Syringomycin and Levan sucrase as potential virulence biomarkers for early detection of the Phytopathogen *Pseudomonas syringae*.

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IN

BIOTECHNOLOGY

Under the supervision of:

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JULY 2022

DECLARATION

I, the undersigned, hereby declare that the research work presented in the thesis entitled "**Syringomycin and Levan sucrase as potential virulence biomarkers for early detection of the Phytopathogen *Pseudomonas syringae***" in partial fulfilment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology (DBT), TIET, Patiala, is an authentic record of my work during the period of February 2022 to July 2022 under the supervision and guidance of Dr. Moushumi Ghosh, Professor, Department of Biotechnology, TIET, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or examining body in India/elsewhere.

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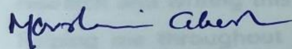
CERTIFICATE

This is to certify that dissertation entitled, "Syringomycin and Levan sucrase as potential virulence biomarkers for early detection of the Phytopathogen *Pseudomonas syringae*" submitted by Ms. Savi Gupta in partial fulfilment of the requirements for the award of M.Sc. Biotechnology at Thapar Institute of Engineering and Technology, Patiala is an authentic work carried out by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in this dissertation has not been submitted to any other university/institute for award of any Degree or Diploma.



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Abstract

Bacterial plant pathogens wreak a huge loss across the globe incurring disruption of economy and nutrition. Of these, infections caused by *Pseudomonas syringae* in plants has been implicated as foremost amongst others. Timely detection and intervention are crucial to prevent loss. For the rapid and reliable clinical treatment of any disease caused by bacteria, a specific diagnosis of the pathogen or its associated toxins is the foremost and essential step. Early detection is a primary step towards treatment of a disease. Traditional techniques rely on culturing of bacteria, biochemical assays, followed by molecular and immunological methods to identify and subsequently decide on further preventive processes. These processes are either time consuming, costly, or less specific and not possible immediately. Therefore, to facilitate rapid diagnosis immunosensors, specifically targeted on virulence markers have been advocated. Several methods in addition for instance Loop Mediated Isothermal Amplification has shown promising results and can be used for amplification of bacterial genes in the sample for detection purposes rapidly with more specificity. A newer approach which targets specific exopolysaccharides released by bacteria as markers for detection. Such exopolysaccharides (LSc) are produced specifically by extracellular enzymes (LVase) released by the pathogen and enables the persistence and proliferation of the pathogen. Appropriate molecular methods can follow thereafter thus assuring accuracy in pinpointing the pathogen. In view of this, the current study aimed to develop a sensitive, specific, and easy-to-use diagnostic platform for rapid early-stage detection of *P.sy*. The major polysaccharide Levan, principal extracellular enzyme Levanucrase (Lsc) and plant toxin specific genes of *P.sy* were used as hallmarks for infection.

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List of Abbreviations and Symbols

S.NO.	ABBREVIATION	FULL FORM
1.	*P.sy*	*Pseudomonas syringae*
2.	AST	Antibiotic Susceptibility Test
3.	LAMP	Loop-mediated Isothermal Amplification
4.	PCR	Polymerase Chain Reaction
5.	EPS	Exopolysaccharides
6.	ZOI	Zone Of Inhibition
7.	mg	milli gram
8.	ml	milli litre
9.	UV	Ultra-Violet
10.	MTCC	Microbial Type Culture Collection
11.	bp	base pair
12.	V	Voltage
13.	mA	milli Ampere
14.	g	gram
15.	mm	milli metre
16.	nm	nano metre
17.	OD	Optical Density
18.	FIP	Forward Internal Primer
19.	BIP	Backward internal Primer
20.	LF	Loop Forward Primer
21.	LB	Loop Backward Primer
22.	L	Litre

23.	µm	micro metre
24.	Mbp	Mega base pair
25.	LOPAT	Levan production, Oxidase activity, Potato soft rot, arginine dihydrolase Activity and tobacco hypersensitive response test
26.	GATT	Gelatin liquefaction, Aesculin hydrolysis, Tyrosinase activity and Tartrate test
27.	rpm	revolutions per minute
28.	ºC	Degree(s) Celsius
29.	w/v	weight by volume
30.	%	Percentage
31.	v/v	volume by volume
32.	NCM	Nitro Cellulose Membrane
33.	PAMP	Pathogen Associated Molecular patterns
34.	NEB	New England Biolabs

1. INTRODUCTION

1.Introduction

Pseudomonas syringae (order *Pseudomonadales* and family *Pseudomonadaceae*) is a gram negative, rod shaped flagellated bacteria having chemohetrotrophic mode of nutrition and requires aerobic environment to grow (Bastas et al. 2012 , Plessis et al. 2012 and Arnold et al. 2019). The size of typical *Pseudomonas syringae* cells are 1.5µm in length and 0.7-1.2µm in diameter requiring optimal temperature between 22ºC and 30ºC to grow. It is a phytopathogen causing infection to diversity of plants having 60 pathovars of different host variety, therefore is used as a model organism to study infection in plants. *Pseudomonas syringae pv. Tomato* has become one of the most important model organism because it can even infect model plant Arabidopsis. This bacteria is usually found as epiphytes growing on huge range of plants, including water bodies (Arnold et al. 2019 and Hofte et al. 2007). *Pseudomonas syringae* grows, multiplies, and colonizes inside the intercellular spaces of plants such as in leaves and other plant tissues thus utilizing nutrition from the host making infection from it an economically important disease. This bacteria releases certain secondary metabolites such as toxins, hormones, pectolytic enzymes, exopolysaccharides, etc. leading to infection, so researchers nowadays from fields like genomics, metabolomics, metabolic modelling, and transcriptomics have been offering new methods to study this host-plant interaction (Rico et al. 2011 and Hofte et al. 2007)). Genome sequencing and molecular characterization of *P.sy* is also done to study the host-plant interaction. The genomic DNA of *P.sy* is 6.09Mbp. It was the years between 2003-2005 when the whole genome sequencing of three pathovars of *Pseudomonas syringae* was published i.e of *Pseudomonas syringae pv. Tomato*, *Pseudomonas syringae pv. syringae* and *Pseudomonas syringae pv. phaseolicola* but with advancement in sequencing now, whole genome sequence of many other pathovars is also known (Arnold et al. 2019).

