	Stem inoculation	0	1	2	3	4	4	4	4
Cmm7	Stem inoculation	0	0	0	1	2	2	3	3
Cmm8	Stem inoculation	0	0	1	1	2	2	2	2
Cmm9	Stem inoculation	0	0	1	1	2	3	3	3
Cmm10	Stem inoculation	0	3	4	5	5	5	5	5

Artificial inoculation of the tomato seedlings with different isolates revealed that the seedlings inoculated with isolates *Cmm*5, *Cmm* 6 and *Cmm* 10 showed first symptom on leaf as marginal necrosis and unilateral wilting on 6th day of inoculation. Isolates, Cmm1, *Cmm*2, *Cmm*3, *Cmm* 8and *Cmm*9 showed symptoms on leaf on 12th day of inoculation, while isolates *Cmm* 4 and *Cmm* 7 showed symptoms on 18th day of inoculation. Isolate *Cmm* 10 was the most virulent which caused complete plant death within 18th day of inoculation, while isolate *Cmm* 8 was observed to be the least virulent amongst the all 10 isolates and showed significant symptoms on 5th week after inoculation. Isolate *Cmm* 10 which show the signs of the most virulent isolate was further taken up for glasshouse and open field studies. The scoring for disease severity was started since the period of inoculation up to a period of 7 weeks.

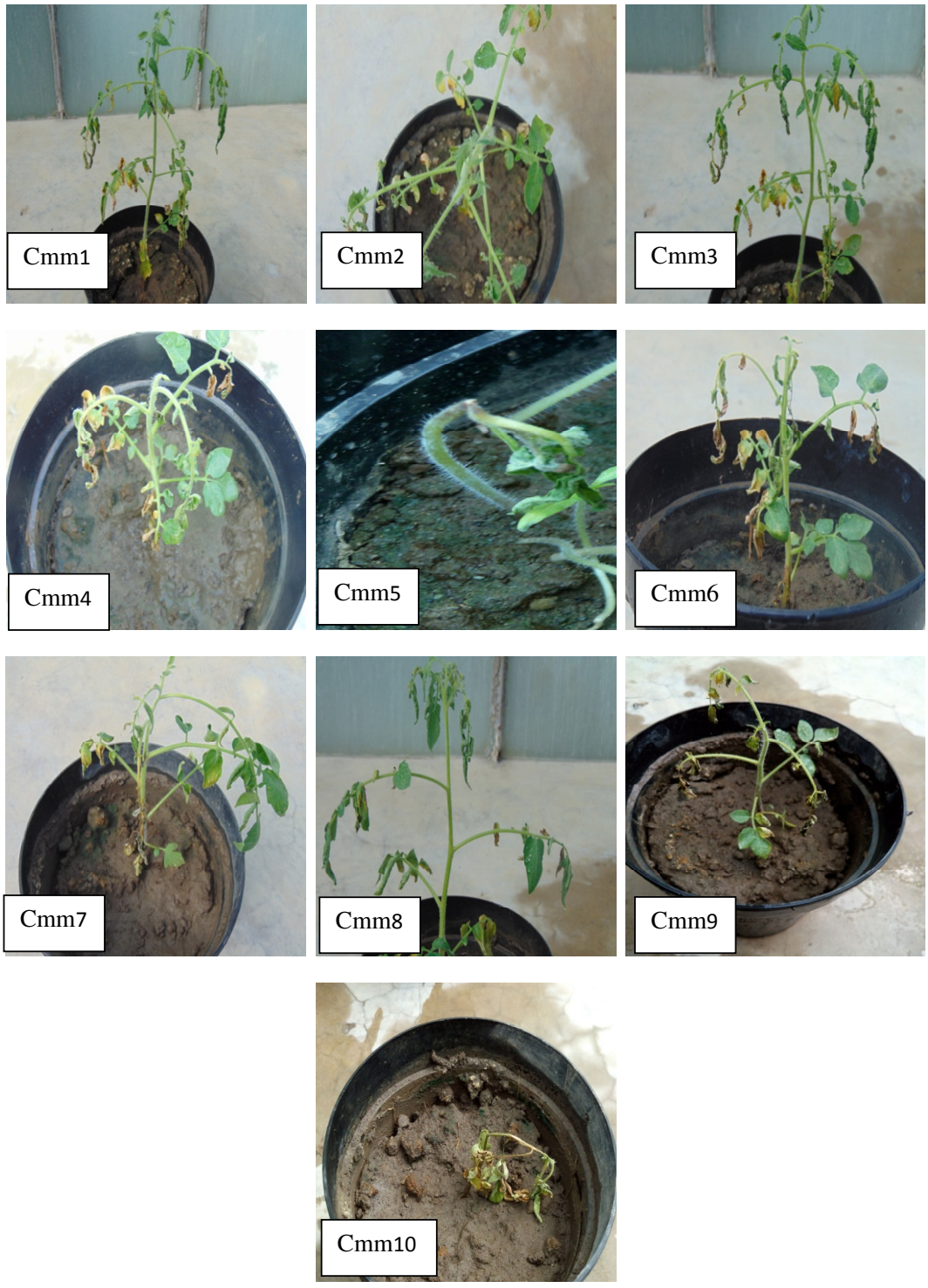


Plate 6: Pathogenic variability of different isolates of the pathogen on variety Arka Vikas

4.5.3. Studies on growth of bacterium in different temperature conditions

For the purpose of identification of the optimum temperature for the growth of *Cmm* the six virulent bacterial isolates *Cmm* (1, 3, 5, 6, 8, 10), were subjected to the following range of temperature.

Table 4.5: Growth of bacterium in different temperature conditions

Isolates	OD value (660nm) of bacterial isolates at different temperature						
	10ºC	**15ºC**	**20ºC**	**25ºC**	**30ºC**	**35ºC**	**45ºC**
Cmm1	0.480	0.946	1.163	1.253	1.280	0.956	0.153
Cmm3	0.946	1.053	1.263	1.336	1.263	1.056	0.190
Cmm5	0.550	0.940	1.140	1.233	1.160	0.850	0.250
Cmm6	0.950	1.146	1.263	1.350	1.256	1.153	0.273
Cmm8	0.450	0.950	1.070	1.176	1.130	0.970	0.970
Cmm10	0.946	1.160	1.256	1.353	1.256	0.940	0.160

CD at 5 %

a	0.0155
b	0.0168
a*b	0.0412
CV	2.625

The studies for observing the most suitable temperature for the growth and multiplication of the six virulent isolates of *Cmm* (1, 3, 5, 6, 8, 10) on a range of temperatures viz., 10ºC, 15ºC, 20º, 25ºC, 30ºC, 35ºC and 45ºC, temperature 25ºC was found to be the optimum temperature for the growth and multiplication of all the isolates (**Table 4.5).** The similar temperature has been reported to be suitable for the growth of the bacterium *Cmm* (EPPO**, 2016 bulletin).**

4.5.4. Studies on growth of bacterium at different pH conditions

For the purpose of identification of the optimum pH for the growth of *Cmm* the six virulent bacterial isolates *Cmm* (1, 3, 5, 6, 8, 10), were subjected to the following range of pH.

Table 4.6: Growth of bacterium in different pH conditions

Isolates	OD value(660nm) of bacterial isolates at different pH				
	4	**5**	**6**	**7**	**8**
Cmm1	0.070	0.80	1.34	1.47	1.26
Cmm3	0.046	0.14	0.81	1.26	0.95
Cmm5	0.083	0.45	1.16	1.34	1.04
Cmm6	0.07	0.36	1.28	1.35	1.16
Cmm8	0.04	0.25	1.24	1.32	1.15
Cmm10	0.073	0.45	1.36	1.43	1.24

CD at 5%

a	0.11
b	0.101
a*b	0.247
CV	13.67

The studies for observing the most suitable pH conditions for the bacterium were conducted on a range of pH viz., 4, 5, 6, 7 and 8 for the six most virulent isolates that are *Cmm*1, 3, 5, 6, 8, 10, Amongst the varied pH conditions observed for the growth of the bacterium pH 7 was found to be the optimum temperature for bacterial growth (Table 4.6). The results are in confirmation with the findings of **Singh *et al.* (2017).**

4.5.5. Host range screening

For the purpose of screening the virulence of the isolates on crops other than tomato, the host range screening for the pathogen was done with 5 solanaceous host and one non solanaceous host.

The study indicated that out of the six different plant species, five belonged to family Solanaceae; four crop host and one weed host while one plant species belongs to family Amaranthaceae, the disease symptom in form of cankerous lesion were observed eighteen days after planting only in the bell pepper stem, while on inoculating the fruit of bell pepper brown colored water discolored spots were formed and no symptoms

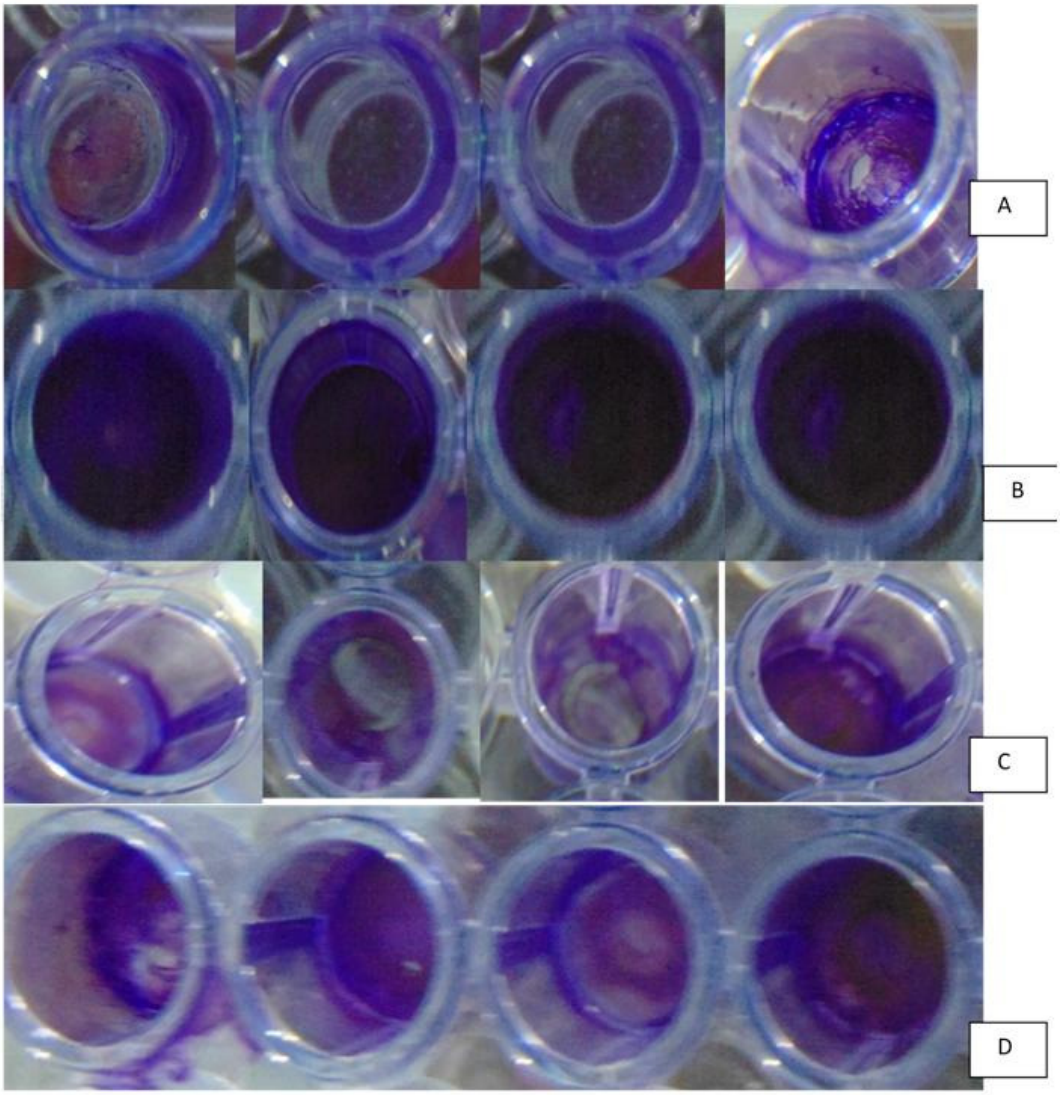


Plate 7: Biofilm formation by the bacterial isolates in different growth medium in the series of *Cmm* 10, *Cmm*6, *Cmm*5 and *Cmm*1.

A NGY medium; B Xylem sap

C Distil water; D M9 minimal medium

were produced in any other solanaceous host (Table 4.7). Very small lesion formation was also observed on the *C. album* plant only at the point of inoculation. The results were in confirmation with the findings of **Kyu *et al.,* 2006** where they observe similar results for bellpepper. However they also confirmed the infection by the pathogen in pepper plant, which was not confirmed in the present study, as no symptoms were seen in pepper plant.

Table 4.7: List of plant species used for studying the host range of *Cmm*

Treatment	Scientific name	Common name	Family	Variety	Disease scoring (Days after Planting)				
					6	12	18	24	30
*Cmm*10	*C. annum*	Bell pepper	Solanaceae	Indra	0	0	1	1	1
*Cmm*10	*C.annum*	Pepper	Solanaceae	P.C.1	0	0	0	0	0
*Cmm*10	*S.tuberosum*	Potato	Solanaceae	K.Jyoti	0	0	0	0	0
*Cmm*10	*S.melongena*	Brinjal	Solanaceae	P.Samrat	0	0	0	0	0
*Cmm*10	*C.album*	Bathua	Amaranthaceae	-	0	0	1	1	1
*Cmm*10	*S.nigrum*	Makoi	Solanaceae	-	0	0	0	0	0

4.6. Biofilm formation by *Cmm* on different media

The biofilm formation by the bacterial isolate *Cmm* (1, 5, 6, and 10) was carried out for the purpose of assessing the affinity of the bacterium towards the growth media screened. The biofilm formation was assessed in 4 different medium viz., the xylem sap of tomato plant, in the minimal medium (M9), in luria bertani broth and sterilized water was used as control. The three mediums were assayed for observing the attachment, aggregation, and biofilm formation by the four most virulent isolates by employing an in vitro assay for biofilm detection in which the effect of XS, LB, and minimal M9media were compared. *Cmm*5 isolate grown in XS developed an apparent biofilm that consisted of large aggregate of adherent cells followed by *Cmm* 10 and *Cmm* 6. The minimum growth in all the four medium was observed in the isolate Cmm 1 In contrast, cells grown in Sterilized water and minimal M9 medium formed a very weak biofilm with no signs of aggregation and those grown in LB showed only few aggregates distributed over the polystyrene well surface. Spectrophotometric

determination (OD590) of the adherent cells assayed by crystal violet staining showed that in Cmm 5 isolate biofilm formation in XS was 0.546 compared with 0.207, 0.198, and 0.109 in M9, LB media and DW, respectively. The results are in the confirmation of the findings of **Chalopowicz *et al.* (2011),** wherein the maximum biofilm formation was observed in the xylem sap of the tomato plant and least in the minimal medium.

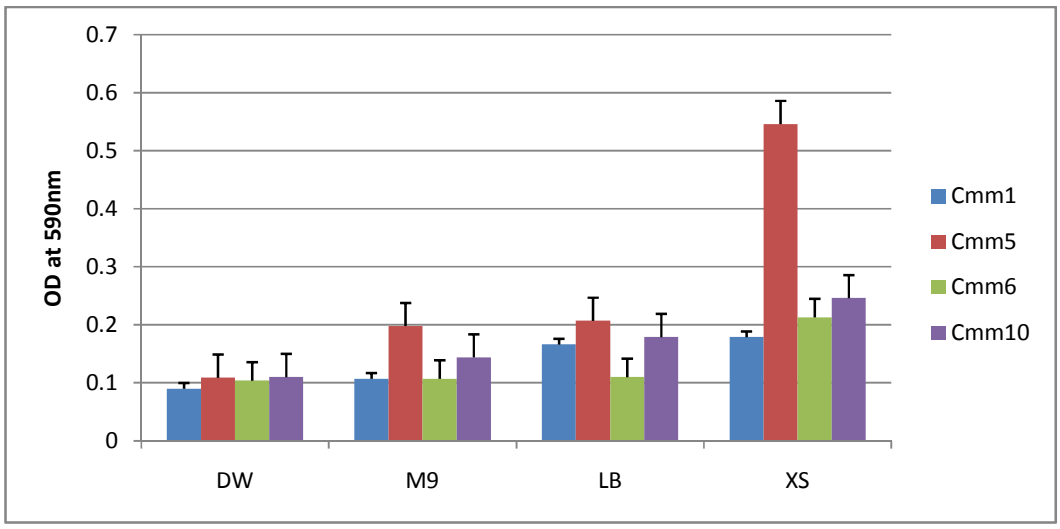


Fig. 3: Quantitative estimation of biofilm formation by the bacterial isolate *Cmm* (1, 5, 6, and 10) in different medium. Bars represent standard error ($p \leq 0.05$)

4.7. Effect of Different Inoculation Methods on Incubation Period of *Clavibacter michiganensis* subsp. *michiganensis* on Tomato Seedlings

To find out best artificial inoculation method for further studies on bacterial canker such as germplasm screening etc., different inoculation methods were tested on young tomato seedlings. The data on symptoms expression/ incubation period was recorded at periodic intervals after each inoculation method and presented in Table 4.8.

Evaluation of disease appearance and development was determined using a 0- 5 arbitrary scale (**Soyulu *et al.*, 2003**). Ratings were as follows:

0 no leaves showing wilting;

1 slight marginal wilting, 1- 10% of leaves with wilt;

2 11- 25% of leaves with wilt;

3 sectored wilting, 26- 49% of leaves showing wilting associated with chlorosis;

4 pronounced leaf collapse, 50- 74% of leaves showing wilting;

5 whole leaf wilted.

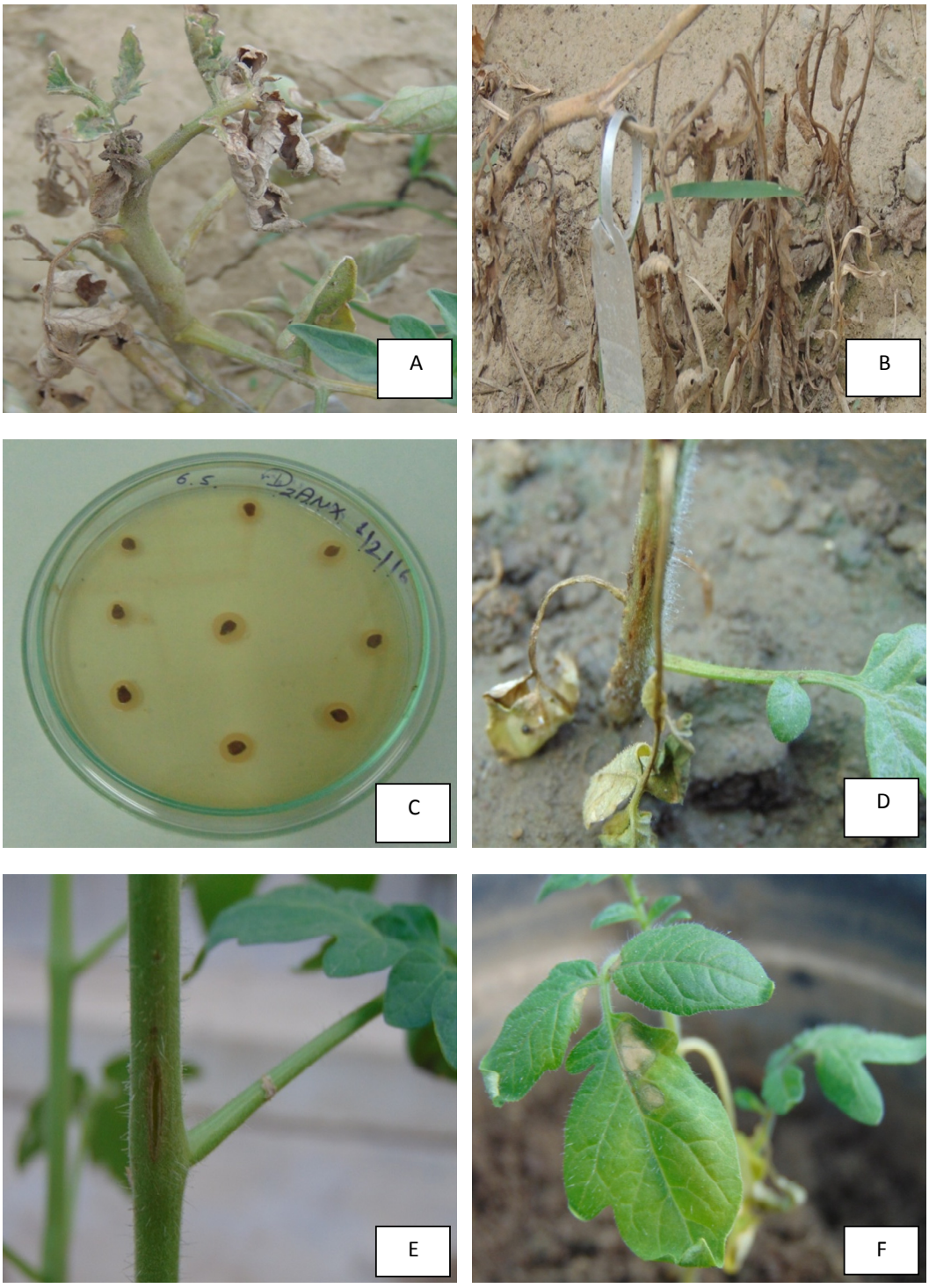


Plate 8: Different inoculation methods and their effect on the *Cmm* 10 isolate incubation period

Table 4.8 Effect of different inoculation methods on incubation period of *Cmm* 10 isolate *on* tomato seedlings

Types of inoculation	Disease Rating (days after inoculation)							
	0	**6**	**12**	**18**	**24**	**30**	**36**	**42**
Seed inoculation	0	2	3	3	3	3	3	3
Syringe inoculation of stem	0	0	1	2	2	3	4	4
Syringe inoculation of leaves	0	0	1	1	1	2	2	2
Toothpick inoculation of stem	0	0	0	1	2	2	3	4
Foliar spray of leaves and stem inoculation	0	1	2	2	3	4	4	5
Syringe inoculation of peduncles	0	0	1	1	2	2	3	3

Persual of the data in Table 4.8, the symptoms were noticed in tomato seeds 5th days after germination, in the tomato seedlings at 8th days of inoculation with syringe inoculation at the junction of first true leaf, on 10th days after inoculation in clipping and stem inoculation with toothpick. Whereas, the symptoms appeared on 11th days of inoculation with foliar spray and syringe inoculation of stem. Syringe inoculation of stem at junction of first true leaf and stem inoculation with toothpick and were adjudged as good methods of artificial inoculation of bacterial canker pathogen *C. michiganensis* subsp. *michiganensis* to the tomato seedlings. The results obtained were in confirmation to the findings of **Singh *et al.* (2017).**

Inoculation methods used:

- A Syringe inoculation of peduncles
- B Foliar spray of leaves and stem inoculation
- C Seed inoculation
- D Toothpick inoculation of stem

- E Syringe inoculation of stem
- F Syringe inoculation of leaves

4.8. Serological Characterization

ELISA technique posses an effective quality for the detection of presence of bacterial isolates. Indirect ELISA testing method was used for the characterization of the bacterial isolates.