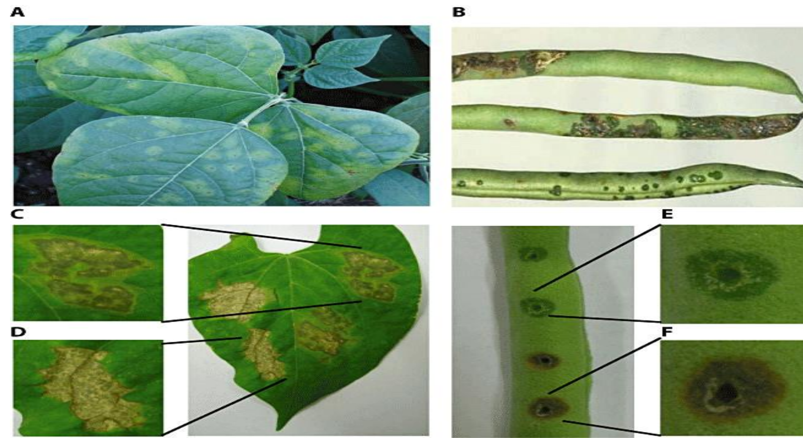


Figure 1.1: Symptoms caused by *Pseudomonas syringae* pv. *phaseolicola* on *beans*. (A) bean pods, (B) Symptoms on artificially inoculated bean, (C) water-soaked necrotic lesions , (D) tissue collapse and browning of the inoculated area, (E) and (F) demonstrate the disease response and HR. (Arnold et al. 2011)

Pseudomonas syringae infect a wide variety of plants such as tomatoes, mangoes, apples, olives, kiwi, almonds, legumes, stone fruits etc. and cause diseases to annual crops, such as halo blight on beans, bacterial speck on tomato, bleeding canker on horse-chestnut to woody plants and trees and a recently occurred epidemic of kiwifruit canker. Symptoms include lesions, blossom necrosis, reddening of tentacles, greenish yellow haloes on leaves, patches on seed coat, stem spots, chlorosis, soft rot, blights, galls, stunting, extensive twig die-backs, distortion of growth and cankers. Ice-nucleation strains of *P.sy* causes frost damage in crops. *P. syringae* colonizes plant tissues such as fruit, seedlings, leaves, seeds, pods, stems, petioles and bark. (Arnold et al. 2019, Hofte et al. 2007, Arnold et al. 2011 and Scortichini et al. 2012).

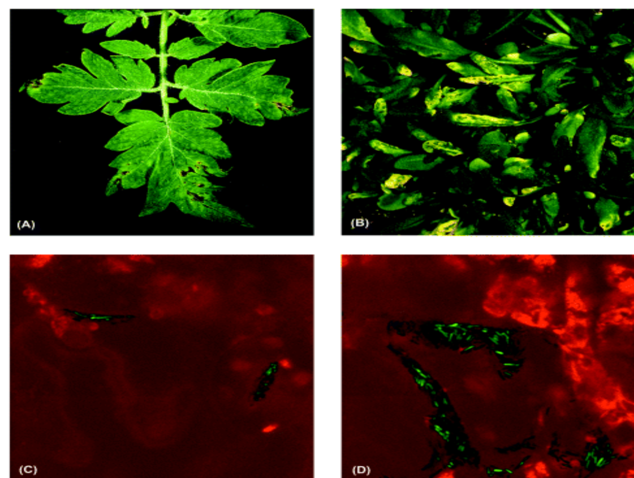


Figure 1.2: (A) and (B) Symptoms caused by *P. s.* pv. *Tomato* (C) and (D) Optical sections obtained using confocal microscopy of a tomato leaf 4 days after infection with *P. s.* pv. *tomato.* (Preston et al. 2000)

Bacterial Canker of green Kiwifruits and yellow Kiwifruits was first time reported in turkey (Bastas et al. 2012). Countries like Italy and New Zealand have experienced a great economic loss due to epidemic of Kiwifruits where Kiwi is the main crop followed by countries like Chile, South Korea, Spain, Japan, France, and Portugal, (Scortichini et al. 2012). Pseudomonas syringae have caused seed decay and Bacterial Umbel blight on Coriander leaves in Germany (Toben et al. 1996). Bacterial canker of plum has caused 30% mortality of plum trees (Geng et al. 2014). Infection of wheat by *P.sy* have decreased the yield in Germany by 50% (Botin et al. 2012). Bacterial leaf spot of squash and cantaloupe by *P.sy* was reported in Georgia (Langston et al. 2003). Infection of tomato plants by *P.sy pv. tomato* has been first time reported in United states and recently has been reported in China. This decreased the tomato yield in China from 30-80% (Chen et al. 2020).

Due to this huge agricultural and economic loss caused by infection from *Pseudomonas syringae* pathovars there is a need to prevent these diseases. Early detection of a disease is therefore the primary step towards prevention of any disease. Several techniques like culturing of bacteria in King's B selective medium, biological assays like LOPAT and GATTs and serological tests have been used for the detection of the infection but these methods have their drawbacks in terms of being less accurate and time consuming (Goudarzi et al. 2020). Early detection is important so that plant could be saved and treated before infection spreads and starts to show symptoms.

Detection tools simple, reliable, specific and less time consuming are warranted. Therefore, the main aim of this study was to use genomic and specific physiological markers which signify virulence for developing a detection tool which can be utilized as a point of care biosensor at later stage following suitable improvisations.

Thus, the objectives of this study were designed as follows:

1. Evaluation of virulence mechanisms of *P.sy*

2. Deciphering the pathogenic /epiphytic fitness of *P.sy*

3. Detection of psy (Syringomycin gene), by a loop-mediated isothermal amplification (LAMP) system.

4. Establishing Levansucrase in *Pseudomonas syringae* as virulence target for designing suitable biosensing marker

2. REVIEW OF LITERATURE

2. Review of Literature

2.1 Taxonomy of *Pseudomonas syringae*

Earlier *Pseudomonas syringae* was referred to as pathogen of lilac plants but after updation of bacterial names in 1980, it was found that *Pseudomonas syringae* actually has around 40 pathovars. Later on with advancement in technology like Multilocus sequence analysis and DNA-DNA hybridization it is now known to have 60 pathovars with 15 species (Young et al. 2010). Taxonomy of *P.sy* was difficult because bacteria of different plants come under the same species name. Classification of bacteria that was done in 1980 was the solution to this problem because now species were divided based on pathovars i.e., the host range to which bacteria infects (Gomila et al. 2017). The identification and classification are not only important for convenient study but also to study epidemiology of this bacteria. Complete genome sequencing of Pseudomonas syringae was done and strains that were misclassified were corrected whereas novel strains were detected (Gomila et al. 2017).

2.2 Classification of *Pseudomonas syringae*

It was thought earlier that all the Pseudomonas species have gram-negative, rod-shaped bacteria with chemoorganotrophic mode of nutrition and have motile properties with polar flagella. Only after 16S rDNA studies it is found that Pseudomonas have fluorescent as well as non-fluorescent species therefore, it was grouped into, gamma, alpha and beta proteobacteria. Fluorescent species were grouped in gamma Proteobacteria while non-fluorescent was grouped in beta Proteobacteria (Young et al. 2010 and Mulet et al. 2010). Pseudomonas has two phylogenetic lineages, one is *Pseudomonas fluorescens* and other is *Pseudomonas aeruginosa* (Mulet et al. 2010). *Pseudomonas fluorescens* have many further lineages out of which one is *Pseudomonas syringae* complex which is the species with the greatest number of phytopathogens (Bull et al. 2010). *Pseudomonas syringae* further have 13 phylogroups (Xin et al. 2018).

2.3 *Pseudomonas syringae*- A phytopathogen

P.sy is one of the most common plant pathogen infecting hundreds to plant species. It exists as epiphytes on the surface of plants causing infestation. To create infection, it penetrates inside the plant through wounds, stomatal openings etc., and multiplies in the apoplast. This makes *Pseudomonas syringae* a model pathogen to study epidemiology, ecology, host plant interaction, microbial ecology, adaption by bacteria to enter inside the host as well as bacterial mechanisms to cause virulence (Xin et al. 2018). There are several factors due to which *Pseudomonas syringae* can cause infection which includes, releasing toxins, exopolysaccharides, cell wall degrading enzymes, effector proteins etc. Since this bacteria can enter inside the plant through stomatal openings, plants have adapted a defense mechanism which includes closure of the stomatal opening called as PAMP induced stomatal closure. To overcome this defense mechanism by plants, *Pseudomonas syringae* releases certain toxins to infect plants. After releasing toxins and EPS, it enters inside the plant, suppresses the plant's immunity by basal defense, nonhost resistance, gene by gene resistance and will create a niche in the apoplast. External environments such as temperature, rainfall and humidity are also an important factor for *Pseudomonas syringae* to cause infection. Due to all this, *P.sy* is an excellent model to study bacteria-host interaction (Xin et al. 2018). When pathogen *P.sy* enters inside the host cell, it divides intracellularly in the absence of host cell death but then in later stages of pathogenesis, cell death occurs, and host cell undergoes necrosis (Nomura et al. 2005).

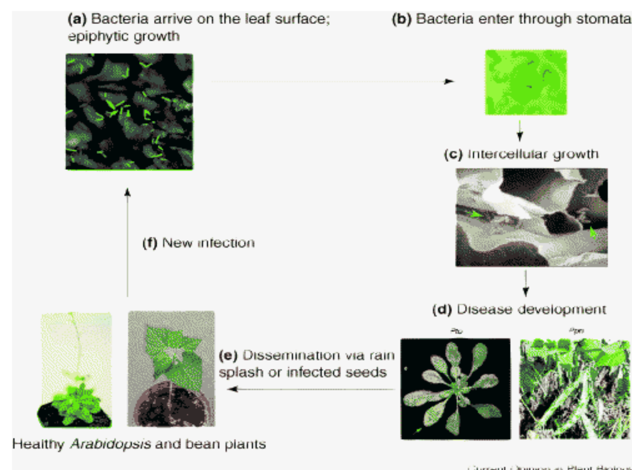


Figure 2.1: Infection mechanism of P.sy (Nomura et al. 2005)

2.4 Symptoms of infection caused by *Pseudomonas syringae*

Symptoms of *Pseudomonas syringae* infection includes frost damage due to ice nucleation activity, bacterial canker, chlorosis, rot, flower and shoot blight, water-soaked lesions, diebacks, necrosis, gall, distortion, and stunting of growth (Arnold et al. 2019, and Arnold et al. 2011).

2.5 Host range of *Pseudomonas syringae*

Pseudomonas syringae infects a wide variety of species of plants such as, rice crops, beets, beans, tomato, olives, stone fruits, nuts, almonds, mangoes, cabbage, plum, okra, cantaloupe, orchids, kiwifruit, broccoli, herbaceous dicots, monocots, woody dicots and many more. Its epidemic is seen worldwide in areas like South America, Netherlands, Oceania, Asia, Northwest Europe, New Zealand, China, Germany etc. (Lamichhane et al. 2014).

2.6 Interaction of *Pseudomonas syringae* with model plant Arabidopsis

Since *Pseudomonas syringae* infects diversity of host ranges and is very specific when it comes to infection of host, it makes it a model phytopathogen (Hirano et al. 2000). Not only *Pseudomonas syringae* itself is a model phytopathogen but also infects model plant Arabidopsis in laboratory conditions making study of interaction of *P.sy* and Arabidopsis feasible for plant pathologists (Mittal et al. 1995). *Pseudomonas syringae* was the first phytopathogen to cause infection to Arabidopsis in laboratory conditions. When 10^8 per ml of P.sy culture was spread onto Arabidopsis it led to formation of water-soaked lesions on leaves which became necrotic later followed by chlorosis, giving appearance of speck disease. *P.sy* and Arabidopsis interaction led to lot of research in plants defense response, signal transduction pathways, host susceptibility, recognition of pathogen by plants and so on (Mittal et al. 1995).