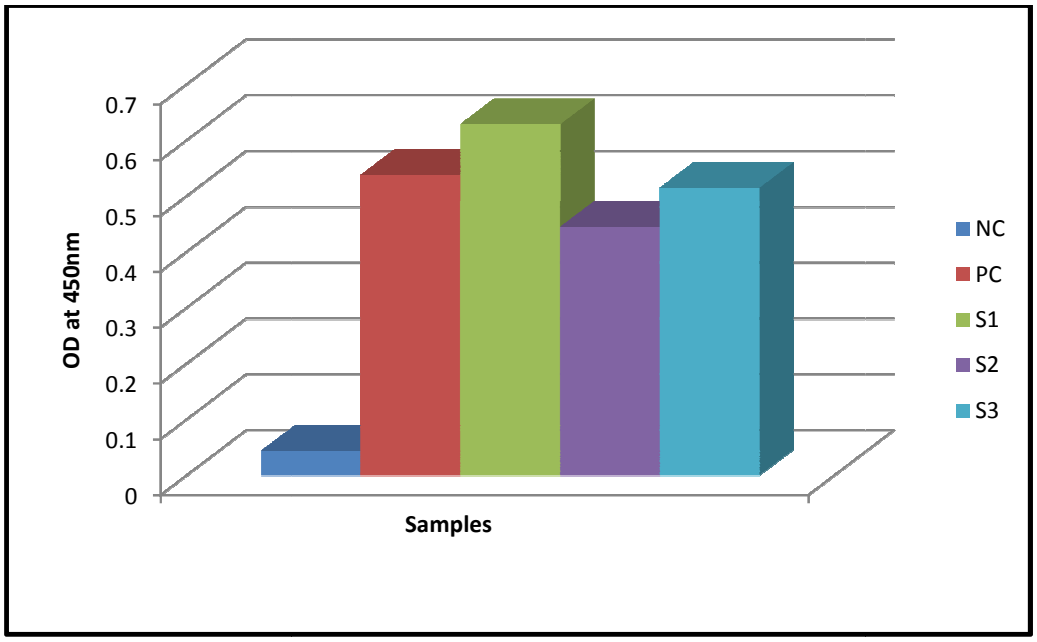


Fig.4: Serological characterization of *Cmm* 10, 6, 5 Isolates

All the three suspected bacterial isolates depicted an OD value close to the positive reference. The OD value was significantly lower in the negative control. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect ELISA technique.. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect ELISA technique. The value of positive control was 0.538nm, while that of *Cmm*10 was 0.629nm; *Cmm*6 was 0.445 and of *Cmm*5 was 0.515. The OD value of negative control was 0.044.

Indirect Elisa testing using MAb Cmm1 antibody was found to be specific for *Cmm* in initial assays (**Alvarez *et al.*, 1993**) The antibody reacted to 99% of 236 *Cmm* strains corresponding to the bacterial population around the globe. MAb Cmm1 was found to be unreactive to several bacteria from seed assays that did not possess *Cmm*-type morphology on semi-selective media. The precise detection nature of Cmm1 MAb was identified by screening 236 *Cmm* strains from varied geographical areas using indirect ELISA. MAb Cmm1 was found to be unreactive with *Cm* subsp. *insidiosus*, *Curtobacterium flaccumfaciens*, *Cf* subsp. *poinsettiae*, *Rathayibacter*

rathayi or *Rhodococcus fascians* (**Kaneshiro *et al.*, 2006**).

4.9. Molecular Characterization

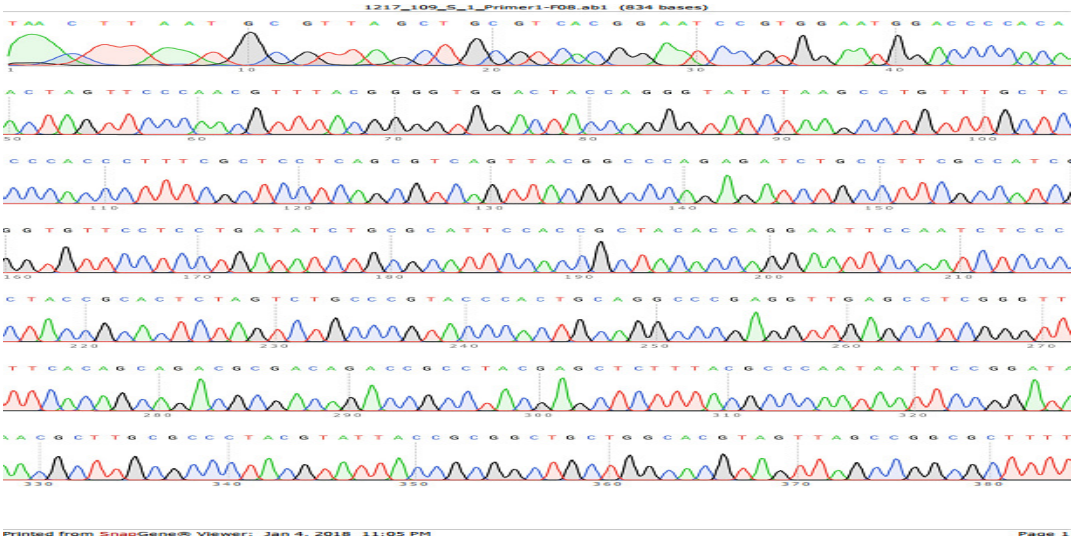
4.9.1. Molecular Identification of the isolates by 16s ribosomal RNA (rDNA) sequencing

The bacterial isolates obtained from two different region of Himachal Pradesh and one region at Uttarakhand were subjected to 16s ribosomal RNA (rDNA) sequencing by universal primer 27f and 1492r, and the results obtained are as follow.

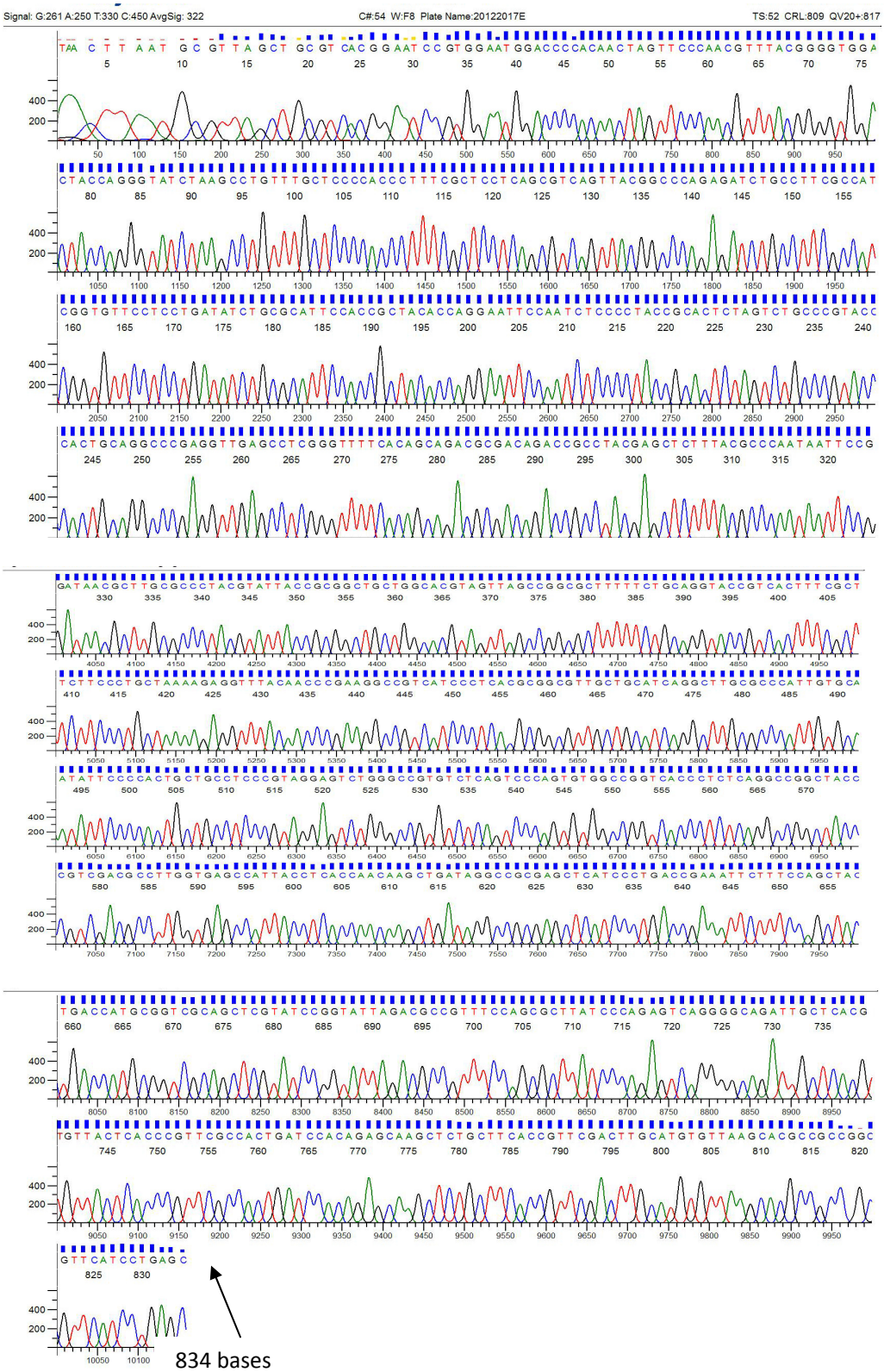
Isolate 1.

a) Chromatogram data Primer forward

Amplicon size: 834 bases



b) Electrophoretogram data primer forward



c) Megablast analysis result output

Sequence Aligned

Microbacterium sp. G1Dc3 16S ribosomal RNA gene, partial sequence

```
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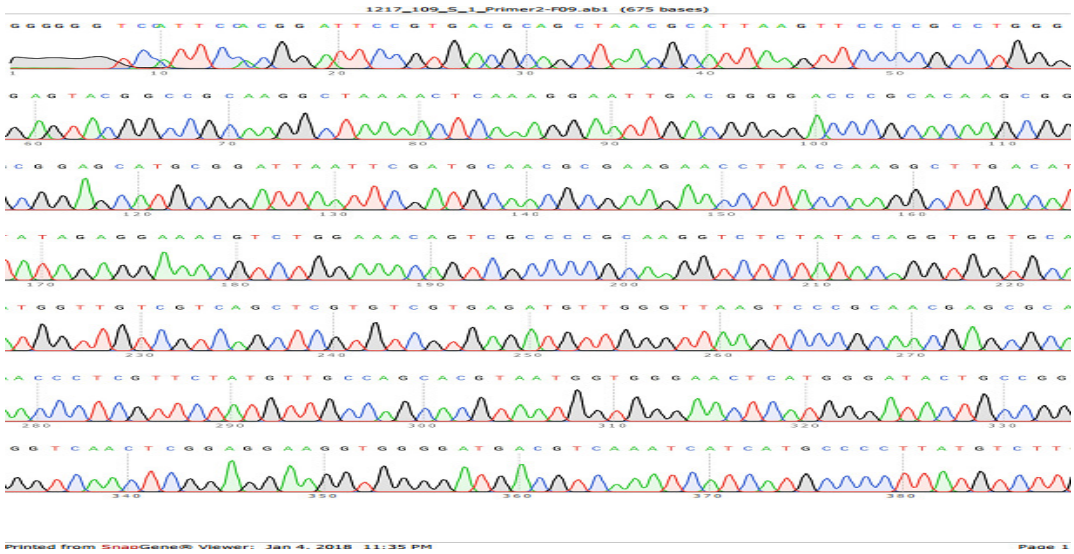
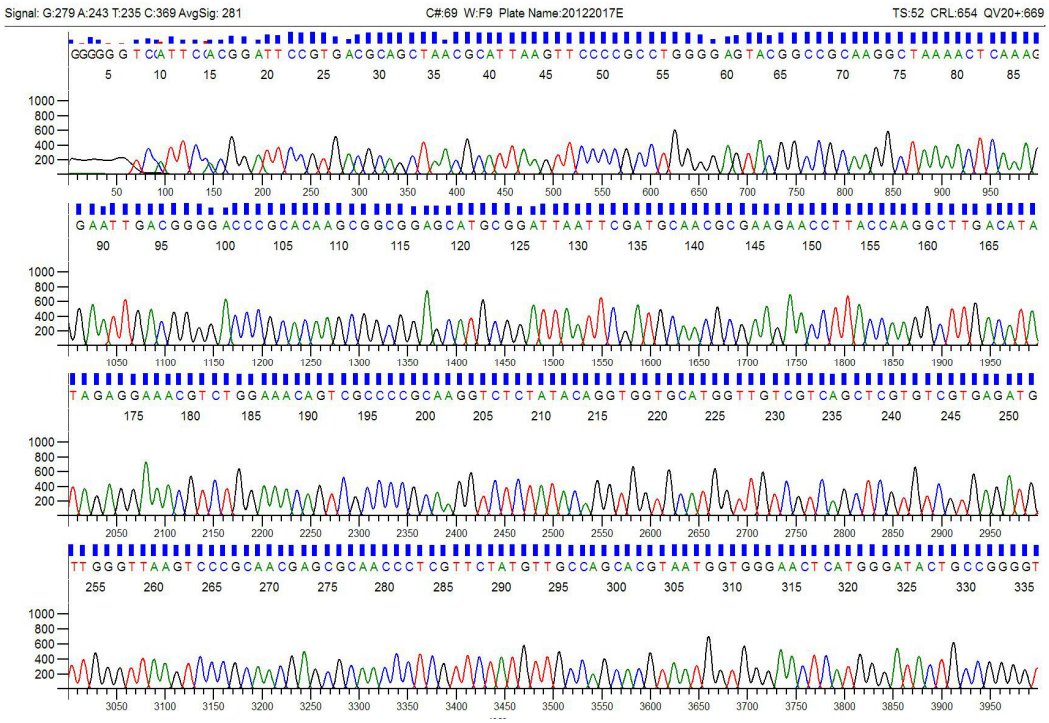
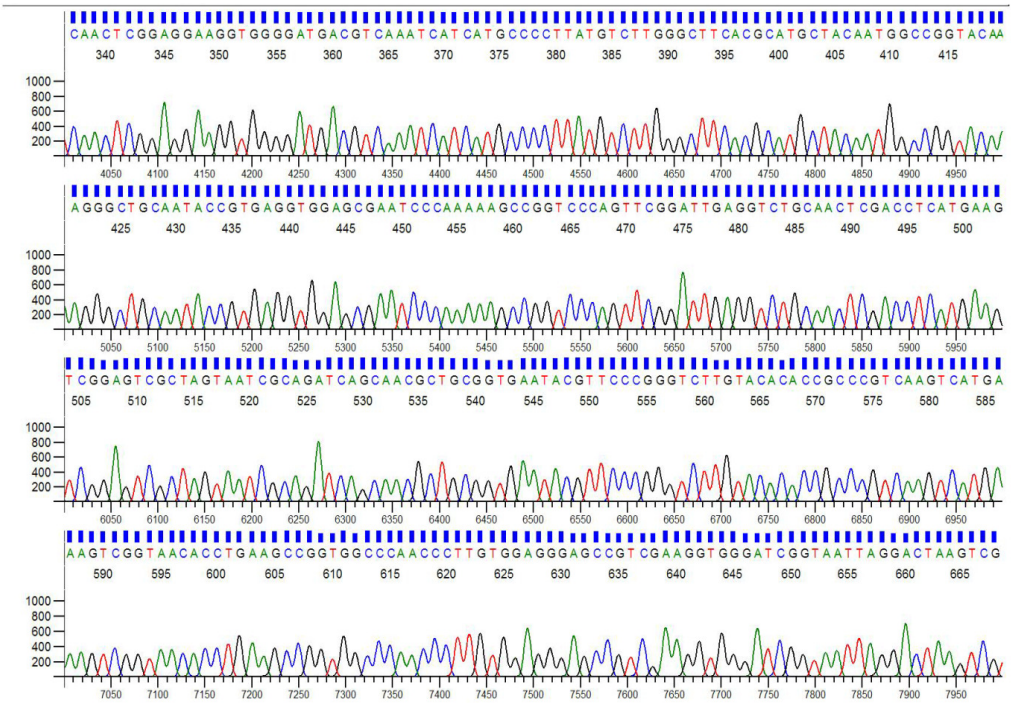
a) Chromatogram data Primer reverse

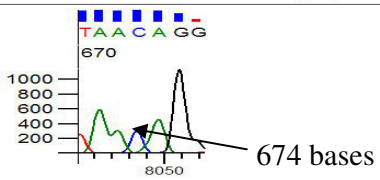
Amplicon size: 675 bases



b) Electrophoretogram data Primer reverse

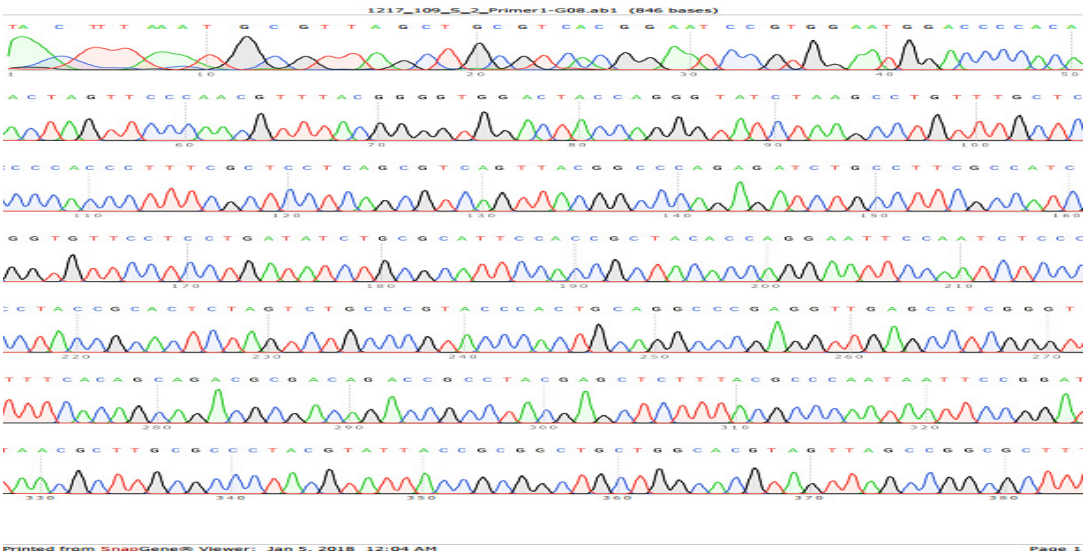






Isolate2

a) Chromatogram data primerF: 846bases



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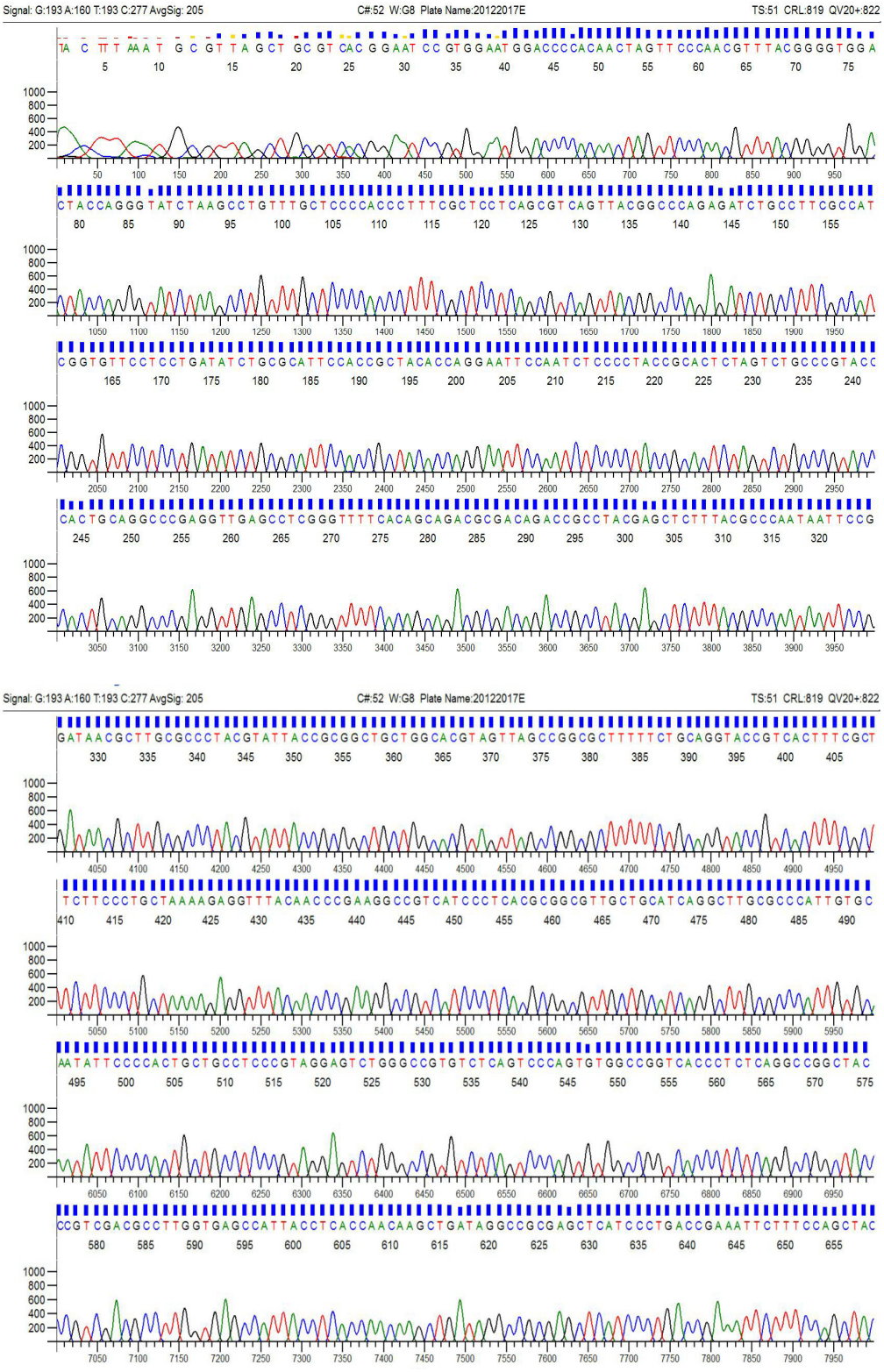

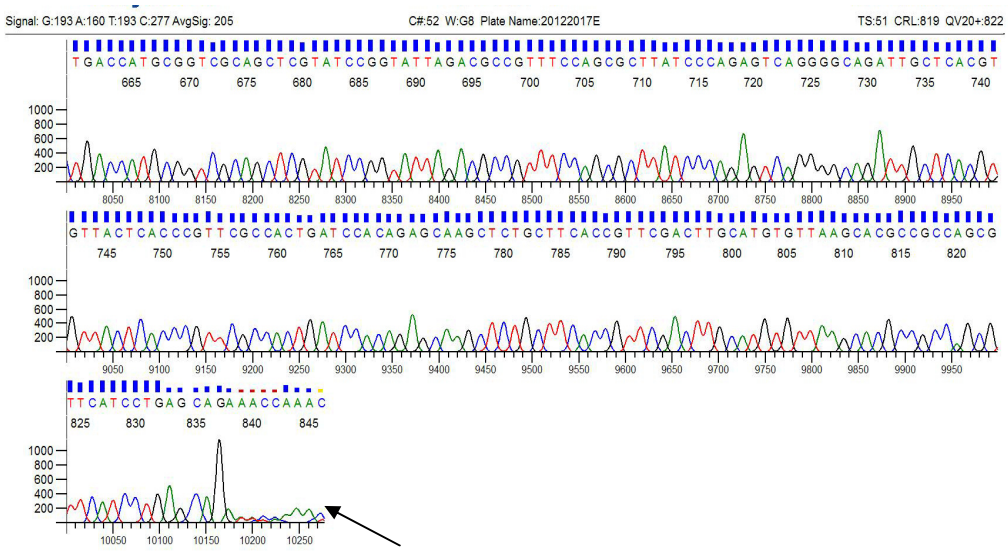

c) Megablast analysis output (Aligned sequence)

Microbacterium sp. G1Dc3 16S ribosomal RNA gene, partial sequence

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AATGCGTTAGCTGCGTCACGGAATCCGTGGAATGGACCCCACAACTAGTTCCCAACGTTT

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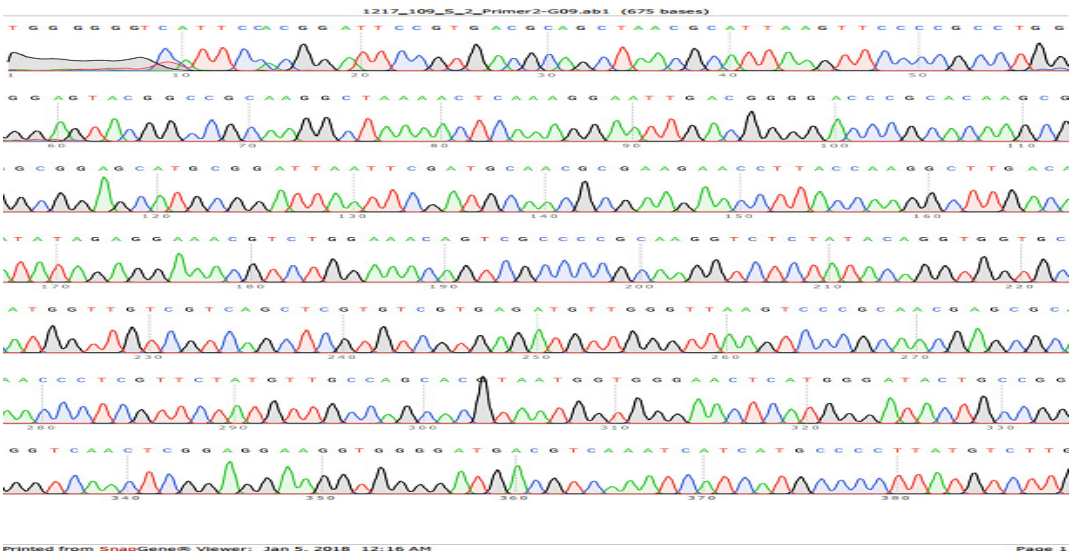
a) Chromatogram data primer R : 675 bases



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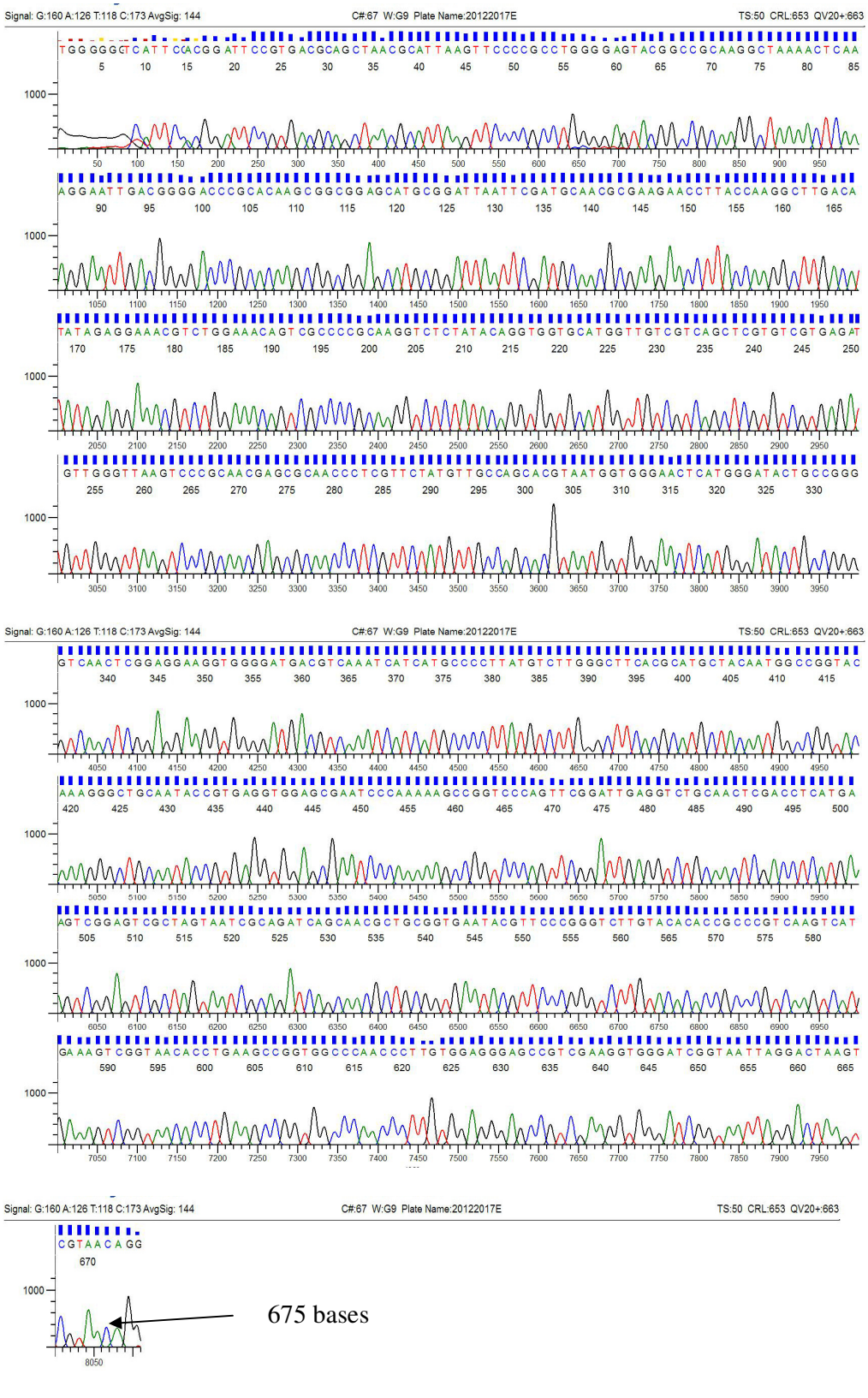

Isolate 3

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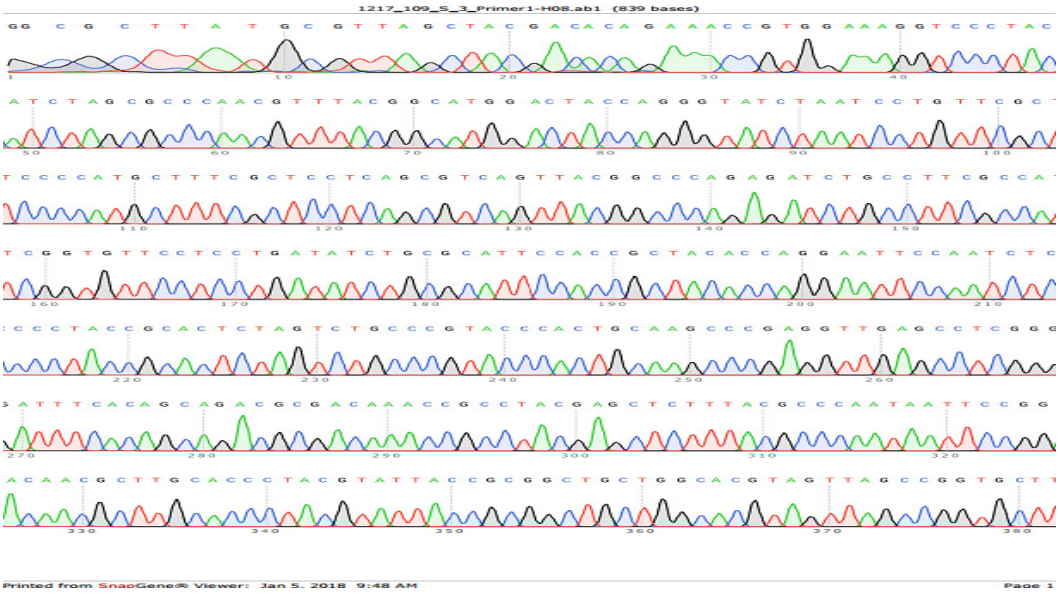
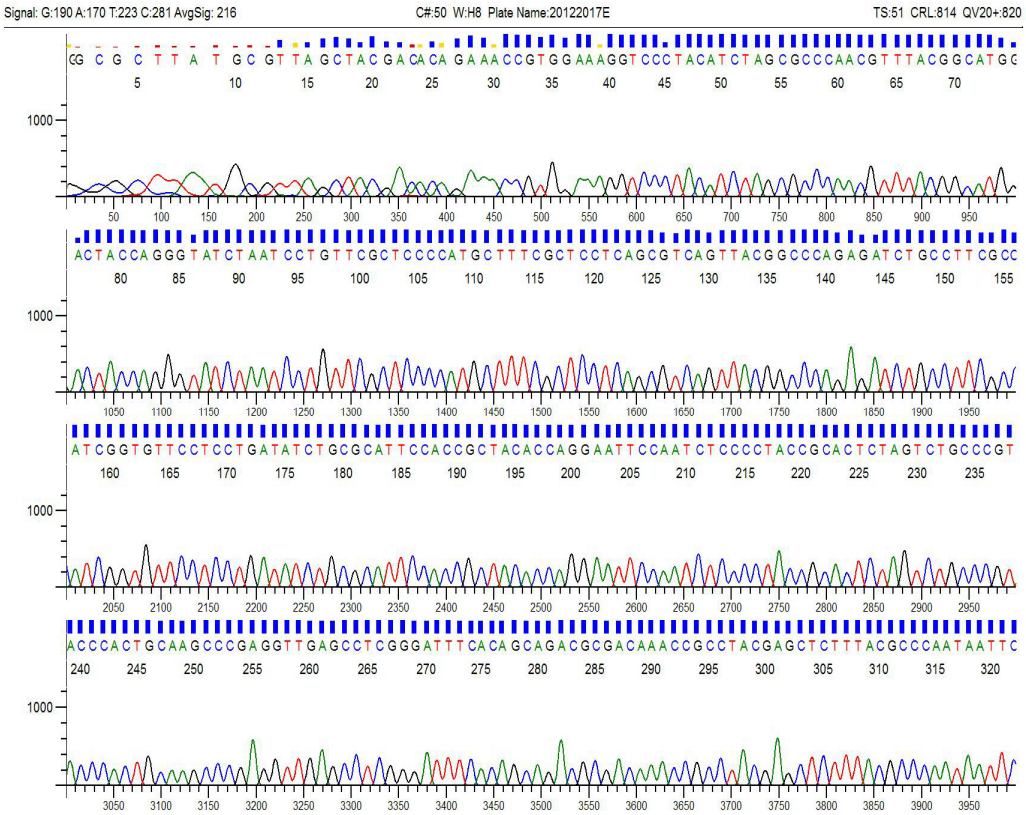
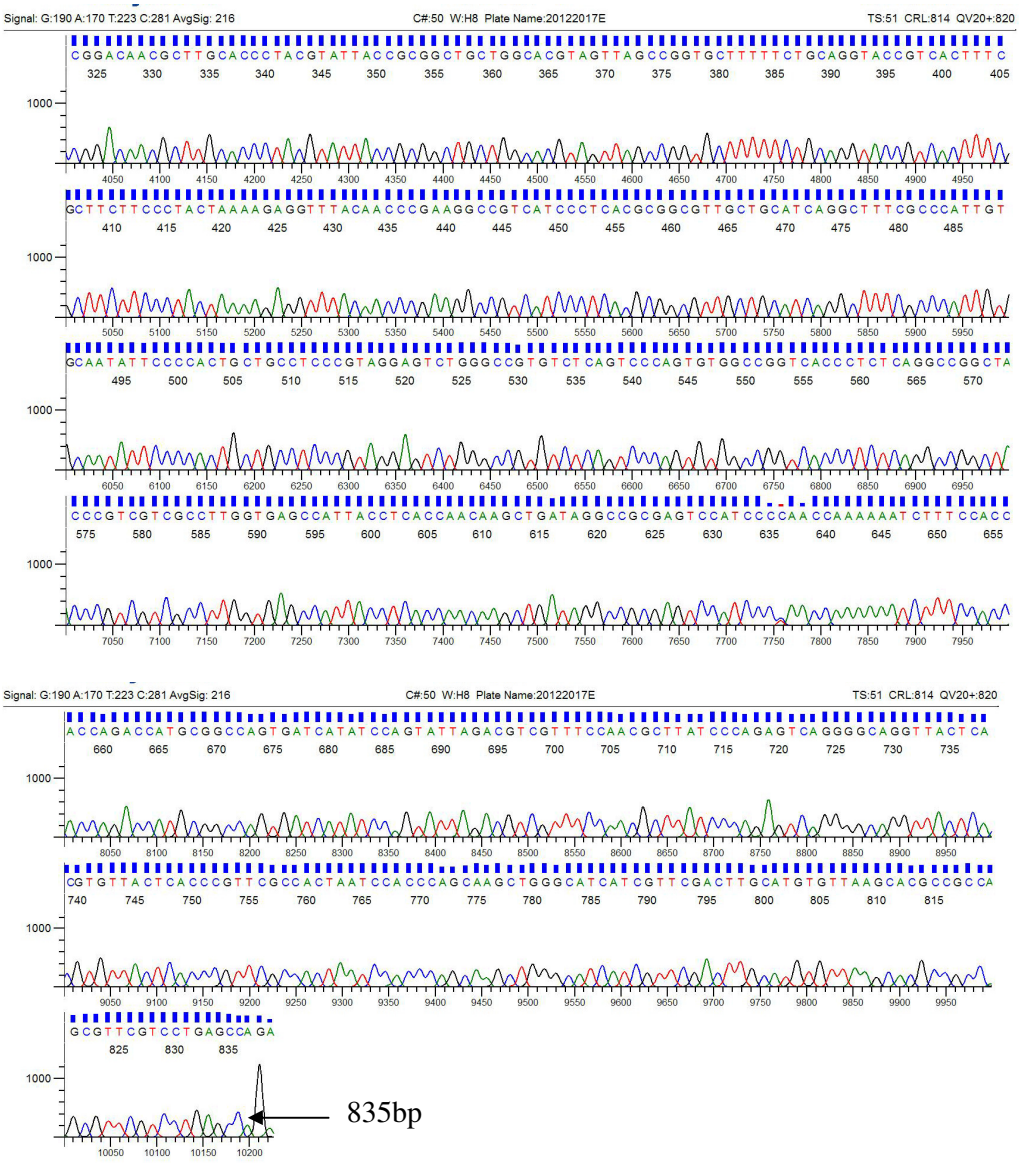
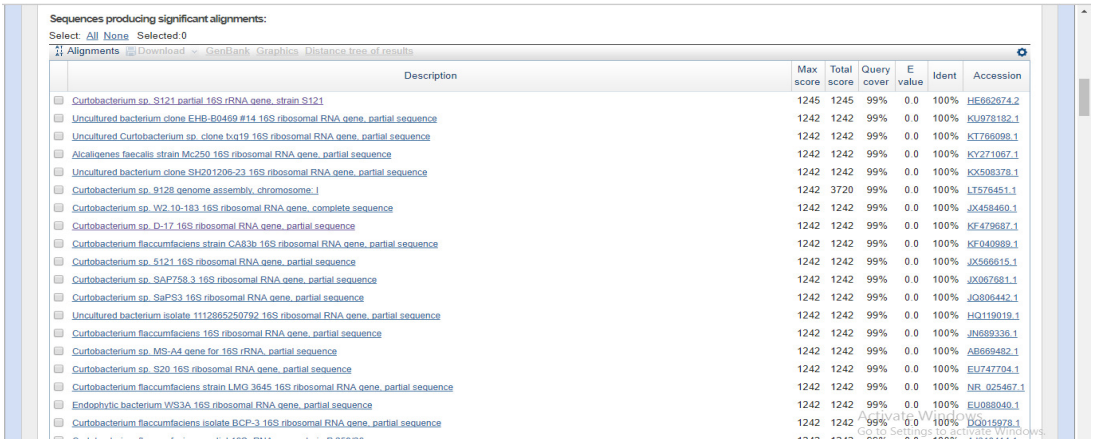


b) Electrophoretogram data primer F: 835bp





c) Output of MegaBlast analysis Forward primer



Sequences producing significant alignments:

Select: All None Selected:0

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
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Uncultured bacterium clone EHB-B0469 #14 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KU978182.1
Uncultured Curtobacterium sp. clone txg19 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KT766098.1
Alcaligenes faecalis strain Mc250 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KY271067.1
Uncultured bacterium clone SH201206-23 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KX508378.1
Curtobacterium sp. 9128 genome assembly, chromosome: I	1242	3720	99%	0.0	100%	LT576451.1
Curtobacterium sp. W2.10-183 16S ribosomal RNA gene, complete sequence	1242	1242	99%	0.0	100%	JX458460.1
Curtobacterium sp. D-17 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KF479687.1
Curtobacterium flaccumfaciens strain CA83b 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KF040989.1
Curtobacterium sp. 5121 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JX566615.1
Curtobacterium sp. SAP758.3 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JX067681.1
Curtobacterium sp. SaPS3 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JQ806442.1
Uncultured bacterium isolate 1112865250792 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	HQ119019.1
Curtobacterium flaccumfaciens 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JN689336.1
Curtobacterium sp. MS-A4 gene for 16S rRNA, partial sequence	1242	1242	99%	0.0	100%	AB669482.1
Curtobacterium sp. S20 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	EU747704.1
Curtobacterium flaccumfaciens strain LMG 3645 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	NR_025467.1
Endophytic bacterium WS3A 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	EU088040.1
Curtobacterium flaccumfaciens isolate BCP-3 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	DQ015978.1

Activate Windows
Go to Settings to activate Windows.

Aligned sequence

***Curtobacterium* sp. D-17 16S ribosomal RNA gene, partial sequence**

GGCGCTT-ATGCGTTAGCTACGACACAGAAACCGTGGAAAGGTCCCTACATCTAGCGCCC
GGCGCTTAATGCGTTAGCTACGACACAGAAACCGTGGAAAGGTCCCTACATCTAGCGCCC
AACGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTC
AACGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCATGCTTTCGCTC
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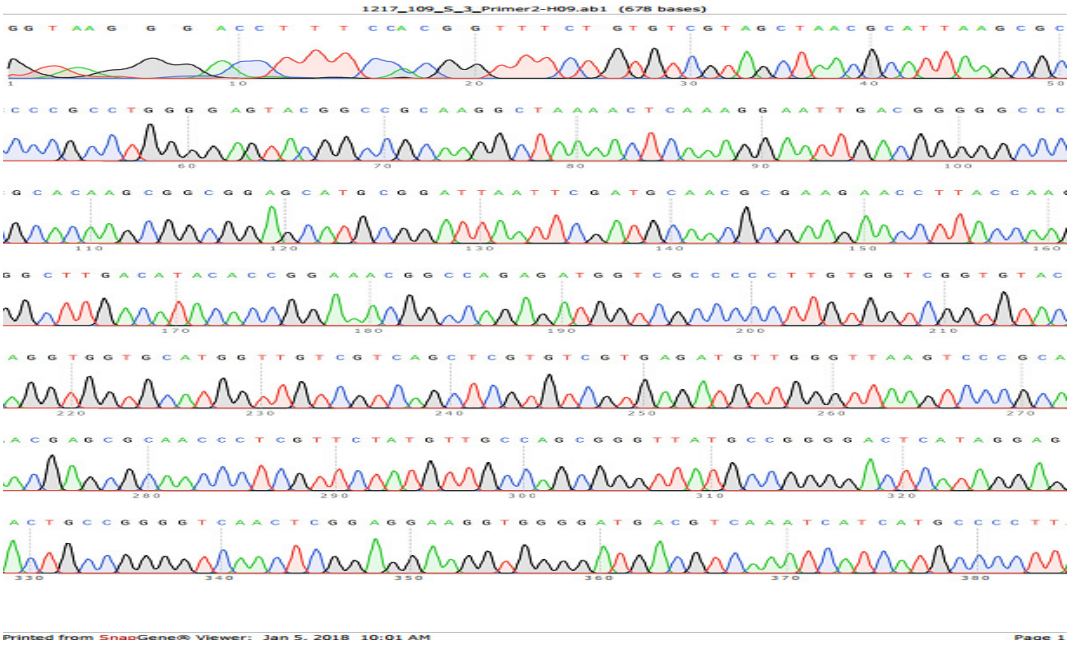
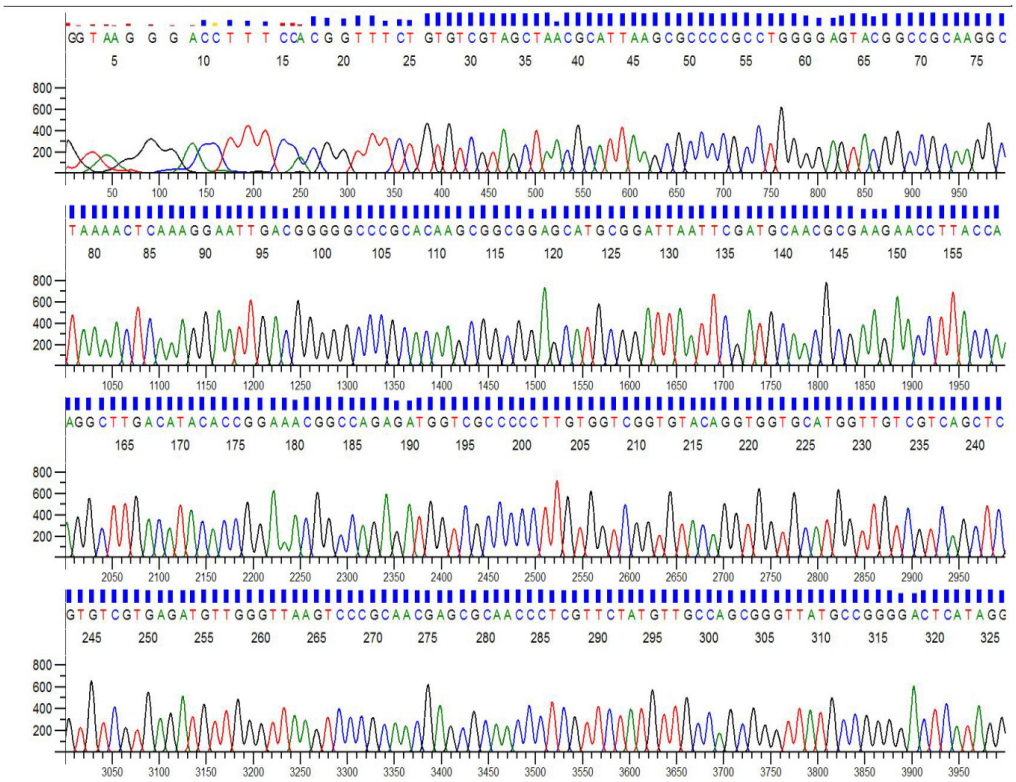
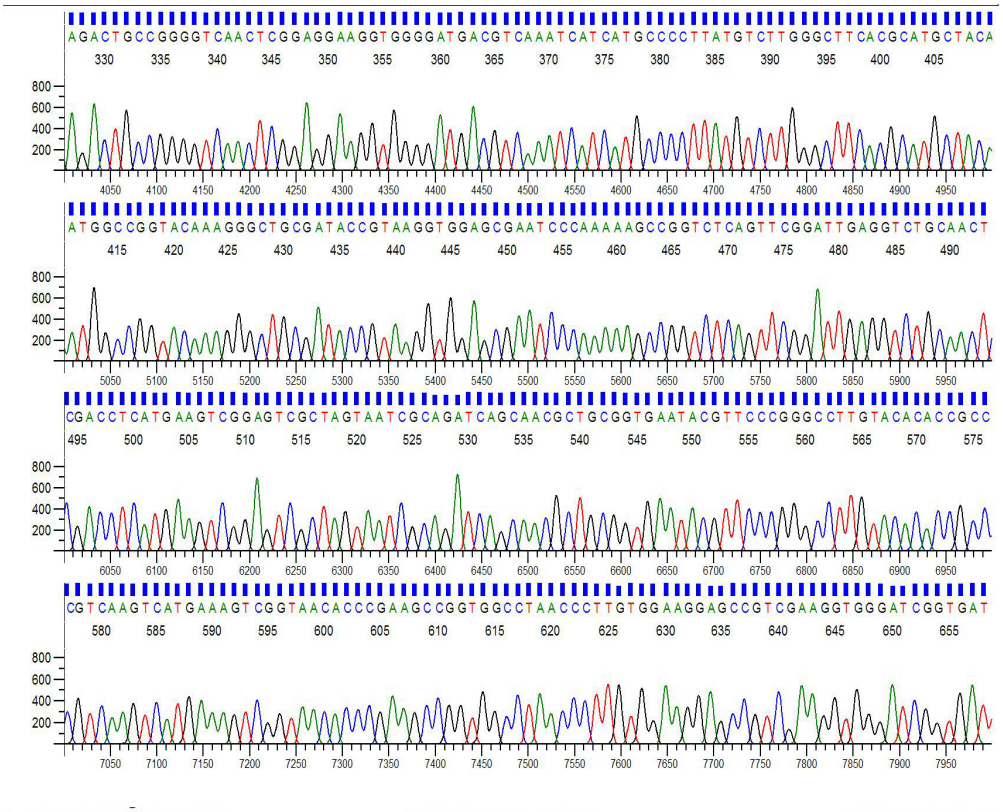
GGGCATCATCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCTGAGCCAG
GGGCATCATCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGTTCGTCCTGAGCCAG

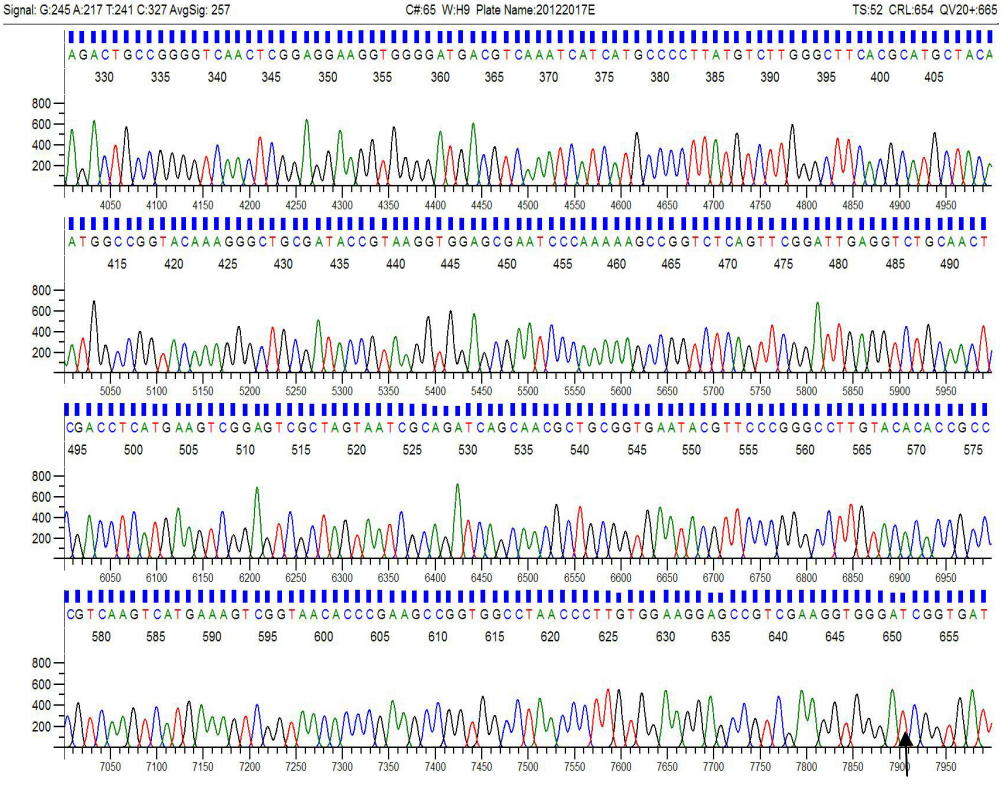
a) Chromatogram data primer R



b) **Electrophoretogram data primer R:** 652bp






Signal: G:245 A:217 T:241 C:327 AvgSig: 257
C#:65 W:H9 Plate Name:20122017E
TS:52 CRL:654 QV20+:665

652 bp

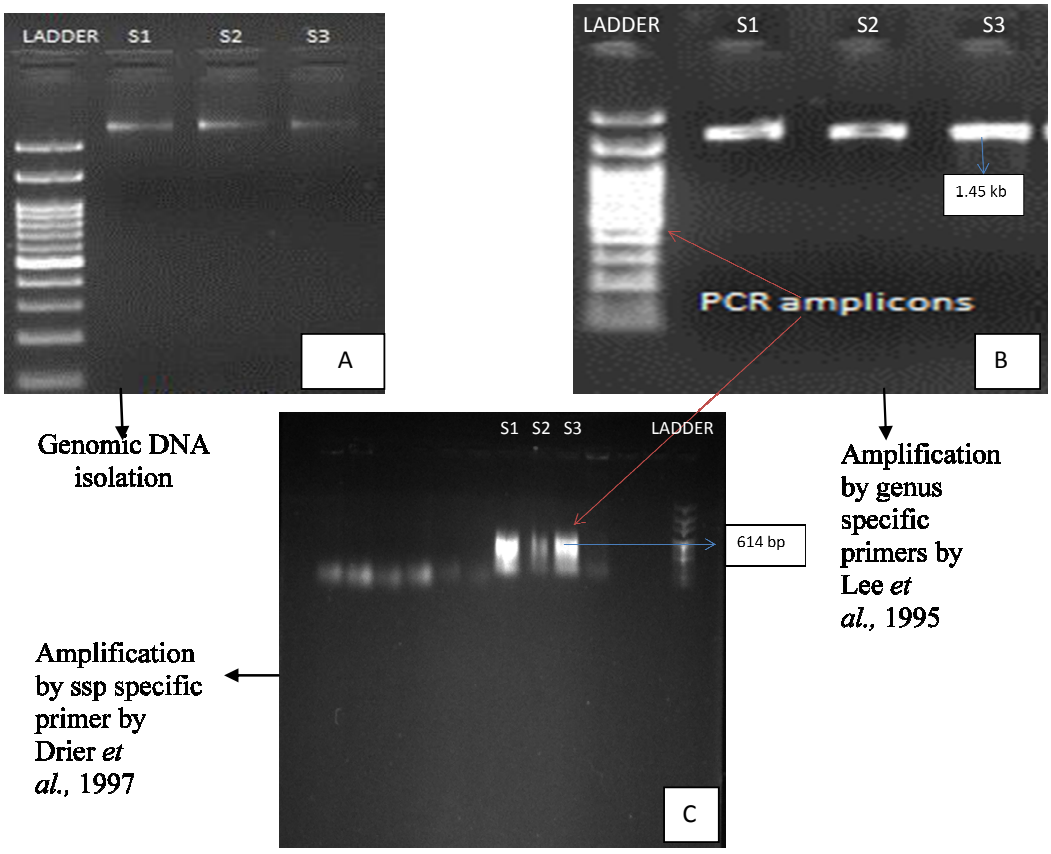
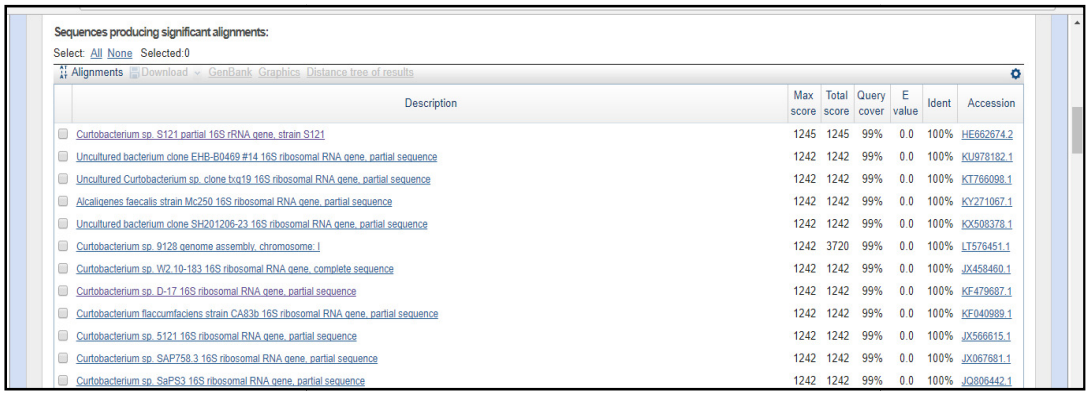


Plate 9: Molecular Characterization of the *Cmm* 5 (S1), 6 (S2), 10 (S3) isolate

c) Megablast analysis output



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Curtobacterium sp. S121 partial 16S rRNA gene, strain S121	1245	1245	99%	0.0	100%	HE662674.2
Uncultured bacterium clone EHB-B0469 #14 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KU978182.1
Uncultured Curtobacterium sp. clone txq19 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KT766098.1
Alcaligenes faecalis strain Mc250 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KY271067.1
Uncultured bacterium clone SH201206-23 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KX508378.1
Curtobacterium sp. 9128 genome assembly, chromosome: I	1242	3720	99%	0.0	100%	LT576451.1
Curtobacterium sp. W2.10-183 16S ribosomal RNA gene, complete sequence	1242	1242	99%	0.0	100%	JX458460.1
Curtobacterium sp. D-17 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	KF479687.1
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Curtobacterium sp. SAP758.3 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JX067681.1
Curtobacterium sp. SaPS3 16S ribosomal RNA gene, partial sequence	1242	1242	99%	0.0	100%	JQ806442.1

Subjecting the three bacterial isolates to 16s ribosomal DNA (rRNA) sequencing by universal primer 27f (59-AGAGTTTGATCCTGGCTCAG-39) and 1492r (59-GGTTACCTTGTTACGACTT-39) (Lane *et al.,* 1985), Forward and reverse nucleotide sequences were edited, assembled, translated, and aligned. 16S rRNA gene sequence data were compared to those referenced in the NCBI database by using BLAST (database of November 2016) with default parameters. The bacterial isolates showed closest belonging to *Microbacterium* sp. and *Curtobacterium* sp. The similar results have been observed in the study conducted by **Chen *et al.,* 2007**and showed a correlation between the genus *Curtobacterium* and genus *Clavibacter*. A high degree of similarity between the genera *Aureobacterium*, *Cellulomonas*, *Clavibacter*, *Curtobacterium* and *Microbacterium*. Several strains of the plant pathogenic coryneform bacteria assigned to the genus *Clavibacter* and *Curtobacterium flaccumfaciens* were found to be within one SJ cluster, indicating the high similarity between these genera (**Kampfer *et al.,* 1993).**

Present study depicts a light on the spread of *Cmm* in tomato growing belt of Himachal Pradesh and the initiation of disease in some areas of Uttarakhand, which is for the first time in this area and detection of the pathogen from the two agroclimatic zone by genus and subspecies specific primer.

4.9.2. Molecular characterization of isolates by genus and susbp specific primers

For the purpose of confirming the pathogen identity at genus and subsp level the isolates were screened by genus and susbp specific primers designed by **Lee *et al.* (1995)**; **Drier *et al.* (1997)** respectively.

A. Genomic DNA isolation

B. PCR amplicons by genus specific primers designed by **Lee *et al.* (1997)**

C. PCR amplicons by subspecies specific primers designed by **Drier *et al.* (1995)**

The characterization of the bacterial isolates was done by two primer pairs; firstly the three most virulent bacterial isolates identified by indirect ELISA were screened with the genus specific primer as described by **Lee *et al.* (1997)**, through which three of the isolates were characterized with the amplicon size of 1.45 kb, while for the second times the three isolates were screened with the virulence specific primers designed by **Drier *et al.* (1995)** based on the virulence genes present in *Cmm*, by this primer pair out of three isolate only two (S1, S3) were characterized at the amplicon size of 614bp.The results are in the good agreement with **Kyu *et al.* (2006)**, where they identified the pepper bacterial strains of *Cmm* by primers designed by **Lee *et al.* (1995)**; while the results for subspecies specific primers are supported by the results of **Milajsevic *et al.* (2012); Umesha (2006)**. There is a scope of further investigation of the spread of the disease in the state in order to examine the other area under the pathogen and device strategy for checking its spread.

4.10 Evaluation of biocontrol agents, chemicals and defense inducers for the management of pathogen

4.10.1 Evaluation of isolates of *Trichoderma* Spp. and *P. fluorescens* against *Cmm* by using dual culture method

The antagonistic potential of nine isolates of *Trichoderma* sp. (B1-B7, PBAT-1 and A1) and two isolates of *P. fluorescens* (one alone and one in consortium of *Trichoderma* sp.) was evaluated against the *Cmm* by the dual culture method (**Table 4.9, Plate 11**). In dual culture test, all the isolates reduced the colony growth of the *Cmm*. PBAT-1 isolate performed best which gave 89.5% inhibition in radial growth followed by B4, B3 and A1 showing (87.8%), (84.5%) and (82.9%), inhibition in the radial growth, respectively. The least inhibition in radial growth was obtained by B2 (32.1%) and B7 (32.2%). The bacterial biocontrol agent PBAT-2 gave an effective control against the *Cmm* with a reduction of 91.34 % in colony dia. when used alone whereas in consortium with PBAT-1, the colony diameter was reduced only up to 74.5%. Those isolates found effective were further screened in the field conditions.

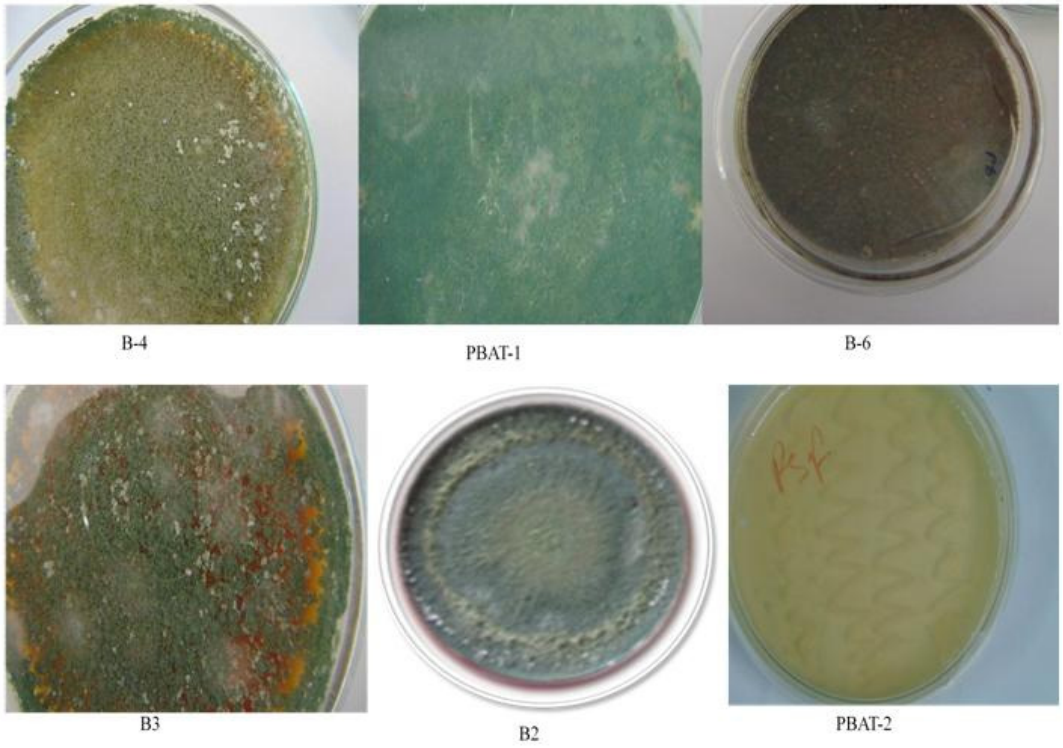

Plate 10: *Trichoderma* sp. and *P. fluorescens* used for screening against the pathogen

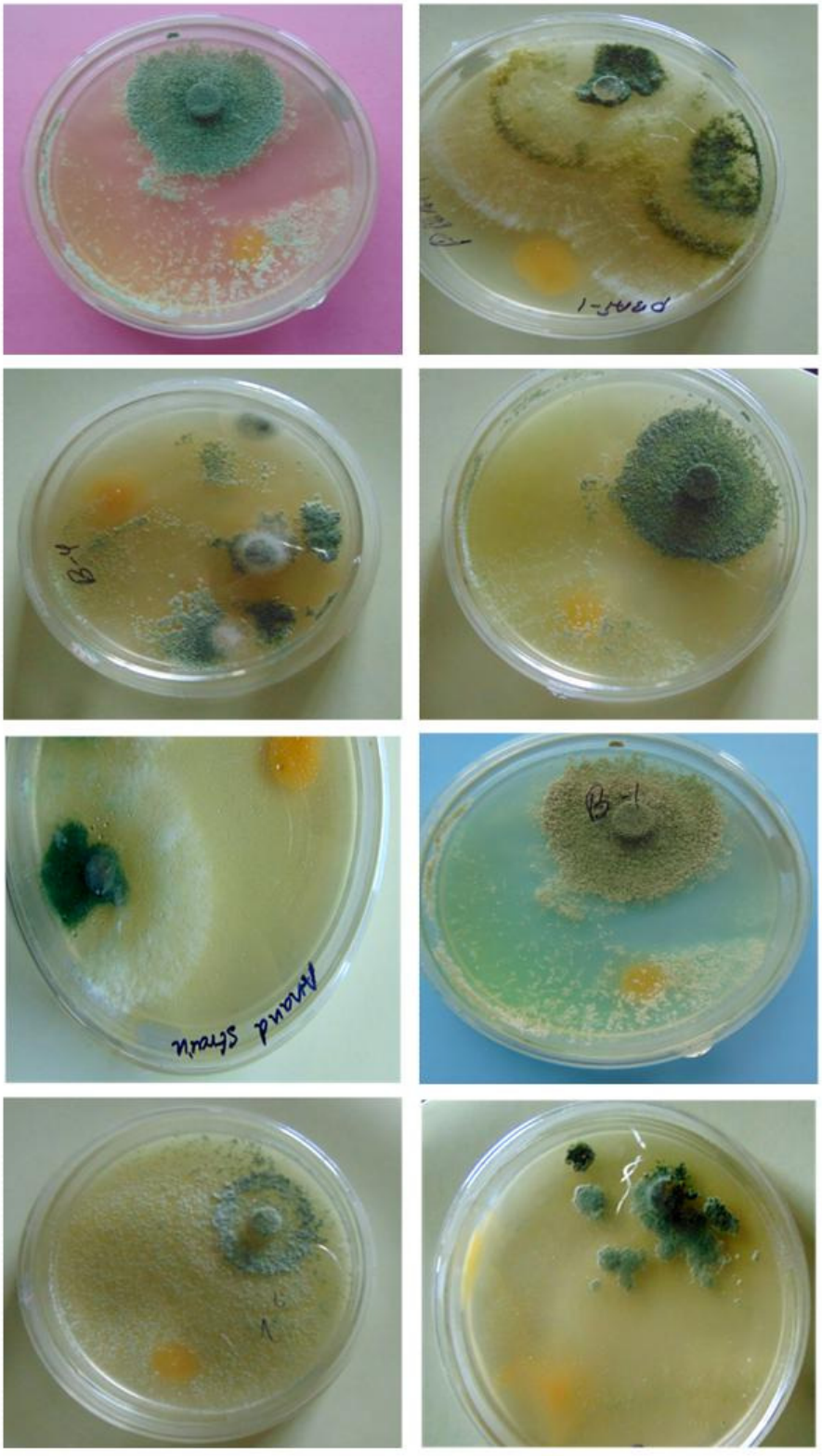

Plate 11: Dual culture screening test

Successful control of bacterial disease in plants through antagonistic bioagents has also been mentioned by **Alivizatos and Pantazis (1992) and Ozaktan *et al.* (1999).**

Table 4.9: Colony diameter and reduction in colony diameter in dual culture screening of biocontrol agents against *Cmm* 10 isolate.

Treatment	Colony Diameter(mm)*	Reduction in colony diameter (%)
PBAT-1	13.15	87.8
B1	35.15	61.3
B2	63.92	32.1
B3	17.21	84.5
B4	11.27	89.5
B5	44.10	54.5
B6	24.10	76.7
B7	57.47	32.2
A1	16.30	82.9
PBAT-2(Psf)	8.13	91.346
PBAT-1+PBAT-2	22.10	74.5

CD at 5% 1.361

SEM 0.46413

CV 2.82609

* Figure represents mean of three replicates

4.10.1.1 Evaluation of BCAs under glasshouse condition through seed treatment method

Seeds (20g) of a susceptible tomato variety "Arka Vikas" were inoculated with the isolates *Cmm* 10, *Cmm* 5 and *Cmm* 6 and then treated with different BCAs at the most effective concentration screened from the *Invitro* testing. The seeds (25/pot) were sown in pots containing sterilized soil+sand+vermicompost mixture (2:1:1) under glasshouse conditions. The germinated seedlings were observed for the symptoms expression at seventh day and subsequently 7 days intervals up to 6 weeks. The

treatment showing highest percent seed germination was PBAT-1(72.25%) followed by PBAT-2 (67.24%) and lowest percent seed germination was found in the seeds treated with PBAT-3(58.35%) **(Table 4.10).**

Table 4.10: BCAs screened under glasshouse condition for Isolates *Cmm10, Cmm5* and *Cmm6* through seed treatment in variety Arka Vikas

Treatments	Seed Germination (%)	Disease rating (days after germination)					
		7	14	21	28	35	42
***Cmm*10**	47.88	0	1	2	4	5	5
***Cmm*5**	52.71	0	0	1	2	2	3
***Cmm*6**	45.92	0	0	1	2	3	4
Cmm10 +PBAT1	72.25	0	0	1	1	2	2
***Cmm*10+PBAT2**	67.24	0	0	1	2	3	3
***Cmm*10+PBAT3**	58.35	0	0	1	2	3	4
***Cmm*10+A1**	69.27	0	0	1	1	2	2
UIC	75.28	0	0	0	0	0	0

CD at 5% 0.130

CV 0.123

SEM 0.043

One of the alternative methods for the management of *Clavibacter michiganensis* subsp *michiganensis* is the use of biocontrol agents. Inhibition of bacterial plant pathogen by *Trichoderma* sp. has been reported by several workers **(Wilhite *et al.,* 2001; Thomashow *et al.,* 2002).** Treatments with RootShield (*T. harzianum*) significantly reduced the incidence of bacterial canker on tomato plants and showed 63% reduction in disease. The study demonstrated that applications of RootShield (*T. harzianum*) applied as preventative foliar sprays were effective to control tomato bacterial canker caused by *C. michiganensis subsp. michiganensis* (**Uthkhede and Koch, 2004**).*Pseudomonas fluorescens* was established as an effective agent for the control of bacterial canker (**Umesha, 2006;Singh, G. 2017**). **Amkraz *et al.* (2010)** also confirmed the antagonistic potential of Fluorescent *psuedomonads*

strains against *Clavibacter michiganensis subsp. michiganensis*. **Wafaa (2003)** also reported the antagonistic potential of plant growth promoting rhizobacterial (PGPRs) strains of *Psuedomonas fluorescens* against bacterial canker pathogen under *in-vitro* conditions.