2.7 Epidemics caused by *Pseudomonas syringae*

Every year there was a remarkable increase in the number of diseases caused by *Pseudomonas syringae*. In the starting of 21st century, 72 reports of *P.sy* infection outbreaks were reported causing infection in 40 plants and 20 countries. 3% of cases were reported in South America, 3% in Oceania, 36% in Asia, 1% in Africa, 26% in Europe, and 31% in North America (Lamichhane et

al. 2015). *Pseudomonas syringae* caused infection to Kiwifruits leading to epidemic in Italy in 2008. Later, the epidemic spread to other areas like South Korea, New Zealand, Spain, South America, Oceania, Asia etc., where kiwifruits were grown. Similarly, it caused infection to horse chestnuts leading to an epidemic in the Netherlands in 2002 which saw spreading of the infection further to Northwest Europe (Lamichhane et al. 2014). Foliar disease caused by *P.sy* is very common in fruits. Every year, diseases caused by *P.sy* infection accounts for 50-100% of the epidemic rates in plants. With the increase in reports of diseases in plants every year, there has been a tremendous increase in the reports associated with *Pseudomonas syringae* infection. Disease outbreaks of few Brassica species have also been reported (Lamichhane et al. 2015). Yield of plant decreases due to flower abortion, decreased photosynthetic capacity, defoliation etc. It was recently reported that there has been a 30-80% decrease of yield of Tomato plants in China due to *P.sy* infection (Chen et al. 2020). Although tomato plants are the most effected by *Pseudomonas syringae*, there have been other instances as well.

1/5th of the coriander yield was reduced in Australia. There was 50% reduction in yield of melons and tomato plants in Greece caused by *P.sy* infection. 10,000 chrysanthemum plants were found destroyed in a Greek nursery, epidemic of sweet onion was reported in Georgia in United States of America, and 80% of cantaloupe fields were destroyed in Southwest France leading to 100% yield loss. There has been 5-50% wheat loss and 40% bean loss reported in United States. Angular leaf spot in cucumber fruit reduced the yield from 37-40% whereas loss of celery infection by *Pseudomonas syringae* was even worse. Further loss of carrots yield was reported in New Zealand and of potato tubers in Brazil from *P.sy* infection.

Noting these incidences, it can be realized that immediate action must take place to eradicate the infection caused by *Pseudomonas syringae* (Lamichhane et al. 2015)

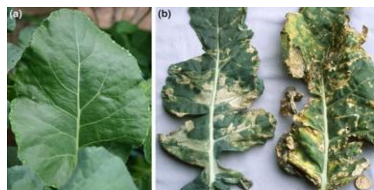


Figure 2.2: Infection of broccoli leaves caused by P.sy (a- represents healthy leaf, b- represents infected leaf) (Lamichhane et al. 2015)

2.8 Resistance of *Pseudomonas syringae* to antibiotics

Highly resistant strains of *Pseudomonas syringae* towards Streptomycin causing infections to kiwifruits were found in Japan and Korea. StrA and StrB genes were found in them encoding enzymes which could inactivate Streptomycin. Strains isolated from Korea had plasmid in them containing Streptomycin resistance genes while strains isolated from Japan had StrA and StrB in their plasmid. Their resistance were analyzed by susceptibility tests, PCR based methods and nucleotide sequence analysis. (Han et al. 2003). It was found that most strains of *Pseudomonas syringae* were resistant to copper and Ampicillin antibiotic while very few strains were resistant to other antibiotics as well. The resistant strains had the ability to nucleate ice (Hwang et al. 2005). Resistance of *Pseudomonas syringae* to copper was the reason that many countries refused the usage of copper as an antimicrobial factor for killing plant pathogens (Cameron et al. 2014).

2.9 Toxins produced by *Pseudomonas syringae*

There are many toxins produced by *P.sy* as mentioned above in order to cause infections to plants. *P.sy* produces four main toxins: syringomycin, coronatine, phaseolotoxin, and tabtoxin (Cameron et al. 2014). Syringotoxins, syringomycin and syringostatins are the complex Syringopeptins (Serra et al. 1999). Syringomycin belongs to the class of lipodepsinonapeptides which leads to electrolyte leakage from pores formed by the bacteria in the host plasma membrane (Cameron et al. 2014). It was seen that production of Syringomycin by *Pseudomonas syringae* in potato dextrose broth (PDB) was almost 20 to 136 times higher than that in potato dextrose agar (PDA) or in PDB shake cultures. With the advancement of technology, the genes responsible for these toxins are known, therefore, this knowledge could be exploited for detection purposes. It was also seen that Syringomycin gene (SyrD) could be used as a marker for detection of *Pseudomonas syringae* (Goudarzi et al. 2020).

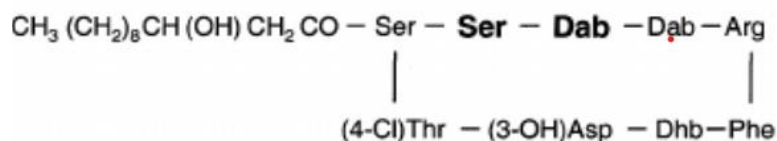


Figure 2.3: Structure of syringomycin

2.10 Exopolysaccharides released by *Pseudomonas syringae*

Exopolysaccharides are the secondary metabolites released by bacterial cultures into the growth medium or found in the extracellular spaces. They are advantageous for the bacteria in terms of absorbing nutrients and minerals, absorption of water etc. (Yu J et al. 1999). When *Pseudomonas syringae* was grown on media containing Glucose or Sucrose it was found that exopolysaccharides could be isolated from the culture (Osman et al. 1986). It was found that production of exopolysaccharides for *Pseudomonas syringae* culture, depends on the type of culture it is grown in to obtain the desired amount of interested EPS (Osman et al. 2012). After the isolation of exopolysaccharides from the *Pseudomonas syringae* culture and further analyzing and characterizing, it was found that EPS contains Levan (a fructan), alginate, mannuronic acid and guluronic acid. When grown on sucrose rich media, it was found that Levan was the major EPS produced but alginate was found majorly in water soaked lesions (Yu J et al. 1999). The slimy layer consisting outside the bacteria is made out of Levan and alginate. Beside Levan and alginate the third lipopolysaccharide present is planta (Rudolph et al. 1997). These exopolysaccharides are required by the bacteria for causing the infection in the plants and for the formation of biofilm (Ponce et al. 2021) therefore, these EPS can be used as a marker for *Pseudomonas syringae* detection.