4.10.2 Evaluation of Chemicals against *Cmm* by using the disk diffusion method *in vitro* conditions

Out of the treatments (six chemicals and two botanicals) alone or in combination when tested for their sensitivity against *Cmm* (Table 4.11, Plate 12) revealed that the maximum zone of inhibition was observed with SS (2.33cm) followed in CS+BP (1.83cm), BP (1.83cm). The minimum zone of inhibition of 0.81cm was observed with CS at 600 ppm concentration. *Invitro* inhibition of *Cmm* in different agrochemicals has been shown by several workers. The use of multiple applications of copper compounds, alone or in mixture with mancozeb for the management of bacterial diseases at every seven to ten days **(Gleason *et al.,* 1993; Hausbeck *et al.,* 2000)** is usually recommended. A good number of copper based chemicals and viz., copper hydroxide, acidified nitrate (**Kasselaki *et al.,* 2011**), copper hydroxide, copper oxychloride, copper sulphate, and antibiotic streptomycin **(Milijasevic *et al.,* 2007; Werner *et al.*, 2002)** have been reported to be effective against the *Cmm*. The use of streptomycin for the effective control of bacterial canker (*Cmm*) has been also supported by **Baysal *et al.* (2005).**

4.10.2.1 Evaluation of chemicals under glasshouse condition through seed treatment method

Seeds of a susceptible tomato variety "Arka Vikas" were inoculated with the pathogen and then treated with different chemicals at the most effective concentration screened from the *Invitro* testing. The seeds (20g) were sown in pots (25seeds/pot) containing sterilized soil+sand+vermicompost mixture (2:1:1) under glasshouse conditions. The germinated plants were observed for the disease symptoms expression at seven days interval after germination upto 6 weeks after germination. From the persual of the data it was observed that highest percent seed germination was found in Streptocycline sulphate (72.34%), followed in COC+BP (68.27%). The minimum percent seed germination was observed in the treatment CS (56.28%) (Table 4.12).

Table 4.11: Screening of chemicals and botanicals at different concentrations against an isolate *Cmm* 10 for zone of inhibition (Diameter in cm*)

Treatment No.	Treatment	Concentration (ppm)	Zone of inhibition (Diameter in cm*)
A	Control	0.00	0.00
B	Copper sulpahate(CS)	100	0.0
		200	0.1767
		400	0.5033
		600	0.8133
C	Bronopol(BP)	100	0.66
		200	1.21
		400	1.59
		600	1.83
D	Streptomycin sulphate(SS)	100	1.01
		200	1.54
		400	2.01
		600	2.33
E	CS+BP	100	1.01
		200	1.25
		400	1.50
		600	1.83
F	Copper oxy chloride(COC)	100	0.46
		200	0.63
		400	0.95
		600	1.21
G	COC+BP	100	0.303
		200	0.50
		400	0.77
		600	0.89
H	Neem Oil	100	0.13
		200	0.15
		400	0.20
		600	0.77
I	Clove oil	100	0.105
		200	0.12
		400	0.77
		600	0.93

CD at 5%

Treatment a 2.15

Treatment b 2.74

a*b 1.72

*Mean of three replications

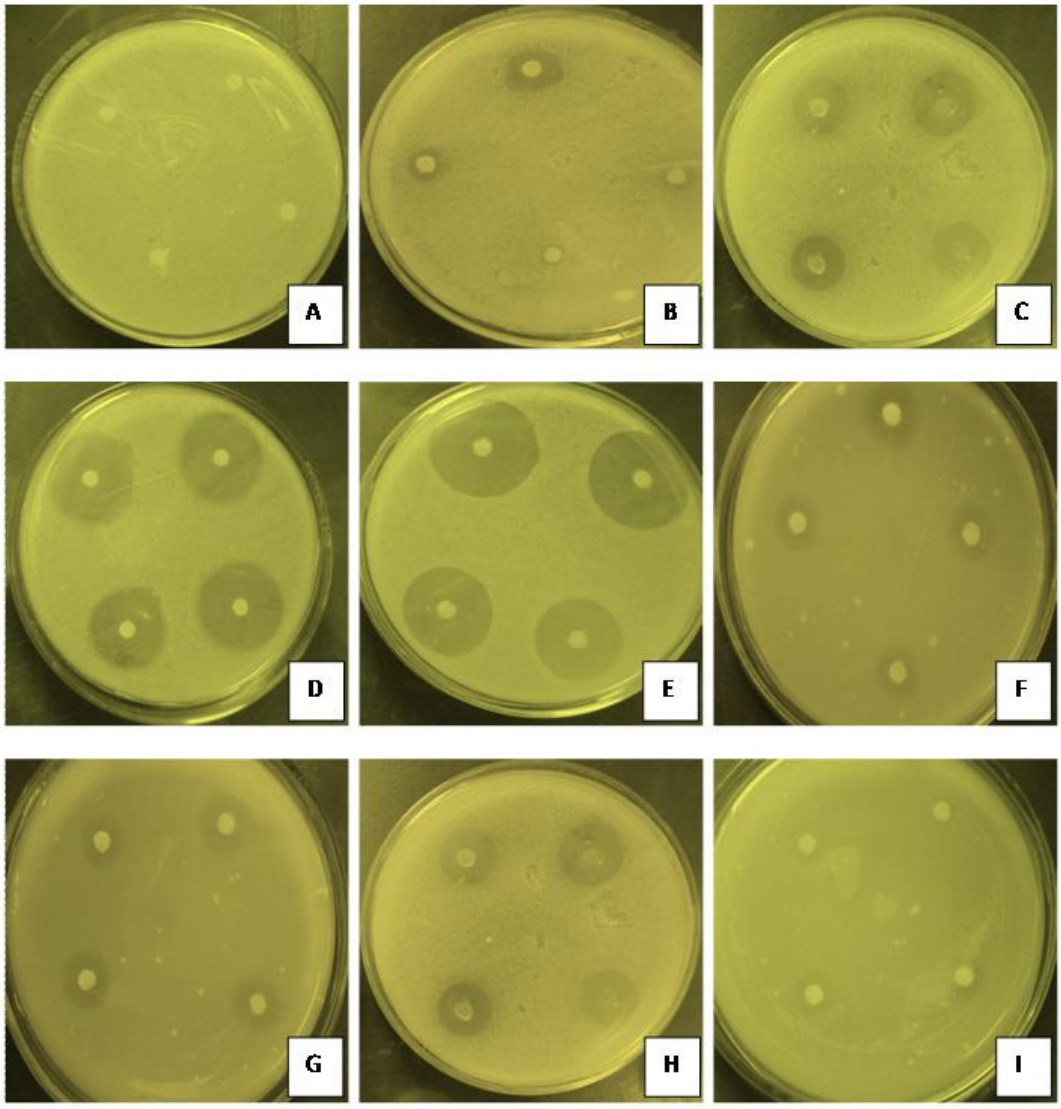

Plate 12: Evaluation of chemicals against *Cmm* 10 under *Invitro* conditions for zone of inhibition

Table 4.12: Chemicals screened under glasshouse condition through seed treatment method for percent seed germination and disease severity

Treatments	Seed Germination (%)	Disease Rating (days after germination)					
		7	14	21	28	35	42
*Cmm*10	47.88	0	1	2	4	5	5
*Cmm*5	52.71	0	0	1	2	2	3
*Cmm*6	45.92	0	0	1	2	3	4
*Cmm*10+CS	56.28	0	0	1	2	2	3
*Cmm*10+COC	58.22	0	0	1	2	3	3
*Cmm*10+BP	65.21	0	0	1	1	2	3
*Cmm*10+SS	72.34	0	0	0	1	2	2
*Cmm*10+CS+BP	68.27	0	0	0	1	2	2
*Cmm*10+COC+BP	63.47	0	0	0	2	2	3
UIC	75.28	0	0	0	0	0	0

CD at 5% 0.21

CV 0.19

SEM 0.067

Evaluation of disease appearance and development was determined using a 0- 5 arbitrary scale (**Soyulu *et al.,* 2003**). Ratings were as follows:

0 no leaves showing wilting;

1 slight marginal wilting, 1- 10% of leaves with wilt;

2 11- 25% of leaves with wilt;

3 sectored wilting, 26- 49% of leaves showing wilting associated with chlorosis;

4 pronounced leaf collapse, 50- 74% of leaves showing wilting;

5 whole leaf wilted.

4.10.3 Evaluation of Chemicals and Biocontrol agents in field condition by estimation of interplant population dynamics of the pathogen

The chemical compounds and biocontrol agents were screened in the field condition at the most effective concentration screened for each treatment from *in vitro* assay and the efficacy of the treatment was estimated in the form of intraplant population dynamics (cfu/mL) recovered from the plant tissue extract after 72 hrs of plating.

Table 4.13: Effect of individual chemical compounds, their consortium and BCAs effect on *C. michiganensis* subsp. *michiganensis*, after 72 h of incubation

Treatments	Initial inoculums (cfu/mL)	Recovered cells(cfu/mL)*		
		P. Ruby	Rohini	US2853
UIC	$1x10^8$	0.00	0.00	0.00
IC	$1x10^8$	$4.53x10^{10}$	$5.61x10^8$	$5.30x10^8$
CS	$1x10^8$	$6.5x10^6$	$4.6x10^5$	$3.4x10^5$
COC	$1x10^8$	$4.3x10^7$	$5.5x10^6$	$5x10^5$
BM	$1x10^8$	$1.2x10^5$	$2.5x10^5$	$2.2x10^4$
SS	$1x10^8$	$1.7x10^4$	$7.36x10^4$	$5.66x10^3$
BP	$1x10^8$	$0.8x10^5$	$0.7x10^5$	$0.5x10^5$
CS+SS	$1x10^8$	$3.2x10^5$	$2.5x10^5$	$2.4x10^4$
CS+BP	$1x10^8$	$2.7x10^4$	$1.6x10^4$	$4.3x10^3$
PBAT-1	$1x10^8$	$7.6x10^4$	$1.4x10^5$	$1.5x10^5$
PBAT-2	$1x10^8$	$3.36x10^7$	$2.5x10^6$	$2.4x10^6$
PBAT-3	$1x10^8$	$1.53x10^6$	$4.3x10^4$	$4x10^4$
A-1	$1x10^8$	$2.3x10^6$	$1.8x10^5$	$1.7x10^5$

*Mean of three replications

CD at 5%

a	0.5022333E+10
b	0.2412651E+10
a*b	0.8698935E+10
CV	449.0363

Amongst all the treatments screened in the field study (Table 4.13), the consortium of CS+BP, inhibited bacterial growth and showed the lowest recovered cfu after 72 h of incubation in all the three cultivars tested with the minimum cfu recovered in Cv. Rohini ($4.3x10^3$) followed in US 2853($1.6x10^{4)}$ and P. Ruby ($2.7x10^{4)}$.The least effective treatment showing the highest recovery in cfu from the plant sap extract of all the three cultivar was CS, showing highest recovered cfu in Cv. P.Ruby ($6.5x10^6$), followed by US2853($4.6x10^5$) and Rohini ($3.4x10^5$). Amongst the BCAs screened the lowest recovered cfu was observed in the treatment PBAT-1, showing highest recovered cfu in Cv. P. Ruby ($2.7x10^4$), followed by US2853 ($1.6x10^4$) and P.Ruby ($4.3x10^3$); the treatment PBAT-2 was observed to be the least effective treatment with highest recovered in cfu in all cvs. viz., P. Ruby, US2853 and Rohini ($3.36x10^7$, $2.5x10^6$, $2.4x10^6$) respectively.

4.10.3.1 Yield data in accordance with the chemicals and biocontrol agents evaluated in the field condition

Out of the thirteen different treatments when subjected to evaluate for their efficacy on yield parameter (Table 4.14), it was observed that the maximum yield (yield/25 plants in plot area of 2 x 2.5 m²) was in treatment Streptomycin sulphate in the cultivar Rohini (2.52kg/25 plants), followed by US2853(2.26 kg/25 plants) and the minimum yield was in the variety P. Ruby (2.52 Kg/25 plants) as compared to inoculated control where the yield was 1.65 kg/25 plants, 1.86kg/25plants and 1.36 kg/25 plants in cultivars P. Ruby, Rohini and US2853 respectively. Lowest yield was observed in treatment copper oxy chloride in Cv. Rohini (1.85kg), US2853 (1.84kg) and in P. Ruby (1.76kg). Amongst the biocontrol agent, A-1 in isolate showed maximum yield/kg in cultivars P. Ruby, Rohini and US253 respectively (1.94kg, 1.96kg, 1.95kg) while lowest yield as observed in treatment PBAT-3(1.82kg, 1.84kg, 1.85kg) in cvs Rohini, US2853 and P. Ruby respectively as compared to inoculated control where the yield was 1.65 kg/25 plants, 1.86kg/25plants and 1.36 kg/25 plants in cultivars P. Ruby, Rohini and US2853 respectively.

Table 4.14: The yield data for three varieties in field condition under different chemical and BCA treatment.

Treatments	Yield/Kg*		
	P.Ruby	Rohini	US2853
UIC	3.16	2.75	3.66
IC	1.65	1.86	1.36
CS	1.86	1.94	1.83
COC	1.76	1.85	1.84
BM	1.87	1.86	1.90
SS	2.21	2.26	2.52
BP	1.88	1.88	1.89
CS+SS	1.86	1.88	1.89
CS+BP	2.26	2.36	2.65
PBAT-1	1.93	1.96	1.96
PBAT-2	1.94	1.96	1.96
PBAT-3	1.82	1.84	1.85
A-1	1.94	1.96	1.95

*Means of three replications

CD at 5%

a	1.87
b	3.11
a*b	1.65

4.10.4. Evaluation of defense inducers for management of Cmm

The highly susceptible cultivar of tomato i.e. P.Ruby was treated with four defense inducers viz., salicyclic acid (SA), Isonicotinic acid (INA), 2.3-Benzothiodiaazole (BTH) and lysozyme for screening of the defense induced by the elicitors. Tomato plants treated with water and with pathogen were taken as control. The enzymatic activity of Peroxidase (Pos), Polyphenol Oxidase (PPO), Total phenol content (TPC) and Pr-2 protein was assessed in two susceptible varieties viz., US2853 and Rohini. The foliar spray was given to tomato plants at 5 week stage in three different concentrations of 200µM, 500µM and 800µM. The spray was given at two

duration first spray was given as prophylactic spray before the inoculation of pathogen and second spray was given after the inoculation of the pathogen.

SAR development is found to be connected with the varied cellular defense responses, such as PR protein synthesis, formation of phytoalexins, and buildup of reactive oxygen species (ROS), Fast changes in cell wall composition and significant increase in the concentration and activity of defence-related enzymes **(Ryals *et al.*, 1996).** The studies exploring AOS during SAR expression showed ample evidence showing that AOS, H_2O_2 in particular, execute numerous significant functions in early defence responses of the plant against pathogens, these responses generally includes mechanism like antimicrobial action, lignin formation, phytoalexin production, and induction of SAR **(Mehdy *et al.,* 1996).** AOS may also render deleterious effects on the cell health. O2, OH**.** and H_2O_2 are some of the AOS species generally produced under stress conditions **(Scandalios, 1993)** act as attacking oxidizing species that can rapidly effect all types of bio-molecules and easily damage them. Oxygen radical detoxifying enzymes such as catalase, peroxidase and superoxide dismutase (SOD) and nonenzymatic antioxidants such as ascorbate peroxidase and glutathione-S-transferase (GST) (**Alscher *et al.*, 1997**). An important role is played by these enzymes in protecting the plant cells by the damage caused due to increased AOS generation (**Kuzniak and Sklodowska, 2001**).

4.10.4.1. Defense inducers activity in relation to Peroxidase

In present study the Peroxidase activity was significantly higher in the plants sprayed with defense inducers at 500µM in both the varieties, followed by 200µM (Fig 5). However, at the concentration of 800µM, phytotoxicity symptom was observed in some plants. The enzymatic activity those which were in plants only sprayed with pathogen was near to that of concentration of 200µM and least enzymatic activity was observed in plants only sprayed with water. It was observed that the when the prophylactic spray of the defense inducers was given the maximum enzymatic activity was observed in the plant sprayed with BTH followed by SA, INA and lysozyme. On comparing the two varieties enzymatic activity was less in US2853 in comparison with Rohini. Significant elevation of the PO activity was observed in the variety Rohini and US2853 receiving BTH foliar spray at the concentration of 500µM (2.97, 2.68) ($p < 0.05$) followed by the plants sprayed with defense inducers at 200µM and 800 µM.

Least enzymatic activity was observed in cultivar US2853 (0.098) sprayed only with water followed in the plants inoculated only with pathogen (0.642) in cultivar US2853.

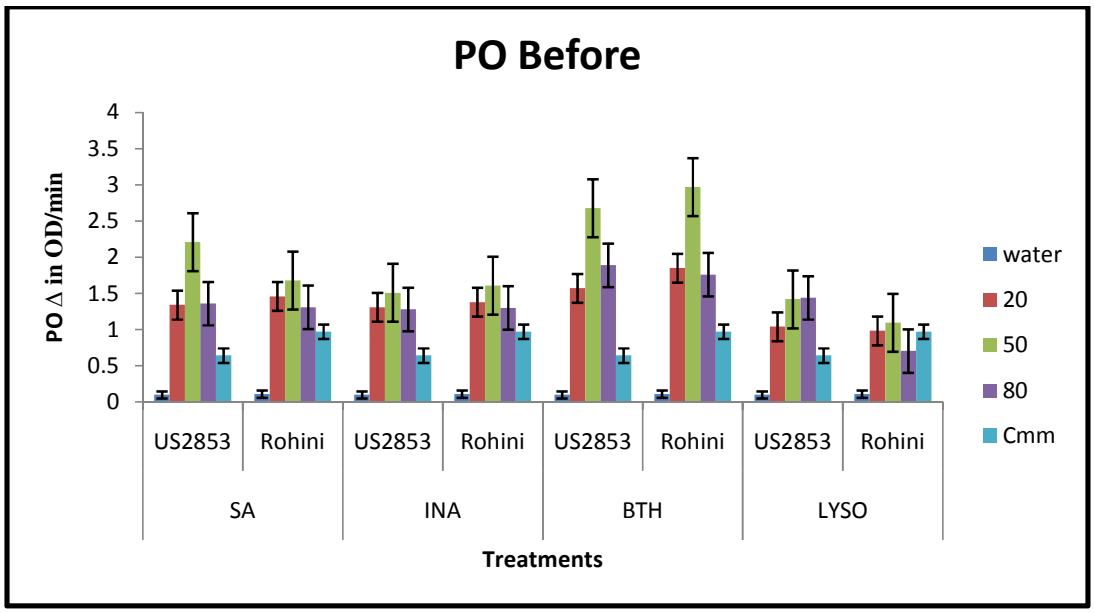

Fig. 5: Peroxidase activity (IUg-1 FW) of tomato plants at 48 h after treatment with prophylactic spray of SA, INA, BTH and Lysozyme.

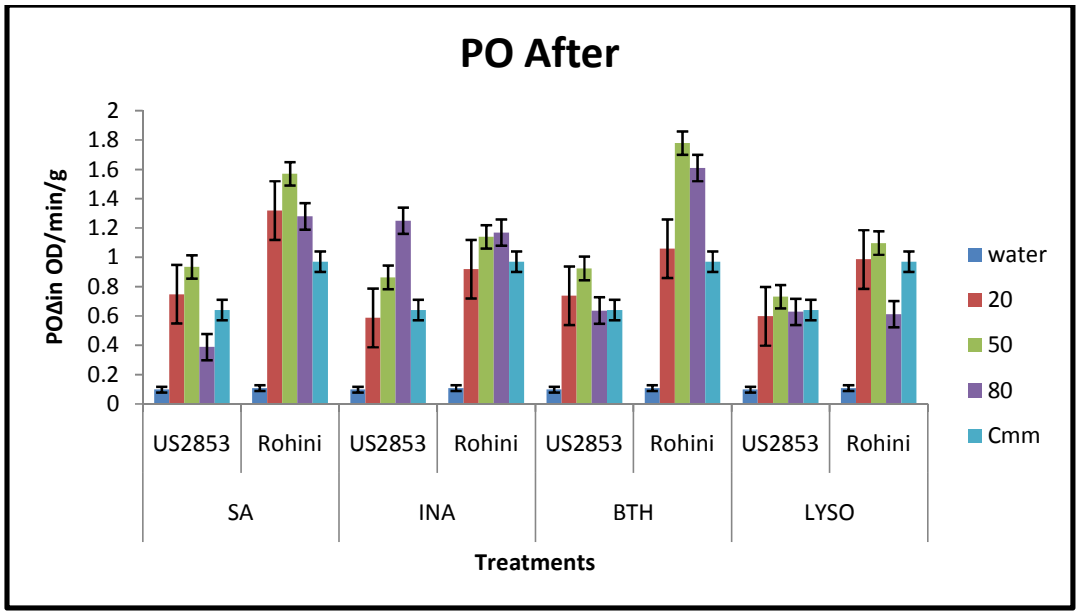

Fig. 6: Peroxidase activity (IUg-1 FW) of tomato plants at 48 h after treatment with after spray of SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$);n = 10 for each treatment

Study reveals that in the plants given the spray of defense inducers after the inoculation of the pathogen (Fig, 6), the enzymatic activity was the maximum in BTH treatment (1.78, 0.925; $p \leq 0.05$) in cultivars Rohini and US2853 respectively at the concentration of 500µM followed by 200µM and 800µM. The enzymatic activity was at par in the plants sprayed with SA and INA, followed by the least activity shown by the plant sprayed with lysozyme (0.613, 0.628; $p \leq 0.05$). Least enzymatic activity was observed in cultivar US2853 (0.098) sprayed only with water followed in the plants inoculated only with pathogen (0.642) in cultivar US2853.

4.10.4.1.1. PO activity assessment in cultivars in Protected conditions

The glasshouse screening of 25 cultivars/lines were done in the protected condition for the purpose of observing the difference in the peroxidases activities before and after inoculation of pathogen.

Studies indicate that he highest PO activity before the inoculation with pathogen was found in cultivar Ankit (3.92µg/g FW), followed by Cv. Suricha (1.53) and Calyx (1.52). The least enzymatic activity after inoculation with pathogen, was observed in the tomato line CLN (0.37µg/g FW), followed in cultivar Himgiri (0.51 µg/g FW) and Noble (0.58 µg/g FW). The maximum decline in the enzymatic activity was observed in the cultivar Ankit, wherein the before inoculation enzymatic activity was recorded to be 3.92 that falls to 1.17 after inoculation and the minimum decline in the enzymatic activity was observed in the variety Phule Raja wherein the before inoculation enzymatic activity was recorded to be 1.23 that falls to 1.13 after inoculation (Table 4.15)

It is established by many workers that pre-treatment of plants with different biotic (pathogens and insect pests) and abiotic inducers (chemicals) induce plant resistance that defends the plants against their subsequent attack **(War *et al.,* 2011; Hu *et al.,* 2009; Lu, 2009).** The plant phytohormones induce plant defense against many biotic and abiotic stresses. Salicylic acid is an important and well-studied endogenous plant growth regulator that generates a wide range of metabolic and physiological responses in plants involved in plant defense in addition to their impact on plant growth and development **(Chen *et al.,* 2009; Zhao *et al.,* 2009**) SA also activates the generation of ROS and other defensive processes such as hypersensitive response and cell death (**Hayat *et al.,* 2009**). Simultaneous inclusion of phenolic compounds in the cell wall during incompatible

Table 4.15: Assessment of PO activities in different cultivars/lines of tomato plants before and after the inoculation with pathogen.

Variety	Before inoculation	After inoculation
Phule Raja	1.23	1.13
Dhanshree	1.19	1.02
Bhagyashree	0.86	0.63
Amrutha	1.17	0.61
Trisha	0.90	0.79
Calyx-248	1.52	1.31
Suricha	1.53	0.91
Pradhan	1.06	0.86
Shivam	0.84	0.81
NTH2350	1.12	0.76
Vaishnavi 2082	0.91	0.85
Lyco	1.14	1.02
Ankit	3.92	1.17
Lakshmi	1.16	0.58
Syngenta(To-1458)	0.83	0.89
PPT-1	1.21	1.03
PPT-2	1.17	0.82
Arka Vikash	1.38	0.91
US 2853	1.35	0.92
P.Ruby	1.38	0.95
Rohini	1.05	0.74
Noble	0.62	0.58
CLN	0.59	0.37
Siroji	0.58	0.48
Himgiri	0.63	0.51

CD at 5%

a	0.11
b	0.031
a*b	0.15
cv	9.42

plant–microbe/elicitor interactions can be associated with increase in POX activity. The enzyme POXs is supposed to catalyse the few last steps of the lignifications pathways in tomato. The resistance of plants to the cell wall degrading enzymes produced by the pathogen can be enhanced by strengthening of the plant cell wall by phenolics and lignin thereby acting as a perfunctory blockade to toxin invasion and to physical penetration toward the protoplast **(Nicholson and Hammerschmidt, 1992).** Low activity of POD and PPO in plants treated with defense inducers at higher concentrations may be due to phytotoxicity experienced by the plant at higher concentration **(Rajjou *et al.*, 2006).**

4.10.4.2. Defense inducers activity in relation to Phenyl ammonia lyase

Studies carried out indicated that in the plants receiving the prophylactic activity of defense inducers, the maximum PAL activity was observed in the plants sprayed with BTH in the cultivar Rohini followed in US2853 (12.02, 7.82, $p \leq 0.05$) at 500µM concentration (Fig.7) followed by enzymatic activity at 200µM and 800µM. The second highest enzymatic activity was observed in the plants sprayed with SA (6.14, 4.13, $p \leq 0.05$) INA and Lysozyme. Amongst the four treatments the least effective treatment was of Lysozyme at 800 µM (2.23, 2.17), ($p \leq 0.05$) in cultivars Rohini and US2853 respectively. The least effective treatment was Lysozyme at 800µM concentration (2.23, 2.17; $p \leq 0.05$) in cultivars Rohini and US2853 respectively.

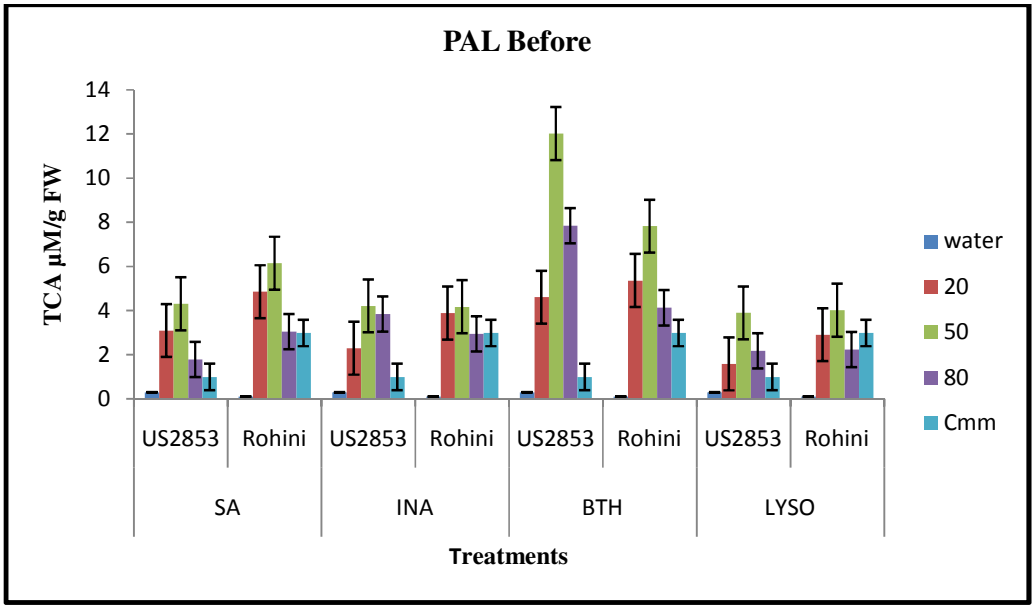

Fig. 7: PAL activity (TCA µMg-1 FW) of tomato plants at 48 h after treatment with prophylactic spray of SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$); n = 10 for each treatment.

Studies indicates that in the plants receiving the spray of defense inducers after the inoculation of the pathogen the highest enzymatic activity was observed in the plants treated with SA at 500µM (5.37, 2.46; p≤.05)(Fig.8). The second highest enzymatic activity was observed in the plants receiving the treatment of BTH and Lysozyme at 500µM concentration. The least enzymatic activity amongst the treatment was observed in the plants treated with INA (3.71, 1.45; p≤0.05) in cultivars Rohini and US2853 respectively.

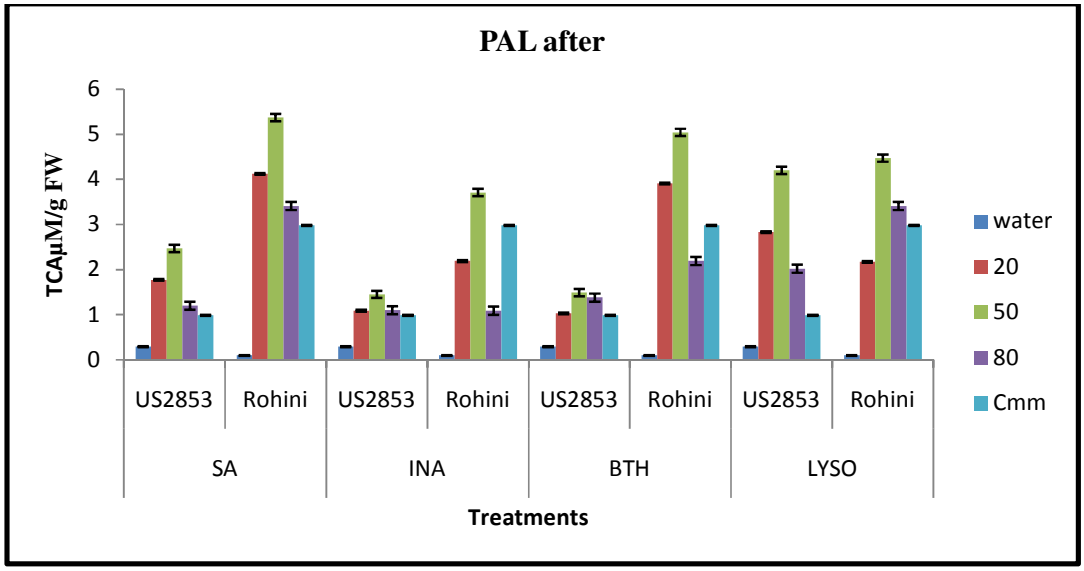

Fig. 8: PAL activity (TCA µMg-1 FW) of tomato plants at 48 h after treatment with after spray of SA, INA, BTH and Lysozyme. Bars represent standard error (p ≤ 0.05); n = 10 for each treatment

Enhanced enzymatic activity of PAL is the foremost response in a number of plant species to pathogen challenge and is very much associated with resistance (**Pallas *et al.*, 1997**). An increase of 7.5 fold in the PAL activity was observed by addition of salicylic acid to *Saussurea medusa* cell cultures at a concentration of 20 μM (Yu *et al.*, 2006). PAL plays an important role in plant defense; it is involved in the biosynthesis of salicylic acid (SA), an essential signal involved in plant systemic resistance (**Nugroho *et al.*, 2002; Chaman *et al.*, 2003**). BTH priming of tea plants before inoculation with *Lasiodiplodia theobromae* leads to an increase in the PAL activity and reduction in the disease severity (**Das *et al.*, 2017**). An increase in the PAL activity following the exogeneous application of SA was also reported by (**Lafuente *et al.*, 2004**).

4.10.4.3. Defense inducers activity in relation to Total Phenolic content

In the present study it is found that the treatments with defense inducers (SA, INA, BTH and Lysozyme) elevated the total phenolic content significantly in plant given a prophylactic spray of defense inducers in relation to control. However, significantly higher phenolic contents were shown by the plants sprayed with BTH at 500 µM in cultivar Rohini and US2853 (5.18, 0.726, $p < 0.05$) followed by enzymatic activity at 200µM and 800 µM in both the cultivars. After BTH, the enzymatic activity was observed in SA treated plants followed by INA treated plants and minimum enzymatic activity amongst the four treatments was observed in the plants treated with lysozyme (2.18, 0.515, $p \leq 0.05$) (Fig. 9) in cultivars Rohini.

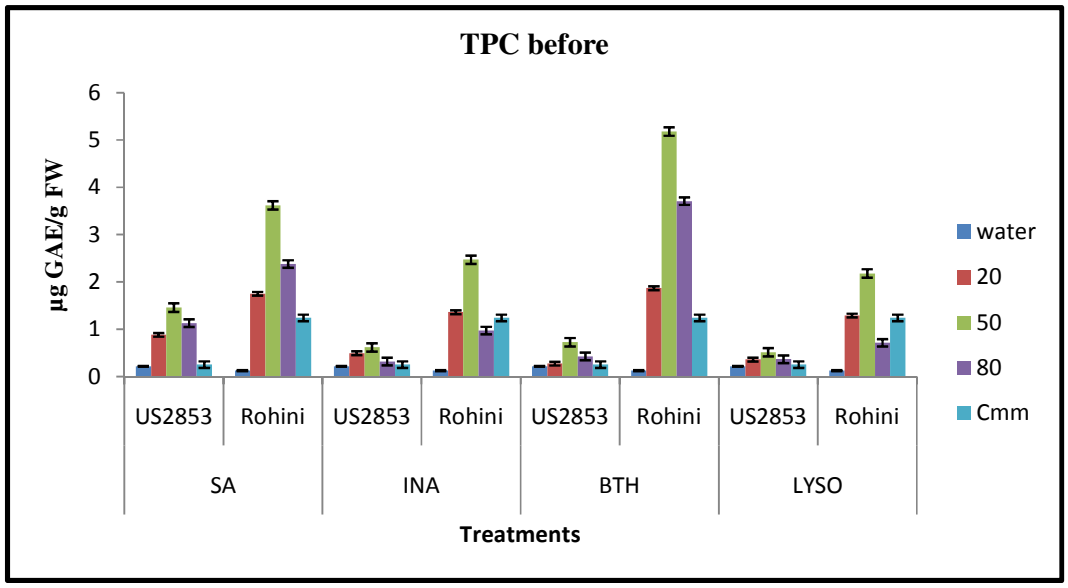

Fig. 9: Total phenols (µg GAE g-1 FW) of tomato plants at 48 h receiving prophylactic treatment with SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$); GAE = Gallic acid equivalents; n = 10 for each treatment

It is revealed that in the plants receiving the after spray of defense inducers (SA, INA, BTH and Lysozyme) at three varied concentration (200µM, 500µM and 800µM) enzymatic activity was more in plants sprayed with SA in both the cultivars viz., Rohini and US2853(3.92, 0.559, $p \leq .05$) (Fig.10) followed by the phenolic concentration in the plants treated with BTH and Lysozyme followed by pathogen inoculation was at par with each other. The minimum phenolic content amongst the four treatments was observed in plants treated with INA (1.36, 0.417; $p \leq 0.05$).

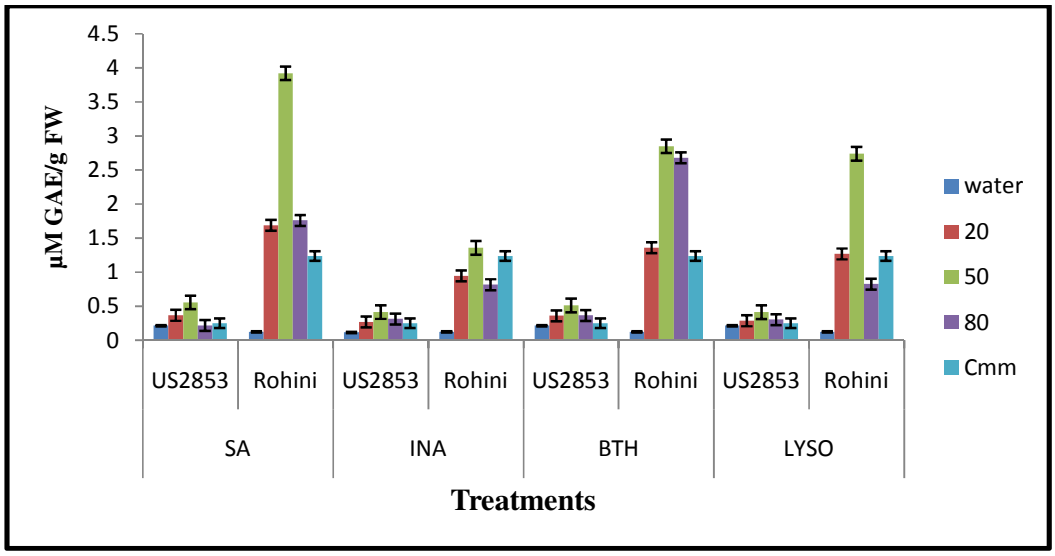

Fig. 10: Total phenols (µg GAE g-1 FW) of tomato plants at 48 h receiving prophylactic treatment with SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$); GAE = Gallic acid equivalents; n = 10 for each treatment

4.10.4.3.1. Assessment of Total Phenolic Content in cultivars in protected conditions

The glasshouse screening of 25 cultivars/lines were done in the protected condition for the purpose of observing the difference in the total phenolic content before and after inoculation of pathogen.

During screening of 25 different tomato cultivars/lines for the TPC (Table 4.16), the maximum phenolic content was observed in the line PPT-1 7084 µg/g FW (fresh weight) however the sudden fall in the TPC after pathogen inoculation to 3471 µg/g FW indicates the susceptible nature of the line. The minimum decline in the phenolic content was observed for the cultivar Phule Raja, wherein, the phenolic content fall from 2981.70 µg/g FW before inoculation to 2509.10 µg/g FW. The other variety showing the susceptible reaction on the basis of enzymatic activities are P. Ruby, A. Vikash and US2853. The stressed posed on the plants by varied biotic and abiotic stress inducers can be defended by phenolic compounds (**War *et al.*, 2011; Sharma *et al.*, 2009**). The physiology of plants and the metabolism displayed can be altered by oxidation of phenols that produces many defensive compounds, that helps the plant in surviving against different stresses either directly or through diverse plant signaling pathways (**Usha Rani and Jyothsna, 2010**) Furthermore, ROS such as

superoxide anion, hydroxide radicals, H_2O_2 and singlet oxygen produced by oxidation of phenols activate plant defense enzymes **(Kawano, 2003; Maffei *et al.,* 2007).**

Table 4.16: Assessment of Total Phenolic Content in different cultivars/lines of tomato plants before and after the inoculation with pathogen

Variety	Before inoculation	After inoculation
Phule Raja	2981.70	2509.100
Dhanshree	2700.89	1914.667
Bhagyashree	3272.40	2805.60
Amrutha	3274.10	3383.20
Trisha	4009.80	2354.80
Calyx-248	2883.20	2750.78
Suricha	3180.20	3300.72
Pradhan	3906.20	3019.72
Shivam	2843.70	2481.19
NTH2350	3174.20	3256.83
Vaishnavi 2082	2931.90	2817.34
Lyco	4114.71	2234.20
Ankit	3444.29	3609.23
Lakshmi	2861.28	2289.15
Syngenta(To-1458)	2420.70	2530.83
PPT-1	7084.50	3471.26
PPT-2	2392.79	3910.63
Arka Vikash	2355.20	1716.27
US 2853	2381.86	1868.63
P.Ruby	3173.70	3003.69
Rohini	5016.84	3521.400
Noble	3840.20	4982.371
CLN	2931.80	3067.83
Siroji	2299.60	2385.61
Himgiri	1321.20	1519.25

CD at 5%

a	2.30
b	0.65
a*b	3.25
CV	0.16

4.10.4.4. Defense inducers activity in relation to Pathogenesis related (PR-2) protein

It is also observed that in the plants receiving the prophylactic spray of the defense inducers, the level of PR-2 protein was significantly higher when treated with BTH at 500 µM concentration (3.58, 2.75, p≤0.05). The second highest enzymatic activity was observed in the plants treated with SA followed by plants treated with INA. The least enzymatic activity was observed in plants receiving the Lysozyme treatment (1.09, 1.42, p≤0.05)(Fig.11) in cultivars Rohini and US2853 respectively.

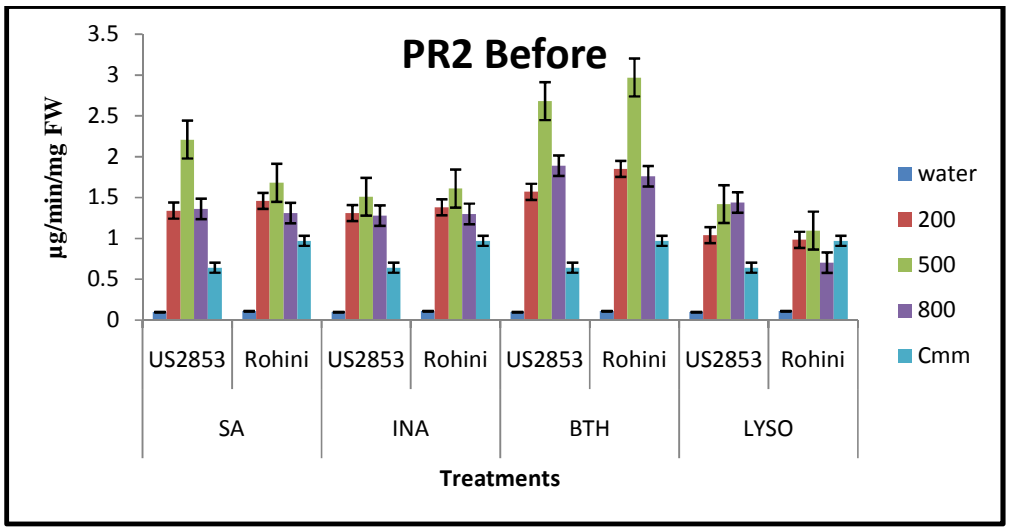

Fig. 11: PR-2 protein activity (µg g-1 FW) in tomato plants at 48 h receiving prophylactic treatment with SA, INA, BTH and Lysozyme. Bars represent standard error (p ≤ 0.05); n = 10 for each treatment

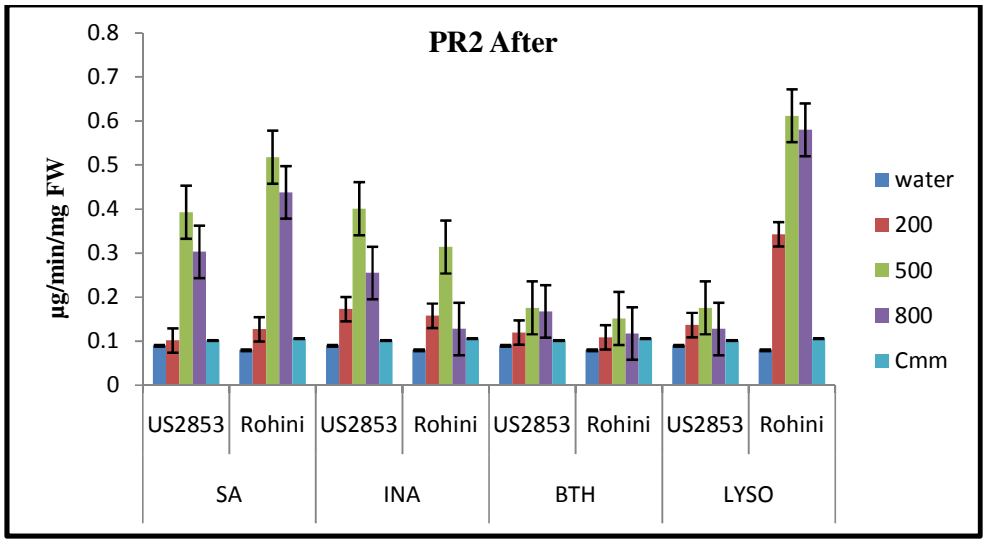

Fig. 12: PR-2 protein activity (µg g-1 FW) in tomato plants at 48 h receiving prophylactic treatment with SA, INA, BTH and Lysozyme. Bars represent standard error (p ≤ 0.05); n = 10 for each treatment

Studies indicated that the plants receiving the treatments of defense inducers at 200µM, 500µM, 800µM for the four defense inducer, after the inoculation of the pathogen maximum activity in the cultivar Rohini was observed at Lysozyme 500µM concentration (0.672, $p \leq 0.05$)(Fig.12) while in cultivar US2853 the maximum enzymatic activity was found at SA 500µm concentration (0.393, $p \leq 0.05$). The PR-2 protein activity was found at par in plants treated with SA and. The minimum PR-2 protein activity was observed in tomato plants treated with BTH (0.11, 0.12, $p \leq 0.05$) in cultivars Rohini and US2853 respectively.

4.10.4.4.1. Assessment of PR-2 protein activity in tomato cultivars in protected conditions

The glasshouse screening of 25 cultivars/lines were done in the protected condition for the purpose of observing the difference in the PR-2 protein activity before and after inoculation of pathogen.

In this study the highest decline in the PR-2 protein activity was observed in the line PPT-1, in which the protein concentration before inoculation with pathogen was 0.85 that dropped to 0.65, followed by the decline in protein concentration in cultivar P.Rubi, Rohini, A.Vikash and US2853 (Table 4.17).

The PR proteins like β-1, 3-glucanases (PR-2) and chitinases (PR-3 have been recognized to posses the enzymatic activities including direct antimicrobial activity by degrading microbial cell wall components. The enzymatic activities of these proteins lead to breakdown of the pathogen and/or plant cell wall components which acts as elicitors to plant defense responses (Van Loon, 1997). Chitinase enzymes in few plants heve been reported to have lysozymal activity leading to bacterial cell wall hydrolyses **(Boller *et al*., 1983; Heitz *et al*., 1994)**. The PR genes expression and the connected accretion of the encoded PR proteins have often been considered as the molecular basis of induced resistance. SA, JA, and ET that stimulate the production of antimicrobial compounds such as phytoalexins and pathogenesis-related (PR) proteins (**Lamb and Dixon, 1997**). Eventually, the HR leads to infected cell death and the containment of the pathogen **(Dangl *et al.,* 1996).**

Table 4.17: Assessment of PR-2 protein in different cultivars/lines of tomato before and after the inoculation with pathogen