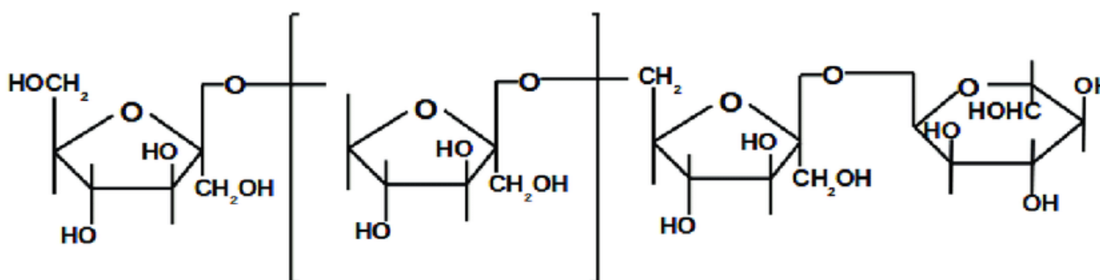


Figure 2.4: Structure of Levan biopolymer

2.11 Detection of *Pseudomonas syringae*

There have been tests performed and tests that can be administered to detect the presence of of *Pseudomonas syringae*. These include culturing of the bacteria on nutrient agar plate, liquid assay test, pathogenicity test, Tobacco-hypersensitivity response test, soft rot test, temperature sensitivity test, potassium hydroxide solubility test, gram staining reaction, Kovac test, sugar utilization test, arginine Di hydrolase activity test, and catalase test (Shila et al. 2013) but all these tests were found to be either time consuming and/or not specific. Also, these methods have disadvantages like false-positivity and cross-reactivity (Nzelu et al. 2019).

Name of the seed sample	Pathogenicity test	Tobacco Hypersensitivity reaction	Gram staining reaction	KOH solubility test	Kovac's oxidase test	Levan test	Sugar fermenttion test				Arginine dehyrolyse Test	Catalase test
							Dextrose	Sucrose	Manitol	Lactose		
Sweet gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Wax gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Bottle gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Bitter gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Snake gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Sponge gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Ridge gourd	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+
Cucumber	+	+	Gram negative	Gram negative	-	+	+	+	+	+	-	+

Figure 2.5: Biochemical tests for detection of *P.sy*

PCR based techniques have been used for the amplification of SyrD gene (1040 bp fragment), which is required for Syringopeptin and Syringomycin secretion (Bultreys et al. 1999). Though molecular based techniques have been there for the detection of *Pseudomonas syringae,* these techniques were not always specific so, multiplex PCR based technology was the new method used for the detection of *Pseudomonas syringae* (Balestra et al. 2013). PCR based techniques have found to be more efficient than techniques like agar plating, serological tests or biochemical tests but if the purpose is the early detection of the bacteria, then relying on PCR based method only, cannot be the method of choice (Goudarzi et al. 2020). This is because PCR based techniques cannot be used for point of care, quarantine, or field detection because it

requires electrical signals and thermal cycler therefore laboratory environment is needed (Goudrazi et al. 2020).

2.12 Levan test for detection of *Pseudomonas syringae*

Bacteria *Pseudomonas syringae* have an enzyme called levan sucrase, that can break down sucrose and form levan (a fructan). Nutrient agar medium, 5% sucrose or sucrose peptone agar is a substrate of choice for the Levan test. For the Levan test a single colony was picked up with a sterilized toothpick and placed on NA medium containing 5% sucrose. After this the plates were incubated at 28ºC for 2 to 4 days to visualize distinctive dome shaped colonies (Shila et al. 2013). Keeping this in mind presence of Levan sucrase can also be used as a marker for detection of *Pseudomonas syringae.*

2.13 Loop Mediated Isothermal Amplification for detection purposes

It is a newly developed method of gene amplification which is widely used for the detection of micro-organisms for it being more rapid, specific, and efficient under isothermal conditions. It is a very simple technique ranging from extraction of nucleic acids to amplification of a gene. (Notomi et al. in 2000). Over the years, LAMP technique has been modified for the detection of a range of diseases. LAMP detection includes methods like fluorescent detection (using SYBR green), calorimetric detection, real-time detection by increase in turbidity etc. (Wong et al. 2018). LAMP method can amplify very little amount of DNA into 10^9 copies within the matter of less than an hour. DNA products are very long (>20 kb) and formed from numerous repeats of the short (80–250 bp) target sequence, connected with single-stranded loop regions in long concatamers. It is a novel method for amplification of nucleic acids involving DNA polymerase and 4-6 primers recognizing 6 distinct regions on the target sequence thus making the technique highly sensitive and specific. There are two types of elongation that occurs in the loop region: one is self elongation of templates at the 3' terminal and other is elongation of new primers to the loop region (Notomi et al. 2000). LAMP reaction takes place at a constant temperature (Chen et al. 2020). This process has a wide range of application including point of care biosensors, rapid testing of environmental and food samples, and amplification in absence

of thermal cycler (where there are less resources or in developing countries). Therefore LAMP technique can easily be commercialized for the detection of plant pathogen like *Pseudomonas syringae*.

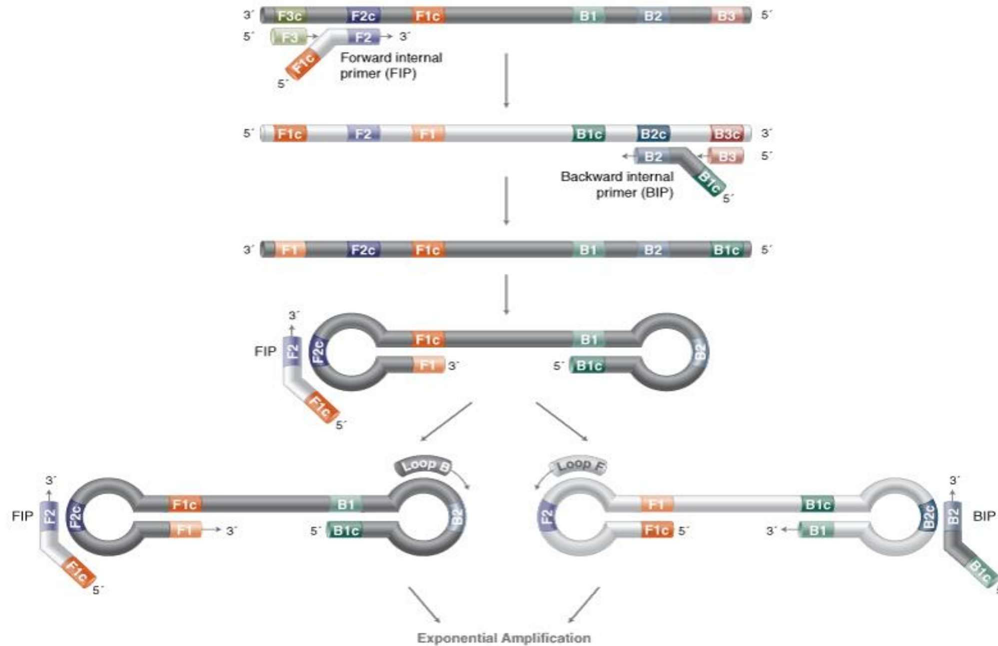


Figure 2.6: Schematic representation of LAMP amplification (New England Biolabs)

The concatamers formed cannot be used directly for downstreaming process, but target amplification is so extensive therefore numerous of detection processes are applicable.