Variety	Before inoculation	After inoculation
Phule Raja	0.21	0.17
Dhanshree	0.24	0.21
Bhagyashree	0.28	0.39
Amrutha	0.46	0.42
Trisha	0.41	0.38
Calyx-248	0.48	0.59
Suricha	0.80	0.72
Pradhan	0.27	0.18
Shivam	0.42	0.34
NTH2350	0.69	0.62
Vaishnavi 2082	0.82	0.78
Lyco	0.49	0.42
Ankit	0.52	0.48
Lakshmi	0.43	0.37
Syngenta(To-1458)	0.29	0.23
PPT-1	0.85	0.65
PPT-2	0.53	0.38
Arka Vikash	0.22	0.18
US 2853	0.71	0.61
P.Ruby	0.26	0.17
Rohini	0.29	0.18
Noble	0.27	0.21
CLN	0.71	0.48
Siroji	0.54	0.32
Himgiri	0.17	0.18

CD at 5%

a	0.021
b	0.0057
a*b	0.028
CV	4.231

4.10.4.5. Defense inducers activity in relation to Polyphenol Oxidase

The findings indicated that the plants receiving the prophylactic spray of defense inducers significant elevation of the PPO activity was observed in comparison to plants sprayed only with water and inoculated only with pathogen. Highest PPO activity was observed in plants sprayed with BTH at 500µM in cultivar Rohini followed in cultivar US2853 (81.68, 61.30, $p<.05$) (Fig.13) followed by enzymatic activity at 200µM and 800µM concentration. Second highest enzymatic activity was observed in plants sprayed with SA followed by INA. The least enzymatic activity was observed in plants treated with Lysozyme (31.82, 19.57, $p\leq0.05$)at similar defense inducers concentration. The minimum enzymatic activity was observed in plants sprayed only with water.

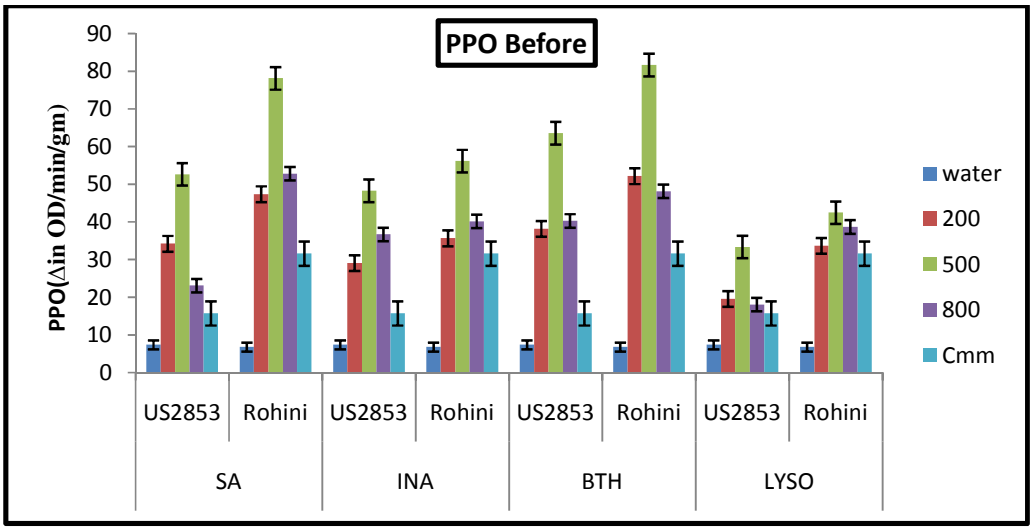

Fig. 13: Polyphenol oxidase activity (IUg-1 FW) in tomato plants at 48 h receiving prophylactic spray with SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$); n = 10 for each treatment.

The findings indicated that plants while receiving treatment of defense inducers after inoculation with pathogen the maximum enzymatic activity was observed in plants treated with SA in both the cultivars viz., Rohini and US2853 (67.39, 38.3, $p\leq0.05$) (Fig.14) at 500µM concentration followed by enzymatic activity at 200µM and 800µM. Second highest enzymatic activity was observed in the plants treated with BTH followed by INA and Lysozyme with similar concentration patterns. Minimum enzymatic activity was observed in the plants sprayed only with water followed by the plants inoculated only with the pathogens.

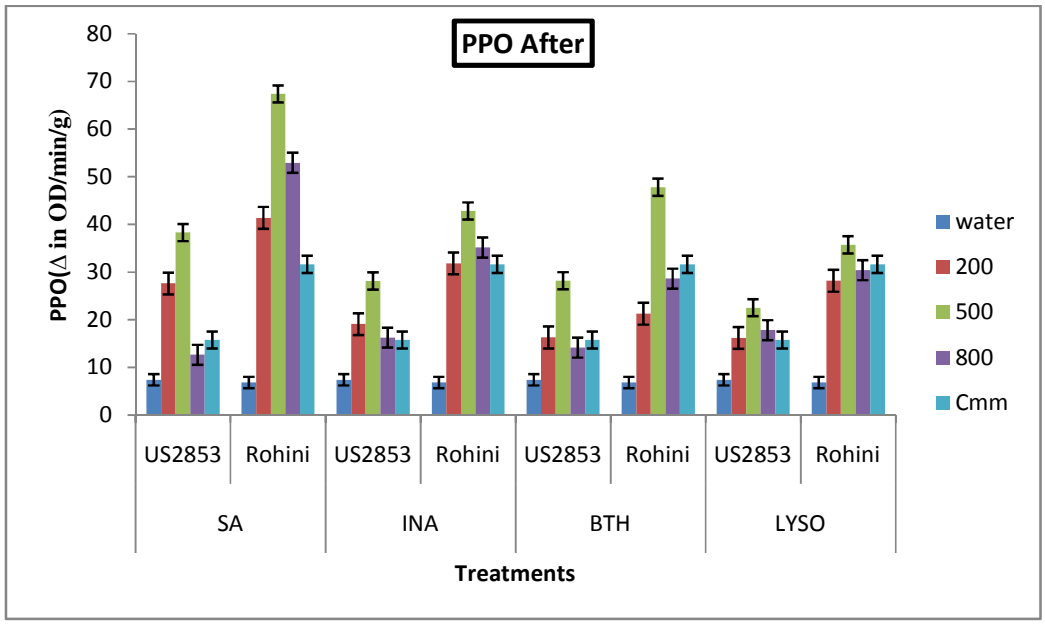

Fig. 14: Polyphenol oxidase activity (IUg-1 FW) in tomato plants at 48 h receiving after spray with SA, INA, BTH and Lysozyme. Bars represent standard error ($p \leq 0.05$); n = 10 for each treatment.

4.10.4.5.1. Assessment of PPO activity in tomato cultivars in protected conditions

The glasshouse screening of 25 cultivars/lines were done in the protected condition for the purpose of observing the difference in the PPO activity before and after inoculation of pathogen

Amongst the 25 different varieties screened for the PPO activity (Table 4.18), the highest decline in the enzymatic activity was observed in the cv. A. Vikas in which the concentration before inoculation with pathogen was 24.57µg/g FW that declined to 9.81 µg/g FW followed by the decline in the cvs. Bhagyashree, US2853 and Calyx-48.

PPOs can be considered as one of the most important enzymes involved in plant defense against many biotic and abiotic stresses (**War *et al.*, 2011**). Defense inducers like jasmonic acid, SA and ethylene have been observed to stimulate such enzymes in plants **(Noreen and Ahraff, 2011**). Induction of these enzymes by exogenous application of SA and their role in plant defense against various stresses including pathogens has been studied in many plants (**Radhakrishnan and Balasubramanian, 2011).**

Table 4.18: Assessment of PPO activity in different cultivars/lines of tomato plants before and after the inoculation with pathogen

Variety	Before inoculation	After inoculation
Phule Raja	17.95	9.87
Dhanshree	9.90	7.36
Bhagyashree	22.8	5.44
Amrutha	8.49	8.26
Trisha	11.17	6.40
Calyx-248	10.95	7.50
Suricha	8.16	7.82
Pradhan	6.65	6.59
Shivam	8.23	7.81
NTH2350	7.4	10.08
Vaishnavi 2082	10.64	8.59
Lyco	9.86	7.84
Ankit	9.19	7.62
Lakshmi	8.65	7.75
Syngenta(To-1458)	8.28	7.62
PPT-1	6.75	6.83
PPT-2	8.95	6.15
Arka Vikash	24.57	9.81
US 2853	28.60	12.50
P.Ruby	10.85	8.62
Rohini	6.71	6.52
Noble	5.86	5.26
CLN	13.19	12.35
Siroji	16.25	14.88
Himgiri	11.20	8.71

CD at 5%

a	0.341
b	0.096
a*b	0.482
cv	2.980

4.10.4.6. Defense inducers activity in relation to Hydrogen peroxide content by DAB staining method

The cut pieces of the leaves easily take the DAB staining and it is distributed uniformally throughout the leaves. A simple test for required peroxidase activity includes the exposure of the plant tissues to DAB and H_2O_2. It is observed that the tomato leaves when given the prophylactic spray of the defense inducers (Plate 13), the peroxidase level was found higher at 48hrs after treatments in plants sprayed with BTH at 500µM concentration followed by the plants sprayed with SA and INA. The minimum hydrogen peroxide activity was observed in plants treated with lysozyme before pathogen inoculation.

Salicylic acid and it's functional analogous has also been reported for to increased H_2O_2 level in treated tobacco leaves (**Wendehenne *et al.,* 1998**). H_2O_2 plays a vital role not only in stimulating hypersensitive cell death, but also in restricting the spread of cell death by inducing the expression of cell protecting genes in surrounding cells (**Levine *et al*., 1994**). Similar histochemical studies carried out by **Iriti and Faoro, (2003)** clearly showed that defense inducers like BTH, ASM induce H_2O_2 accumulation in bean plants. The elevated levels of H_2O_2 which result from inhibition of ROS would serve as a second messenger for the induction of defense responses (**Conrath *et al.,* 1995**).

4.10.4.7. Defense inducers activity in relation to Lignin content

The tomato plants when given the prophylactic treatments of the defense inducers (Plate14) and screened for the lignin production at 48 h after the treatment by cutting the transverse section of the stem 1cm above the point of inoculation. It was observed that lignin content was higher in plants sprayed with SA at 500µM concentration followed by the plants sprayed with BTH and INA. Least hydrogen peroxide activity was observed in plants leaf treated with lysozyme before pathogen inoculation. The transverse section of stems, (Plate 10) exhibiting the lignin deposition in stem cells may block the pathogen penetration as observed by **Luna *et al.* (2011); Park and Ekida (2008)**.

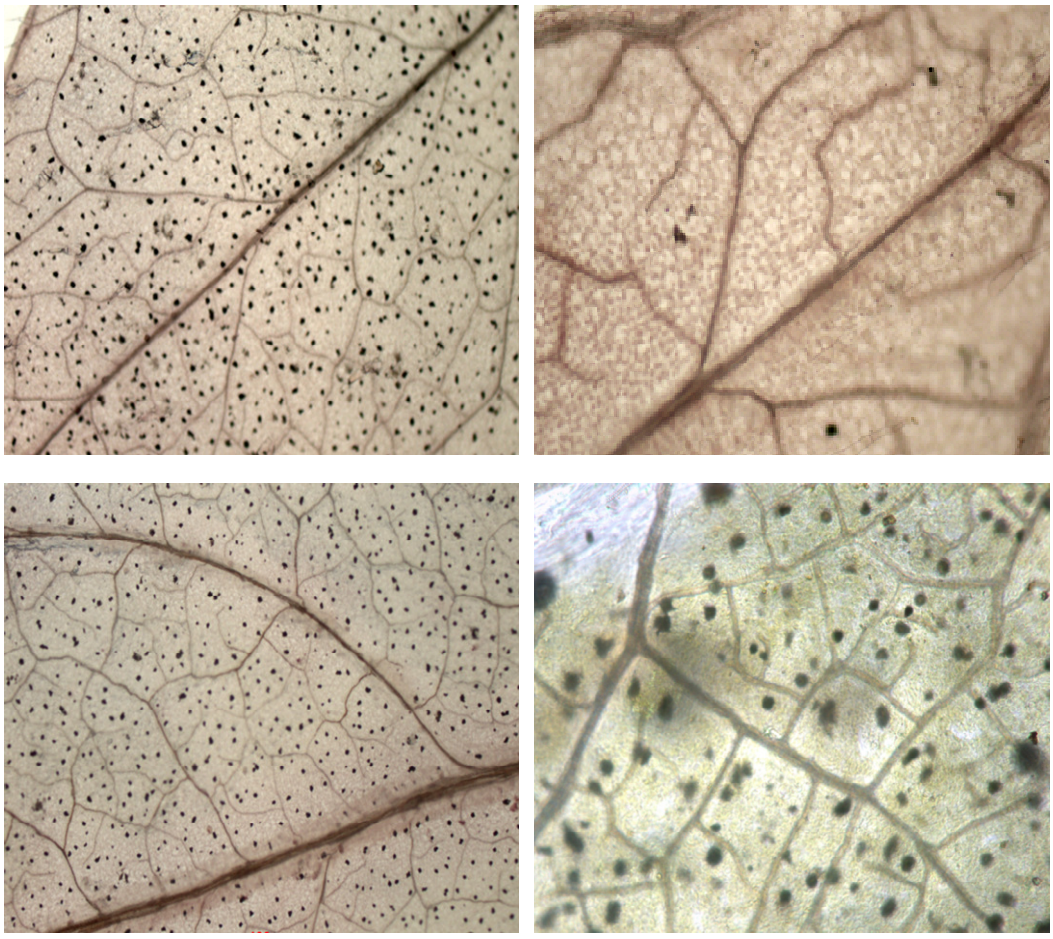

Plate 13: Hydrogen peroxide formation in the leaves of the plants receiving the prophylactic spray of defense inducers (SA, INA, BTH and Lysozyme) at 500µM after 48h of treatment. Pictures show a 100X magnified image of the leaf tissue section.

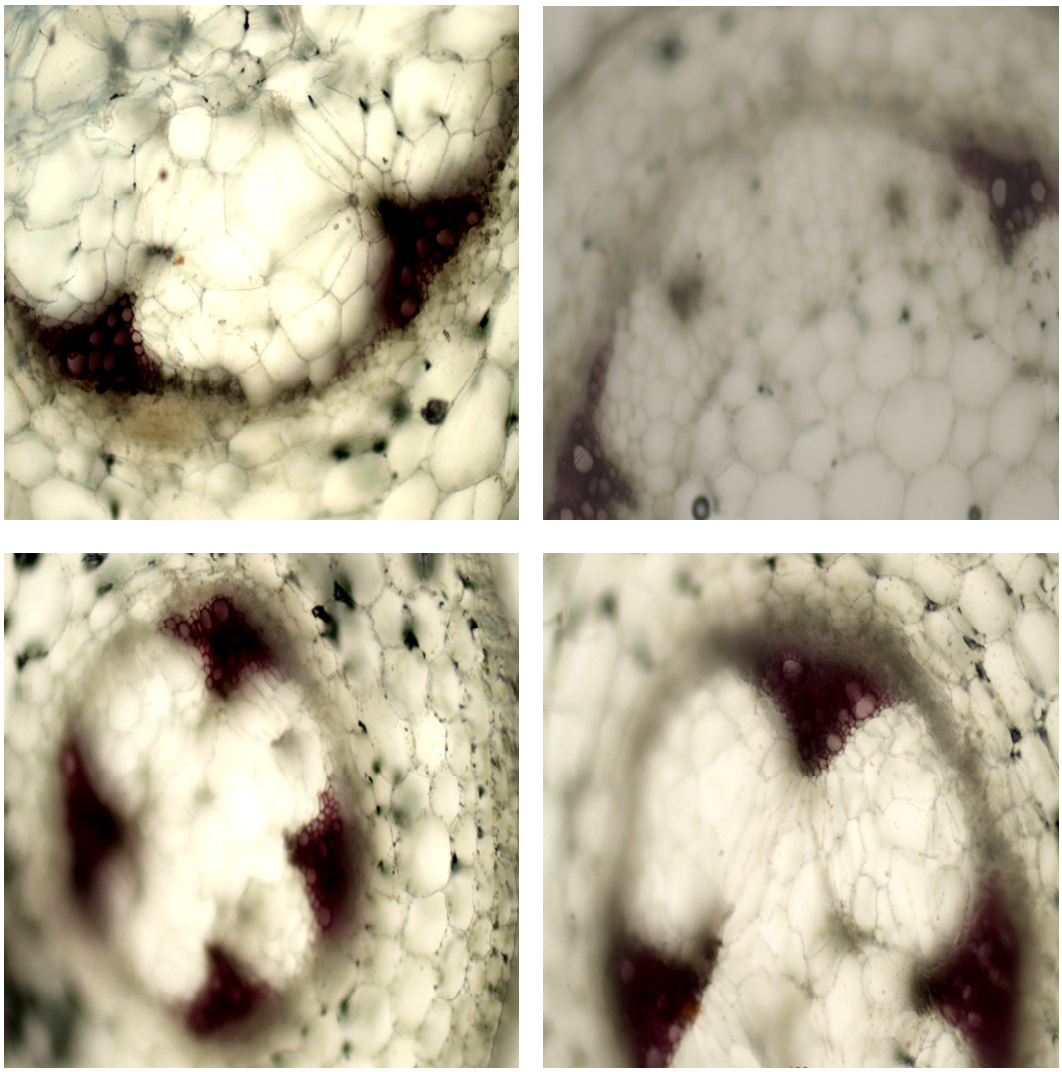

Plate 14: Lignin formation in the xylem vessels of the tomato plants stem receiving the prophylactic treatment of defense inducers (SA, INA, BTH and Lysozyme) at 500µM after 48h of treatment. Pictures show a 100X magnified image of the leaf tissue section.

4.10.4.8. Disease severity per cent in the plants receiving the defense inducers treatments

While comparing the timings of application of defense inducers on effective disease management, it was found that the disease was the minimum in BTH when applied before the pathogen inoculation (27.88%) and it was highest in lysozyme (49.72%) (Table 4.19) When the plants received the treatments with defense inducers after the inoculation of the pathogen, the treatment showing lowest disease severity was BTH (53.62%) and the treatment showing highest disease severity was INA (62.94%). The application of lysozyme at 500µM concentration before or after the inoculation did not show much difference in disease severity however, in the case of the other three defense inducers; the prophylactic spray was more effective in the management of the pathogen. It indicates that the priming of plants by defense inducers before the inoculation of pathogen activates, faster and more pronounced defense response in plants **(Conrath *et al.,* 2006; Goellner and Conrath, 2008).**

Table 4.19: Disease severity in different defense inducers treatment in glasshouse conditions

Pathogen inoculation	Disease severity (%)			
	SA	INA	BTH	Lysozyme
Before	38.68	46.8	27.68	49.72
After	58.38	62.94	53.62	52.62

CD at 5 %

a	4.49
b	3.61
a*b	4.19

4.11. Screening of the cultivars/ lines/ germplasm in response to ambient temperature conditions in two growth seasons and locations.

In contrast to Gram-negative plant-pathogenic bacteria, an incompatible reaction between *Cmm* and a tomato cultivar has not yet been found and all efforts to obtain resistant tomato cultivars by breeding so far have not been satisfactory.

Table 4.20 Reaction of tomato cultivars/ lines/ germplasm to bacterial canker of tomato (*Clavibacter michiganensis subsp. michiganensis*) in (a) open field and (b) polyhouse condition in rabi season

Season of planting1- September 2015 – Jan 2016

(a) Location1: Open field

S. No.	Variety/lines	Disease severity (%)	Disease reaction	Temperature (ºC)	RH (%)
1.	Phule Raja	7.70(16.1)	R	9.4-26.4	32-82
2.	Dhanshree	8.40(16.8)	R	9.4-26.4	32-82
3.	Bhagyashree	7.600(16.01)	R	9.4-26.4	32-82
4.	Amrutha	13.50(21.55)	MR	9.4-26.4	32-82
5.	Trisha	14.0(21.97)	MR	9.4-26.4	32-82
6.	Calyx-248	13.80 (21.81)	MR	9.4-26.4	32-82
7.	Suricha	13.80(21.80)	MR	9.4-26.4	32-82
8.	Pradhan	15.50(23.18)	MR	9.4-26.4	32-82
9.	Shivam	16.40(23.88)	MR	9.4-26.4	32-82
10.	NTH2350	17.73(24.91)	MR	9.4-26.4	32-82
11.	Vaishnavi 2082	16.20(23.733)	MR	9.4-26.4	32-82
12.	Lyco	17.40(24.65)	MS	6.8-22.2	48-96
13.	Ankit	16.63(24.06)	MR	9.4-26.4	32-82
14.	Lakshmi	13.50(21.55)	R	9.4-26.4	32-82
15.	Syngenta(To-1458)	19.40(26.25)	MS	8.3-23.3	46-93
16.	PPT-1	15.33(23.05)	MR	9.4-26.4	32-82
17.	PPT-2	14.46(22.35)	MR	9.4-26.4	32-82
18.	Arka Vikash	19.56(26.13)	MS	9.4-26.4	32-82
19.	US 2853	16.70(24.12)	MR	9.4-26.4	32-82
20.	P.Ruby	21.60(27.69)	MS	6.8-22.2	48-96
21.	Rohini	20.60(26.99)	MS	8.3-23.3	46-93
22.	Noble	13.70(21.72)	MR	9.4-26.4	32-82
23.	CLN	14.60(22.46)	MR	9.4-26.4	32-82
24.	Siroji	15.20(22.94)	MR	9.4-26.4	32-82
25.	Himgiri	16.73(24.14)	MR	9.4-26.4	32-82

*Figures in parentheses are angular transformed values

CD at 5%	0.418
CV	1.67
SEM	0.147

(b) Location 2: Polyhouse

S. No.	Variety/lines	Disease severity	Disease reaction	Temperature (ºC)	RH (%)
1.	Phule Raja	8.59 (17.05)	R	12.1-28.8	37-88
2.	Dhanshree	11.90 (20.17)	MR	11.8-26.4	44-87
3.	Bhagyashree	12.83(20.99)	MR	11.8-26.4	44-87
4.	Amrutha	13.10 (21.21)	MR	11.8-26.4	44-87
5.	Trisha	13.70(21.72)	MR	11.8-26.4	44-87
6.	Calyx-248	15.20 (22.94)	MR	11.8-26.4	44-87
7.	Suricha	15.16 (22.91)	MR	11.8-26.4	44-87
8.	Pradhan	16.10 (23.65)	MR	11.8-26.4	44-87
9.	Shivam	15.80(23.41)	MR	11.8-26.4	44-87
10.	NTH2350	14.06(22.02)	MR	11.8-26.4	44-87
11.	Vaishnavi 2082	12.80(20.96)	MR	12.1-28.8	37.88
12.	Lyco	17.56(24.77)	MS	9.4-26.4	32-82
13.	Ankit	15.40(23.10)	MR	10.2-27.8	38.4-91
14.	Lakshmi	18.70(25.61)	MR	11.8-26.4	44-87
15.	Syngenta(To-1458)	18.70(25.61)	MS	9.4-26.4	32-82
16.	PPT-1	12.900(21.04)	MR	11.8-26.4	44-87
17.	PPT-2	13.700(21.72)	MR	11.8-26.4	44-87
18.	Arka Vikash	24.100(29.40)	MS	11.3-29	36-90.6
19.	US 2853	13.36 (21.44)	MR	11.8-26.4	44-87
20.	P.Ruby	18.00(25.10)	MS	11.3-29	36-90.6
21.	Rohini	19.50(26.20)	MS	11.3-29	36-90.6
22.	Noble	13.10(21.21)	MR	11.8-26.4	44-87
23.	CLN	13.80(21.80)	MR	11.8-26.4	44-87
24.	Siroji	15.10(22.86)	MR	11.8-26.4	44-87
25.	Himgiri	17.40(24.65)	MS	11.3-29	36-90.6

*Figures in parentheses are angular transformed values

CD at 5%	0.72
CV	2.90
SEM	0.25

From the persual of the data presented in Table 4.20 (a), it is evident that the seedlings of all the lines/cultivars of tomato expressed limited disease symptoms on artificial inoculation with *Cmm*. Most of the lines/ cultivars showed resistant and moderately resistant reaction against the bacterial canker disease. However, five cultivars viz., Lyco, Syngenta (T0-1458), Arka Vikash, Pusa Ruby and Rohini exhibited moderately susceptible reaction amongst all the tested germplasm. Maximum disease expression was observed in cultivar P. Ruby (21.60%) followed by Rohini (20.60%) and Arka Vikash(19.56%). The minimum disease severity was observed in cultivar Phule Raja (7.70%), followed by Dhanshree (8.40%) and Bhagyashree (7.60%).

Results indicates that the different cultivars in the polyhouse condition when screened one variety i.e. Phule Raja exhibited resistant disease reaction with no symptom expression and least disease severity (8.59%) (Table 4.20, b) while most of the variety exhibited moderately resistant disease reaction. The variety showing maximum disease expression was Arka Vikas with a disease severity of 24.1% followed by Rohini (19.50%) and P. Ruby (18.00%). The expression of limited disease symptoms can be due to lower range of temperature during the growth period as the optimum temperature for the symptom expression has been reported to be between 25-30ºC by many workers **(EPPO Bulletin, 2016; Eichenlaub *et al.,* 2006). Chang *et al.* (1992)** reported that with the decline in temperature, the incubation period of the bacterium increases that leads to a slower development of disease. Expression of symptoms in some of the varieties may be due to relative humidity (87-97%) enhanced the symptoms in 2-3 week-old tomato seedlings **(Basu, 1966)**.