Designing of primers for LAMP reaction can be a challenging process, but software tools greatly facilitate this process. NEB LAMP Primer Design Tool can be easily used for the designing of LAMP primers. After inputting a DNA or RNA sequence of interest, the LAMP Primer Design tool will identify suitable target regions and create the outer F3/B3 and looping inner FIP/BIP primers in a single step. The LoopF/LoopB primers, that accelerate the LAMP reaction, are created in a second step and are strongly recommended for best performance (NEB).

3. MATERIALS AND METHODOLOGY

3. Materials and Methodology

3.1 Revival and subculturing of *Pseudomonas syringae*

Pseudomonas syringae culture MTCC 11950 was retrieved from glycerol stocks by inoculating in 100 ml of sterile King's B Broth followed by incubation at 28ºC for 2 days. The cultures were streaked on King's B agar plate, gram stained and observed microscopically to confirm culture purity (Fig- 3.1). Subculturing of the media was carried out routinely after every 15 days (The culture conditions were followed given in the CSIR-IMTECH, Chandigarh catalogue).

Figure 3.1-Streaking of bacterial culture to obtain pure colonies

3.2 Growth Kinetics of *Pseudomonas syringae* in King's B media

Well isolated colonies of *P.sy* were inoculated to sterile King's B media. Overnight cultures were then inoculated in Erlenmeyer flasks containing sterile Kings B media and incubate at 37^0C with agitation for growth kinetic experiment. 2 mL aliquots were removed asceptically and absorbance was checked at 660 nm after every one hour and readings were noted down for 10 hours (Rahme et al. 1992).

3.3 Carbon utilisation by *Pseudomonas syringae* in M9 media

Culture of *P.sy* with cells in log phase was inoculated to autoclaved M9 media where sucrose, fructose, glucose, and glycerol were used differently as sole source of carbon at different concentrations. The combinations were incubated at 28ºC with agitation (120 rpm). Aliquotes were withdrawn aseptically after every one hour and absorbance was checked at 660nm; readings were noted down for upto 12 hours (Kim et al. 2009).

3.4 Antibiotic profiling test

50µl of grown culture of *P.sy* was spread on King's B agar plates and kept undisturbed for 15 minutes. Stock solution of 1mg/ml was made for three different antibiotics like Ampicillin, Chloramphenicol, Rifampicin and their dilutions were further made. Autoclaved discs of Whatman filter paper were dipped into the different dilutions of antibiotic and were placed on the plate with the control (discs were dipped in sterile distilled water). The plates (in triplicates) were incubated at 28ºC for 1-2 days and presence of zone of inhibition was checked (Biemer et al. 1973).

3.5 DNA isolation

QIAamp DNA mini kit (250) by QIAGEN was used for the process of DNA isolation. The procedure was followed given in the handbook of mentioned DNA isolation kit which is as follows:

1 ml of overnight grown *P.sy* culture was added to the microcentrifuge tube and centrifuged at 5000g/5mins/25ºC to discard the supernatant. Washing of the pellet was done with the saline and again centrifuged at 5000g/5mins/25ºC. ATL buffer was added to the tube to make up the volume to 180 µl followed by addition of 20 µl of Proteinase K and mixing the solution by vortexing and then incubating the tube at 56ºC for 1-3 hours. Again tube was briefly centrifuged and 4 µl of RNase A was added. Further it was incubated at room temperature for 2 minutes and 200 µl of AL buffer was added and vortexed briefly. Tube was incubated at 70ºC for 10 minutes and 200 µl of absolute chilled ethanol was added to it. This solution was shifted to a column containing nitrocellulose membrane and centrifuged at 6000g/1min. The leftover on the NCM was collected whereas filtrate was discarded. 500µl of AWI was added to it and again centrifuged at 6000 g/1min and again filtrate was discarded. This was followed by addition of 500 µl of AW2 to the leftover and centrifugation at 20,000g/3mins. Filtrate was discarded and 200 µ AE buffer was added to the remaining solution and centrifuged at 6000g/1min. Filtrate was collected and stored at -20ºC.

3.6 Polymerase Chain reaction

Polymerase chain reaction was carried out for the amplification of target Syringomycin gene. The designed forward and reverse primers were used to amplify the gene. Psy/PCR/F-AAACCAAGCAAGAGAAGAAGG represents the forward primer for PCR and Psy/PCR/R-GGCAATACCGAACAGGAACAC represents the reverse primer for PCR. The sequence of these primers was ordered from Sigma-Aldrich. Composition of PCR reaction is mentioned in Table 3.1. Thermal cycler was adjusted according to Table 3.2 for 35 cycles. PCR product was then run on a 2% Agarose gel at 60V and current was 20-25mA. 100bp DNA ladder was used to trace the length of bands under UV transilluminator (Lorenz et al. 2012).

Table 3.1: Composition of polymerase chain reaction

Composition	**Working**
Master mix (2X)	25 microlitres
FP(10 micromolar)	0.1-1.0 micromolar
RP (10 micromolar)	0.1-1.0 micromolar
Template (Genomic DNA)	10 pg- 1 microgram
Sterile nuclease free water	Makeup to 50 microlitres

Table 3.2: Gradient PCR cycle for amplification of Syringomycin gene

Process	Temperature	Time
Initial denaturation	95ºC	3 min
Denaturation	95ºC	30 secs
Annealing	55-65ºC	30 secs
Extension	72ºC	1 min
Final extension	72ºC	15 mins

3.7 Loop Mediated Isothermal Amplification reaction

The designed six primers for LAMP reaction were ordered from Sigma-Aldrich. All the components mentioned in Table 3.3 were added into the reaction mixture which includes amplification buffer, designed primers specific to the target region, dNTP mix, thermoresistant Bsm DNA polymerase was used with template concentration of 2µl and optimised $MgSO_4$ concentration at 8mM was used. Nuclease free water was used to make up the volume to 25µl. LAMP reaction was carried out between a temperature gradient of 58-65ºC for 60 mins. SYBR green was also added to the reaction mixture to increase the visual detection. LAMP product was then run on 2% Agarose gel at 60 V and current used was 20-25mA (Chen et al. 2020).