Season 2: Feb-June 2016

From the persual of data, Table 4.21a it also observed that most of the varieties/ lines exhibited susceptible and moderately susceptible reaction however five cultivars Viz., Trisha, NTH2350, Lakshmi, Arka Vikash and P. Ruby exhibited highly susceptible disease reaction. The variety showing maximum disease severity was Arka Vikash (66.10%) followed by Trisha (66.00%) and P. Ruby(64.80%). The variety showing least disease severity was Phule raja(23.90%) followed by Suricha (32.53%) and Calyx(33.90%).

Table 4.21 Reaction of tomato cultivars/ lines/ germplasm to bacterial canker of tomato (*Clavibacter michiganensis subsp. michiganensis*) in (a)open field and (b) polyhouse condition in Feb-June growth season

(a) Location 1: Open field

S. No.	Variety/lines	Disease severity	Disease reaction	Temperature (ºC)	R.H. (%)
1.	Phule Raja	23.90 (29.26)	MS	22.6-34.1	44.6-71.7
2.	Dhanshree	62.9 (52.47)	HS	14.0-31.3	28-80
3.	Bhagyashree	54.06 (47.33)	S	16.2-33.5	31-75
4.	Amrutha	49.50 (44.71)	S	16.2-33.5	31-75
5.	Trisha	66.0 (54.33)	HS	14.0-31.3	28-80
6.	Calyx-248	39.90 (39.17)	MS	24.5-33.8	52.9-72.7
7.	Suricha	32.53 (34.77)	MS	24.5-33.8	52.9-72.7
8.	Pradhan	46.10 (42.76)	S	22.6-34.1	44.6-71.7
9.	Shivam	56.63 (48.81)	S	22.6-34.1	44.6-71.7
10.	NTH2350	65.30 (53.90)	HS	13.5-29.1	37-83
11.	Vaishnavi 2082	55.70 (48.27)	S	19.2-36.0	30-66.7
12.	Lyco	47.10 (43.33)	S	19.2-36.0	30-66.7
13.	Ankit	23.00 (28.65)	MS	22.6-34.1	44.6-71.7
14.	Lakshmi	62.70 (52.35)	HS	13.5-29.1	37-83
15.	Syngenta(To-1458)	33.90 (35.60)	MS	23.7-33.8	48.4-72.1
16.	PPT-1	39.60 (38.99)	MS	23.7-33.8	48.4-72.1
17.	PPT-2	28.80 (32.45)	MS	23.7-33.8	48.4-72.1
18.	Arka Vikash	66.10 (54.39)	HS	16.2-33.5	31-75
19.	US 2853	47.70 (43.68)	S	16.2-33.5	31-75
20.	P.Ruby	64.80 (53.61)	HS	14.0-31.3	28-80
21.	Rohini	30.70 (33.64)	MS	25.5-32.9	67.7-86.3
22.	Noble	34.90 (36.21)	MS	25.5-32.9	67.7-86.3
23.	CLN	44.09 (41.61)	S	16.2-33.5	31-75
24.	Siroji	52.60 (46.49)	S	16.2-33.5	31-75
25.	Himgiri	58.90 (50.12)	S	25.9-31.2	74.9-91.1

*Figures in parentheses are angular transformed values

CD at 5%	0.33
CV	0.47
SEM	0.11

(b) Location 2: Polyhouse

S. No.	Variety/lines	Disease severity	Disease reaction	Temperature (ºC)	R.H. (%)
1.	Phule Raja	29.90 (33.14)	MS	26-35.3	55.7-68.3
2.	Dhanshree	65.20 (53.84)	HS	16.2-33.5	31-75
3.	Bhagyashree	56.00 (48.44)	S	26.-35.1	51.7-79.4
4.	Amrutha	46.80 (43.16)	S	21.3-36.3	29.6-66.6
5.	Trisha	70.10(56.87)	HS	16.2-33.5	31-75
6.	Calyx-248	40.10 (39.27)	MS	26.6-34.1	44.6-71.7
7.	Suricha	45.90(42.64)	S	17.9-37.8	29-64
8.	Pradhan	53.70(47.12)	S	17.9-37.8	29-64
9.	Shivam	57.90(49.54)	S	26.-35.1	51.7-79.4
10.	NTH2350	65.70(54.15)	HS	21.8-38.7	33-68
11.	Vaishnavi 2082	58.70(50.010)	S	17.9-37.8	29-64
12.	Lyco	58.70(50.01)	S	17.9-37.8	29-64
13.	Ankit	48.80(44.31)	MS	26.6-34.1	44.6-71.7
14.	Lakshmi	62.59(52.29)	HS	16.2-33.5	31-75
15.	Syngenta(To-1458)	48.70(44.255)	S	26.-35.1	51.7-79.4
16.	PPT-1	38.30(38.23)	MS	26-35.3	55.7-68.3
17.	PPT-2	41.80(40.28)	MS	25.5-32.9	67.7-86.3
18.	Arka Vikash	67.80(55.42)	HS	16.2-33.5	31-75
19.	US 2853	58.70(50.010)	S	17.9-37.8	29-64
20.	P.Ruby	65.90(54.27)	HS	16.2-33.5	31-75
21.	Rohini	60.30(50.95)	S	22.6-34.1	44.6-71.7
22.	Noble	33.80(35.54)	MS	26-35.3	55.7-68.3
23.	CLN	48.60(44.19)	S	22.6-34.1	44.6-71.7
24.	Siroji	52.80(46.60)	S	22.6-34.1	44.6-71.7
25.	Himgiri	57.80(49.48)	S	26.-35.1	51.7-79.4

*Figures in parentheses are angular transformed values

CD at 5% 1.948604

CV 2.223402

SEM 0.6852805

From the screening of the 25 different cultivars/lines of tomato in polyhouse it was observed that six of the varieties exhibited moderately susceptible disease reaction, thirteen of them exhibited susceptible disease reaction and six of them exhibited highly susceptible disease reaction, Table 4.21b. Highest disease susceptibility was observed in the variety Arka Vikas (67.80%), followed by P. Ruby (65.90%) and NTH2350 (65.70%). The variety exhibiting the minimum disease severity is Phule Raja (29.00%), followed by line PPT-1(38.30%) and variety Calyx-248(40.10%). In comparing the extent of disease severity in both location it was observed that more number of varieties tend to be susceptible in protected cultivation condition. The results are in confirmation of the findings of **(Carlton *et al.,* 1998)** as they have reported that the pathogen can inflict high economic damage in polyhouse condition investigated by many workers, the lack of resistant genotypes has hampered the successful management of *Clavibacter michiganensis* subsp. *michiganensis* **(Sen *et al.,* 2014).**

Summary and Conclusions

Chapter 5 SUMMARY AND CONCLUSION

Tomato (*Solanum lycopersicon* L.) is one of the most popular vegetable crops worldwide. Owing to its special nutritive value, it is considered as poor man's apple. Tomato, in India ranks third in production after potato and onion. Tomato is grown in an area of 776'000 ha with a production of 18,732 thousand MT (NHB database, 2016). However the crop is affected by various diseases caused by fungi, bacteria and viruses, resulting into significant losses in the crop production (Akram and Arjun, 2011). Tomato crop is severely affected by different bacterial diseases amongst them the bacterial wilt and canker disease of tomato caused by *Clavibacter michiganenis subsp michiganensis* is on increase in several states of India.

The present study was carried out during 2015-17 at GBPUA&T, Pantnagar with the an objective of acquiring sufficient knowledge related to the extent of the distribution spread of the bacterial canker and wilt disease of tomato in the lesser and outer Himalayan tomato growing belt of Himachal Pradesh and Uttarkhand, which could certainly help in identifying the location of pathogen spread. The bacterium is a quarantine pest according to EPPO classification and seed borne in nature therefore, the bacterium becomes more significant. The bacterium Cmm was isolated from two different agroclimatic zone of Uttarakhand and Himachal Pradesh. Five different tomato growing regions in Uttarakhand and nine tomato growing regions in Himachal Pradesh are surveyed to assess the spread of disease. The findings can be summarized below:

1. Amongst the two different agroclimatic regions surveyed for the presence of *Cmm* it was observed that the extent of the disease spread was very high in Himachal Pradesh with a mean disease incidence of 33.59% and disease severity was recorded to be 27.71% while in Uttarakhand the spread of disease is very with the disease incidence recorded to be 5.55% and the disease severity found to be 4.52%. In the entire region surveyed, to check the spread of *Cmm* in Uttarakhand the bacterium was isolated only from one region i.e. Gaulapar, that too with varied symptoms from the characteristics symptom produced by the pathogen. The cumulative mean disease incidence of both the state was 19.57% and disease severity was 16.11%.

2. The symptom of bacterial canker and wilt of tomato were observed on the different parts of tomato plant, in leaves the symptoms were observe in the form of marginal chlorosis followed by necrosis, canker formation in stem, bird's eyespot formation in fruit, unilateral wilting in branches followed by complete plant wilting and eventual death of the plant.Out of the 10 bacterial isolates of Cmm collected from different agrocliamatic region 16s rDNA sequencing was done and the isolates were identified as belonging to *Microbacterium* and *Curtobacterium* sp.

3. The 10 bacterial isolates were inoculated into cultivar A. Vikash, and all the isolates produced symptoms typical to *Cmm*. The cultivar *Cmm*10 was identified as the most virulent one from HP region and isolate *Cmm* 5 from Uttarkhand region. Hence, the pathogenecity of the bacterium was established and proved to be a potential pathogen.

4. Ten isolates of the test bacterium were established by biochemical, physical and morphological characterization.

5. Cultural variability was observed in the bacterial isolates in the form colony pigmentation, isolates *Cmm* 1, 3, 4, 5 showed yellowish pigmentation; Cmm2 showed creamish yellow pigmentation while isolates *Cmm* 6, 7, 8, 9, 10 showed orangish pigmentation.

6. The bacterial isolates were subjected to seven different nonselective and semiselective growth medium and NGY was the medium showing the fastest growth amongst the nonselective medium and D2ANX showing the fastest growth between the semiselective medium at four and ten days after plating.

7. The antagonistic potential of nine isolates of *Trichoderma* sp (B1-B7, Pant Biocontrol Agent-1, Anand isolate1). and two isolates of *P. fluorescens* were evaluated against *Cmm* by dual culture assay. PBAT-1 performed best which gave 89.5% inhibition of radial growth followed by B4 (87.8%), B3 (84.5%) and A1 (82.9%), whereas least inhibition was obtained by B2 (32.1%) and B7 (32.2%). *P. fluorescens* isolate PBAT-2 provide an effective control against the pathogen with a reduction of 91.34 % in colony diameter when used alone while in consortium with PBAT-1 it reduced the colony diameter upto74.5%.

8. In sensitivity test of *Cmm* to chemicals Streptomycin sulphate was found to be the most effective followed by the combination of Copper Sulphate+Bronopol .Maximum zone of inhibition was observed with Streptomycin sulphate (2.33cm) followed by Copper Sulphate+Bronopol (1.83cm), Bronopol (1.83cm). Least zone of inhibition (0.81cm) was observed with Copper sulphate at 600ppm concentration

9. In evaluating the defense inducers for the management of bacterial wilt and canker Salicylic acid (SA), 2,3- Benzothiodazole (BTH), Isonicotinic acid(INA) and Lysozyme were used as defense inducers in three different concentrations and at two different duration. Amongst the four treatments used, BTH followed by SA exhibited significant disease reduction as compared to control.

10. On biochemical analysis a significant increase in the Peroxidase (PO) activity was observed, comparing the two varieties enzymatic activity was less in US2853 in comparison to variety Rohini. Significant elevation of the PO activity was observed in plants in the variety Rohini and US2853 at 500µM) (2.21, 1.68)($p < 0.05$) followed by the plants sprayed with defense inducers at 200µM and 800 µM. Least enzymatic activity was observed in plants sprayed only with water followed by plant inoculated only with pathogen. In the plants given the spray of defense inducers after the inoculation of the pathogen, the enzymatic activity was the maximum in SA treatment at the concentration of 500µM followed by 200µM and 800µM.

11. On biochemical analysis a significant increase in the Phenyl ammonia lyase(PAL) activity was observed, the plants receiving the prophylactic activity of defense inducers, the maximum PAL activity was observed in the plants sprayed with BTH in the cultivar Rohini followed by variety US2853 (7.82,4.21, $p<0.05$) at 500µM concentration followed by enzymatic activity at 200µM and 800µM. In the plants receiving the spray of defense inducers after the inoculation of the pathogen the highest enzymatic activity was observed in the plants treated with SA at 500µM (5.37, 0.935).

12. On biochemical analysis a significant change in the Total Phenol Content was observed, higher contents were shown by the plants sprayed with BTH at 500

µM in cultivar Rohini and US2853 (2.47, 0.617, $p < 0.05$) followed by enzymatic activity at 200µM and 800 µM in both the cultivars. In the plants receiving the after spray of defense inducers at enzymatic activity was more in plants sprayed with SA in both the cultivars viz., Rohini and US2853(2.85,0.559,$p \leq .05$).

13. On biochemical analysis a significant change in the PolyPhenol Oxidase activity was observed, highest enzymatic activity was observed in plants sprayed with BTH at 500µM concentration in cultivar Rohini followed by US2853 (81.68, 61.30, $p \leq 0.05$) followed by enzymatic activity at 200µM and 800µM. In plants receiving treatment of defense inducers after inoculation with pathogen the maximum enzymatic activity was observed in plants treated with SA in both the cultivars viz., Rohini and US2853 (67.39,38.3, $p<0.05$) at 500µM concentration followed by enzymatic activity at 200µM and 800µM .

14. Amongst the four treatments given to the tomato plants at 500µM concentration it was observes that treatment with lowest disease severity was BTH (27.68%) and with highest disease severity was lysozyme (49.72%). When the plants received the treatments with defense inducers after the inoculation with the pathogen the treatment showing lowest disease severity was BTH (53.62) and the treatment showing highest disease severity was INA (62.94%).

15. Evaluation for most suitable inoculation technique for artificial disease condition on inoculation in tomato under glasshouse condition, syringe inoculation of stem at junction of first true leaf and stem inoculation with toothpick are judged as suitable methods for inoculation of bacterial canker pathogen *C. michiganensis* subsp. *michiganensis* to the tomato seedlings.

16. For establishing the most appropriate season for disease development, 25 variety are screened for disease reaction in the open field and protected condition in ambient temperature conditions during Sep.-Jan. and Feb.-June, the growth season Feb-June was found to be more appropriate for the spread of pathogen as all the 25 varieties screened showed a susceptible disease reaction for *Cmm.*

17. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect

ELISA technique. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect ELISA technique. The value of positive control was 0.538nm, while that of *Cmm*10 was 0.629nm; *Cmm*6 was 0.445 and of *Cmm*5 was 0.515. The OD value of negative control was 0.044.

18. For the molecular characterization all the three isolates were screened by the primer specific for genus *Clavibacter* giving an amplicon of size 1.45kb, while by the subsp specific primer out of three isolates only two isolates that is *Cmm*10, *Cmm* 6 are identified as *Cmm* giving an amplicon of size 614bp while *Cmm*5 was not detected.

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Appendices

APPENDIX I

Medium used in studies: Composition and preparation

All the medium used in this study were sterilized by autoclaving at 121ºC for 15 min. The pH of the medium was adjusted using NaOH or HCl before autoclaving. After autoclaving the medium was cooled down to 40-45ºC for pouring into petriplates.

1. **Nutrient broth yeast agar (NGY) medium**

Ingredients	**gms / Litre**
Nutrient Broth	8.0g
Yeast extract	2.0g
K_2HPO_4	2.0g
KH_2PO_4	0.5g
Glucose	2.5g
Agar	15.0g
DW	1000mL

After autoclaving add 1.0 ml of sterile 1M $MgSO_4.7H_2O$

2. **Sucrose Peptone Yeast Agar (SPY)medium**

Ingredients	**gms / Litre**
Yeast extract	4.0g
Sucrose	20.0g
KH_2PO_4	1.0g
$MgSO_4$	0.5g
Agar	15.0g

Suspend in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates or tubes as desired

3. **Yeast Dextrose Calcium carbonate Agar (YDCA) medium**

Ingredients	**gms / Litre**
Yeast extract	10.0g
Glucose	20.0g
Calcium carbonate	20.0g
Agar	15.0g
DW	1000mL

4. **Yeast Peptone Dextrose Agar (YDCA) medium**

Ingredients	**gms / Litre**
Peptone	10.0g
Yeast extract	5.0g
Dextrose	20.0g
Agar	15.0g
DW	1000mL

5. **Nutrient Agar (NA) medium**

Ingredients	**gms / Litre**
Peptone	10.0g
Beef extract	5.0g
Agar	15.0g
DW	1000mL

6. **SCM medium**

Sucrose	10g
K_2HPO_4	2g
KH_2PO4	0.5g
$MgSO_4.7H_2O$	0.1g
Yeast extract	0.1g
Nicotinic acid	0.1g
Boric acid	1.5g
Agar	15.0g
Nalidixic acid	30mg
K Tellurite	0mg
Cycloheximmide	200mg

The antibiotics were added separately after autoclaving and cooling the media in the laminar air flow.

7. D2ANX Medium

Glucose	10.0g
Casein hydrolysate	4.0g
Yeast	2.0g
Ammonium chloride	1.0g
Trizma base	1.2g
Agar	15.0g
Nalidixic acid	3mg
Cycloheximide	100 mg
Polymixin B Sulphate	13mg

The antibiotics were added separately after autoclaving and cooling the media in the laminar air flow.

APPENDIX II

Reagents used in Bacterial genomic DNA isolation

1M Tris buffer pH 8.0

Dissolve 12.11 g of tris base in 80 mL autoclaved distilled water. Adjust the Ph to 8.0 with 1N HCl. Adjust to 100mL with distilled water. Autoclave the buffer then use at the room temperature.

0.5M EDTA

Dissolve 18.621g Na_2EDTA (by adding 2g of NaOH pellets). Stir vigorously on a magnetic stirrer for several hours to ensure that all the solutes have dissolved. Adjust the volume upto 100mL.

5M NaCl

Dissolve 29.0g of NaCl in 80mL of deionized water and volume was adjusted finally to 100mL.

Working Solutions

DNA extraction buffer	**500ml**
1M Tris Buffer	50mL
0.5M EDTA	50mL
NaCl	14.61g

Adjust the pH to 8.0 with HCl. Make up volume upto 500mL. Autoclave at at 121ºC, 15psi for 15-20 min and store at 4ºC.

70% Ethanol

Absolute ethyl alcohol	70 mL
SDW	30mL

10% (w/v) SDS

SDS	10g
SDW	100mL

Heat at 60ºC to dissolve it properly and adjust the pH to 7.2 by HCl. Make up the volume to 100mL. Autoclave and store at room temperature.

CTAB solution	**10mL**
CTAB(10%)	2g
NaCl(0.7M)	14mL(1M stock)

Stored at room temperature

TE Buffer	**100mL**
1M Tris Buffer	1.0 mL
0.5M EDTA	0.2mL

Make up to 100mL. Autoclave and store at room temperature.

Isopropanol	**100mL**

Stored in -20ºC in dark colored bottles.

Phenol:Chloroform:Isoamyl alocohol	**500mL**
Chloroform	240mL
Phenol	250mL
Stored in brown colored reagent bottle at	-20ºC

Agarose Gel Electrophoresis

Reagents used and preparation

DNA loading dye	**10mL**
Bromophenol Blue (0.25%w/v)	0.025g
Sucrose (40%w/v)	4.0g

Dissolve properly; makeup volume to 10ml. Store at -20ºC.Use sterilized distilled water.

Electrophoresis Buffer (5X TBE)	**300ml**
Tris base	270g
Boric acid	137.5g
EDTA(0.5M)	100ml
SDW	200mL

Ethidium Bromide (10,000X)	**10mL**
EtBr	100mg
DW	10mL

Store at 4ºC. Ethidium Bromide is highly carcinogenic. Use gloves while handling.

VITA

The authoress, Ruchi Tripathi was born on 18th January, 1990 in Haldwani, Uttarakhand. She passed her High School Examination in 2005 and Intermediate Examination in 2007 from St. Paul's Senior Secondary School, Kathgodam. She earned her B.Sc. degree in 2012 from G B Pant University of Ag. & Tech., Pantnagar & M.Sc. degree in Mycology and Plant Pathology in 2014 from B.H.U.., Varanasi. In August 2014, she joined G.B.P.U.A.& T., Pantnagar for her Ph.D. programme with major in Plant Pathology.

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Semester & Year of Admission: I[st] & 2014-15 **Degree** : Ph.D.
Major : Plant Pathology **Department** : Plant Pathology
Minor : Entomology
Thesis Title : **"Exploration of different methods for detection of *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis, the cause of bacterial wilt and canker of tomato (*Lycopersicon esculentum* M.) and its management"**
Advisor : Dr. K. Vishunavat

ABSTRACT

Solanum lycopersicon (L.) is one of the prime vegetable crops grown in different parts of the world. The yield potential of this crop is affected by a number of diseases. Bacterial wilt and canker caused by *Clavibacter michiganensis* subsp *michiganensis (*Smith) Davis is one of the most destructive bacterial disease of tomato. Considering the devastating nature of the pathogen and availability of little knowledge on the spread and successful management of *Cmm* through chemical compounds , biocontrol agents and defense inducers, present investigation was carried out with with the following objectives to study ; (1) To record the incidence and severity of bacterial canker of tomato in Middle and Lesser Himalayan regions in Uttarakhand and Himachal Pradesh., (2)To detect the isolated bacterial pathogen through morphological, physiological, biochemical, serological and molecular characterization, (3)To evaluate available germplasm (cultivars, lines) against the bacterial pathogen in protected (glasshouse and polyhouse) and open field condition, (4)Study of pathogenic variability existing among *Clavibacter michiganensis subsp michiganensis* isolates under glasshouse conditions through different inoculation techniques, (5)Screening of test chemicals and biocontrol agents against the test pathogen under *invitro* and *invivo* condition and assessment of population dynamics of the pathogen, (6) Study of defense inducers by explicating the biochemical basis of resistance in plant in *in vitro* conditions. The antagonistic potential of nine isolates of *Trichoderma* sp (B1-B7, Pant Biocontrol Agent-1, Anand isolate1). and two isolates of *P. fluorescens* were evaluated against *Cmm* by dual culture assay. PBAT-1 performed best which gave 89.5% inhibition of radial growth followed by B4 (87.8%), B3 (84.5%) and A1 (82.9%), whereas least inhibition was obtained by B2 (32.1%) and B7 (32.2%). *P. fluorescens* isolate PBAT-2 provide an effective control against the pathogen with a reduction of 91.34 % in colony diameter when used alone while in consortium with PBAT-1 it reduced the colony diameter upto74.5%. In sensitivity test of *Cmm* to chemicals Streptomycin sulphate was found to be the most effective followed by the combination of Copper Sulphate+Bronopol .Maximum zone of inhibition was observed with Streptomycin sulphate (2.33cm) followed by Copper Sulphate+Bronopol (1.83cm), Bronopol (1.83cm). Least zone of inhibition (0.81cm) was observed with Copper sulphate at 600ppm concentration .In evaluating the defense inducers for the management of bacterial wilt and canker Salicylic acid (SA), 2,3- Benzothiodazole (BTH), Isonicotinic acid(INA) and Lysozyme were used as defense inducers in three different concentrations and at two different duration. Amongst the four treatments used, BTH followed by SA exhibited significant disease reduction as compared to control. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect ELISA technique. In serological characterization of the *Cmm* isolates by ELISA it was observed that out of ten isolates three isolates were characterized to be *Cmm* by indirect ELISA technique. The value of positive control was 0.538nm, while that of *Cmm*10 was 0.629nm; *Cmm*6 was 0.445 and of *Cmm*5 was 0.515. The OD value of negative control was 0.044. For the molecular characterization all the three isolates were screened by the primer specific for genus *Clavibacter* giving an amplicon of size 1.45kb, while by the subsp specific primer out of three isolates only two isolates that is *Cmm*10, *Cmm* 6 are identified as *Cmm* giving an amplicon of size 614bp while *Cmm*5 was not detected. After evaluation for most suitable inoculation technique for artificial disease condition on inoculation in tomato under glasshouse condition, syringe inoculation of stem at junction of first true leaf and stem inoculation with toothpick are judged as suitable methods for inoculation of bacterial canker pathogen *C. michiganensis* subsp. *michiganensis* to the tomato seedlings. For establishing the most appropriate season for disease development, 25 variety screened for disease reaction in the open field and protected condition in ambient temperature conditions during Sep.-Jan. and Feb.-June, the growth season Feb-June was found to be more appropriate for the spread of pathogen as all the varieties showed a susceptible disease reaction for *Cmm.*

(K. Vishunavat)
Advisor

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सत्र एवं प्रवेष का वर्ष	: **प्रथम, 2014–15**	**उपाधि** :	**पी0 एच0 डी0**
प्रमुख विषय	: **पादप रोग विज्ञान**	**विभाग** :	**पादप रोग विज्ञान**
गौण विषय	: **कीट विज्ञान**		
शोध का विषय	: *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis **द्वारा टमाटर में जीवाणु जनित उकठ्ा एवं छाला रोग के कारक की विभिन्न तकनीकों द्वारा जाँच एवं उसका प्रबन्धन**		
सलाहकार	: **डा0 के0 वीशुनावत**		

सारांश

लाइकोपर्सिकोन एस्क्यूलेंटम (एम0) विश्व की प्रमुखतम् सब्जी वर्गीय फसलों में से एक है। इस फसल की उत्पादन क्षमता को विभिन्न कवक जनित, जीवाणु जनित एवं विषाणु जनित रोग प्रभावित करते हैं। *Cmm* (Smith) Davis द्वारा जनित टमाटर का जीवाणविक उक्ठा एवं छाला रोग टमाटर की फसल के लिए अत्यन्त विनाशकारी है। इस रोग की हानिकारक प्रवृत्ति एवं (1) रसायनिक, जैविक नियंत्रण, रोग प्रतिरोधिता उत्पन्न करके एवं सीएमएम के विलमों का लक्षण वर्णन, (2) कांचहार परिस्थितियों में रोगजनक क्षमता एवं कृत्रिम महामारी उत्पन्न करने की विधियाँ, (3) कांचहार परिस्थितियों में सीएमएम के विलमों में उपस्थित रोगजनक क्षमता की विविधता, (4) सीएमएम के विलमों के उपस्थित आंणविक विविधता एवं सीरम विविधता, (5) पात्रे एवं जीवे परिस्थिति में जैव नियंत्रकों की रोगजनक के विरूद्ध एन्टागोनिस्टिक क्षमता, (6) खुले मैदान एवं कांचहार परिस्थिति में दो विभिन्न मौसमों में टमाटर की 25 किस्मों की जाँच, (7) प्रतिरोधी उत्पन्न उपचार में यथा रसायनों SA, INA, BTH and Lysozyme की रोगजनक के विरूद्ध प्रबंधन क्षमता की कांचहार परिस्थिति में जाँच। टेरिको एसपी. के विलमों का सीएमएम के विरूद्ध ड्यूअल कल्चर विधि द्वारा परीक्षण किया गया। सर्वाधिक त्रिज्यक वृद्धि को PBAT-1 ने हटाया (89.5 प्रतिशत) तत्पश्चात् B_4 (87.8 प्रतिशत), B_3 (84.5 प्रतिशत) एवं A_1 (82.8 प्रतिशत) जबकि न्यूनतम B_2 (32.1 प्रतिशत) हटाया। सीएमएम की कालौनी को अवरूद्ध करने में PSP प्रभावी (91.34 प्रतिशत) पाये गये। सीएमएम की रसायनों के प्रति संवेदनशीलता परीक्षण में 600 पीपीएम सांद्रता पर सर्वाधिक अवरोध क्षेत्र (2.23 सेमी0) S.S. तथा CS+BP (1.83 सेमी0), BP (1.83 सेमी0) पाया गया। हिमाचल प्रदेश एवं उत्तराखण्ड के विभिन्न भागों से एकत्रित सीएमएम के विलमों की सीरम परीक्षण द्वारा जांच करने पर सीएमएम विलग 10, 6, 5 सही पाये गये एवं सीएमएम 10, 5, 6 विलगों का जीनस विशिष्ट प्राइमर द्वारा जांच करने पर तीनों ही विलग सही पाये गये। सीएमएम के विलगों सीएमएम 10, 6, 5 का उपप्रजाति विशिष्ट प्राइमर से आंणविक लक्षण वर्णन करने पर सीएमएम विलग 10, 5 सही पाये गये। कांचहार में कृत्रिम टीकाकरण प्रशिक्षण द्वारा कृत्रिम बीमारी की स्थिति उत्पन्न करने हेतु सबसे उपयुक्त टीकाकरण तकनीक की जांच करने पर सिरिंज टीकाकरण तकनीक एवं दंतखुदमी के साथ तना टीकाकरण तकनीक को सर्वाधिक उपयुक्त पाया गया। रोगजनक की आक्रामकता विश्लेषण में सीएमएम के तीन विलग 10, 5, 6 सर्वाधिक आक्रामक पाये गये। खेत की परिस्थितियों में रसायनों एवं जैव नियंत्रकों की रोग नियंत्रण क्षमता का परीक्षण करने पर CS+BP को सर्वाधिक उपयुक्त पाया गया।

(के0 वीशुनावत)
सलाहकार

(रूचि त्रिपाठी)
लेखिका