Table 3.3: Composition of LAMP

Component	25 microlitre (total)	Final conc.
10X Isothermal Amp buffer	5 µl	1X (contains 2 mM MgSO4)
Magnesium sulphate (100micromolar)	3 µl	6 mM (8mM total)
dNTP (10micromolar)	7 µl	1.4 mM each
FIP/BIP (25X)	2 µl	1.6 µM
F3/B3 (25X)	2 µl	0.2 µM
Loop F/B Primers (25X)	2 µl	0.4 µM
Bsm 2.0 DNA pol (8000U/ml)	2 µl	320 U/ml
DNA sample	2 µl	

Table 3.4: Gradient LAMP cycle

Initial denaturation	95ºC	3 minutes
Annealing	55-65ºC	60 minutes
Storage	4ºC	∞

3.8 Detection of Levan Biopolymer

Cell free supernatant was spotted on 8.5% (w/v) sucrose and 1.35% (w/v) agar plate. The plate was then incubated overnight at 40ºC and whitish slimy spots formed were photographed the next day (Ağçeli et al. 2020).

3.9 Laboratory scale production of Levan biopolymer

1L of *Pseudomonas syringae* culture was grown. The fully grown culture solution was centrifuged to separate the cells, and cell free supernatant was collected. The centrifugation step was repeated so that supernatant have no cells in it. Further batches of supernatant were transferred to round bottom flask where each batch had 200 ml of supernatant. Supernatant containing round bottom flasks were frozen at -80ºC overnight. This followed the process of concentrating the supernatant by process of freeze drying (Lyophilization) where 200 ml of supernatant was reduced to $1/10^{th}$ of its volume that is 20 ml. Now, double the volume of chilled alcohol was added into the concentrated supernatant and was refrigerated overnight. White coloured powder precipitated down were collected, subjected to the process of dialysis and then dried to obtain the yield (Srikanth et al. 2015).

3.10 Solubility Test for Levan biopolymer

Stock solution of Levan biopolymer powder was taken in concentration of 1 mg/ml. Different dilutions of the stock solution were made like 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. The solubility of stock solution and its dilutions were checked by both sonication and by dissolving the stock solution in dH_2O (Manandhar et al. 2009).

3.11 Biochemical tests for Levan biopolymer

3.11.1 Phenol Sulphuric acid test

To make Glucose standard: Glucose standard of concentration from 0.01-0.1 g/L was prepared. Stock solution of 1 mg/ml of glucose was diluted to different concentrations. 25 µl of 80% phenol was added to all the dilutions and vortexed briefly. 2.5 ml of concentrated H_2SO_4 was

then added to all the test tubes and then incubated at 25ºC for 10 minutes. Absorbance of all dilutions were recorded at 490 nm and graph was plotted.

Sucrose standards: Sucrose standards of concentration from 0.001-0.01 g/L was prepared. Stock solution of 1 mg/ml of sucrose was taken and its dilutions were made of different concentrations. 25 µl of 80% phenol was added to all the dilutions and vortexed briefly. 2.5 ml of concentrated H_2SO_4 was then added to all the test tubes and then incubated at 25ºC for 10 minutes. Absorbance of all dilutions were checked at 490 nm and graph was plotted.

Determination of sugar in the Levan biopolymer: Stock solution of 1 mg/ml of Levan biopolymer was taken. 25 µl of 80% phenol was added to it and vortexed briefly. This was followed by addition of 2.5 ml of concentrated H_2SO_4 and incubation at 25ºC for 10 minutes. Absorbance was checked at 490 nm (Rao et al. 1989).

3.11.2 Folin Lowry method

Preparation of Reagent A- 2% Na_2CO_3 in 0.1 N NAOH, Preparation of reagent B – 0.1% Copper sulphate in 1% potassium sodium tartrate, Preparation of Reagent C – mixing of reagent A and B, and preparation of reagent D – Folin Ciocalteu reagent was done.

BSA standard: Stock solution of 1 mg/ml of BSA was prepared and its multiple dilutions were made of different concentrations (0.05 mg/ml, 0.1 mg/ml, 0.15 mg/ml, 0.2 mg/ml, 0.25 mg/ml) to which 5 ml of reagent C was added. After adding reagent C, test tubes were incubated for 10 minutes at room temperature. This was followed by addition of 0.5 ml of reagent D to each tube. All the tubes were incubated in dark for 30 minutes. Absorbance was taken at 660 nm and graph was plotted.

Determination of protein in the Levan biopolymer: Stock solution of 1 mg/ml of Levan biopolymer produced was prepared to which 5 ml of reagent C was added. After adding reagent C, test tubes were incubated for 10 minutes at room temperature. This was followed by addition of 0.5 ml of reagent D to each tube. All the tubes were incubated in the dark for 30 minutes. Absorbance was taken at 660 nm (Waterborg et al. 1994)..

3.11.3 Bradford method

To make BSA standard: Stock solution of 1 mg/ml of BSA was prepared and its multiple dilutions were made of different concentrations (0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, and 0.5 mg/ml). 1X Bradford reagent was taken in 1X PBS. 980 µl of Bradford reagent in PBS was added to microcentrifuge tubes to which 20 µl of BSA of different concentrations was added. Absorbance was taken at 595 nm and graph was plotted.

To check concentration of protein in the Levan biopolymer: Stock solution of 1 mg/ml of Levan biopolymer produced was prepared. 1X Bradford reagent was taken in 1X PBS. 980 µl of Bradford reagent in PBS was added to microcentrifuge tubes to which 20 µl stock solution of Levan biopolymer was added. Absorbance was taken at 595 nm (Kruger et al. 2009).

3.12 Seliwanoff's test (1% Resorcinol assay)

Stock solution (1 mg/ml) of Levan biopolymer was incubated at 40ºC for 12 hours. Levan was separated by adding 4 volumes of chilled 70% (v/v) ethanol and then centrifuged at 17,400 g/5ºC. The pellet was suspended in ddH_2O and then the 3 ml solution of concentrated HCl with 1% resorcinol was added to the solution (Gerwig et al. 2021).

3.13 Test for Levansucrase activity

Stock solution of 1 mg/ml of lyophilized extract was made which was divided into two parts namely A and B. Test tube A was used to determine Levansucrase activity in which 1M sucrose and 50 mM phosphate buffer at pH-6.0 was added while nothing was added to test tube B. EPS in both the tubes (A and B) were precipitated using 95% ethanol and concentration in both the tubes were subsequently quantified and expressed by phenol sulphuric acid method. Sucrose as a standard was used for Phenol sulphuric acid test. Concentration in test tube A was expressed as $[EPS]_A$ and concentration in test tube B was expressed as $[EPS]_{B.}$

Levansucrase activity was calculated from the equation: $[EPS]_L = [EPS]_A - [EPS]_B$, where $[EPS]_L$ represents the concentration of EPS produced during the Levansucrase assay (Castilo et al. 2004).

3.14 Zymography

A 10% native gel was prepared to observe the Levansucrase enzyme activity. The procedure follows cleaning the notched and un-notched glass plates, spacers and comb with 70% (v/v) ethanol. The plates were assembled by placing spacers at the sides and sealed it using adhesive tape. Clamps were used to support the gel plates.

Preparation of 10% native separating gel and stacking gel was done (as per mentioned in table 3.5). Slowly separating gel was added in the space between the two glass plates till it fills 2/3rd of the space and was left undisturbed for it to polymerize. Comb was then placed between the glass plates and stacking gel was added to it to fill the remaining 1/3rd of the space. Again it was left undisturbed for stacking gel to polymerize. Comb was slowly removed.

Table 3.5: Composition of Native separating and stacking gel

	Native/separating Gel	**Native/Stacking Gel**
dH_2O	4.11 ml	6.2 ml
1.5 M Tris pH: 8.8	2.5 ml	
0.5 M Tris pH: 6.8		2.5 ml
30% Bis- Acrylamide	3.33 ml	1.33 ml
10% APS	50 µl	50 µl
TEMED	5 µl	5 µl

5X running buffer (1L): contained Tris 15 gms pH:8.3 and Glycine- 72 gms was prepared. Adhesive tapes around the glass plates were removed and PAGE setup was filled up with running buffer. Stock solution of 1 mg/ml of Levan biopolymer was prepared which was mixed in loading dye and all the wells of the gel were filled with the mixture of Levan biopolymer and loading dye. PAGE setup was attached to the electrical power supply and time taken for the gel to run was about 4 hours. The gel was run at 90 V at 4^0C (inside refrigerator).

After the loading dye was about to reach the buffer, electrical supply was discontinued and gel was incubated in 8.5% (w/v) buffered sucrose at 50ºC for 60 minutes and bands were observed in visible light (Frederiks et al. 2004).

3.14 Mango leaf adhesion assay

Disease free, undamaged Mango leaves were wiped with 70% ethanol. Overnight grown cultures of *P.sy* was adjusted to 10^8 cfu mL^{-1} and drops (10µl) of *P.sy* culture was inoculated on Mango leaves, incubated aseptically and washed off after 30 minutes. Infected mango leaves were homogenised in sterile distilled water, and the solution was diluted in startle PBS (Phosphate Buffered Saline) and thereafter spread onto King's B agar plate to calculate the cfu on 0 day. Samples were obtained likewise after two and four days (Ponce et al. 2020)

3.15 Swarming motility assay

0.3% semisolid King's B agar plate was prepared. Centrifuged overnight culture (10µl) was then placed carefully in the centre of the plate and incubated at 37ºC. The agar plate was left undisturbed for 2-3 days. Motility was scored from the appearance of concentric rings extending outwards from the centre (Ha et al. 2014)

Statistical analysis: Data are presented as means per group ± standard errors of the means. The statistical significance of differences between means was calculated using analysis of variance (ANOVA) ($P \leq 0.05$).

4. Results

SyrD gene used as a marker for early detection of Phytopathogen *Pseudomonas syringae* in plants.

Primary control in prevention of diseases in plants includes monitoring the presence of infection causing bacteria in the plant and one of the best approaches towards it is early detection of the infection. Developing markers that can detect early infection in plants and which can amplify raw, crude, and low signal to give the best signal with high sensitivity, specificity and accuracy is the crucial step towards prevention of the disease.

Infection from *Pseudomonas syringae* is a threat to wide variety of plants therefore there is a need to develop a marker that can detect the early infection of bacteria in the plants before it starts to show symptoms so that necessary actions could be taken to save the plant.

SyrD gene is responsible for secretion of Syringomycin in *Pseudomonas syringae*. Syringomycin is a peptide toxin which is unique to this bacteria. SyrD gene is one of the major causes of infection caused by these bacteria out of other factors. Therefore, SyrD gene can be used as a marker for early detection of infection caused by *Pseudomonas syringae*. SyrD gene can be used as a target sequence which can be amplified to many folds to achieve the best biosignal. One of the accurate methods to achieve specific amplification of target gene is achieved with polymerase chain reaction.

Though PCR-based techniques are sensitive and accurate in detection of the disease, early detection could not be done in the absence of a thermal cycler. Thus, early detection of the disease, high efficiency, reproducibility, and specificity in the absence of thermal cycler is desirable. Loop Mediated Isothermal Amplification is used to amplify the target region. LAMP technique has an edge over PCR technique when it comes to detection of a disease for it being more simple, rapid, specific, and cost effective as compared to PCR. Visual detection of the amplicons by naked eye is quite simple and reliable which cannot be done by PCR method.

Amplification of the target sequence requires primer designing which is discussed further.

4.1 Primer specificity of syringomycin gene by gradient polymerase chain reaction.

Gradient polymerase chain reaction was used for Syringomycin specific amplification. PCR mix was run at gradient of annealing temperature ranges between 55ºC to 65ºC in a temperature gradient thermocycler and then the PCR product which was expected to be 446 bp in length was

made to run on Agarose gel. After running the gel, it was visualized in UV transilluminator and was observed that band of PCR product lies between 400 bp and 600 bp ladder. High quality of bands was seen at temperature ranges of 62ºC and 65ºC while comparatively fade bands were seen at 55ºC and 58ºC. This proves the accuracy of primers designed.

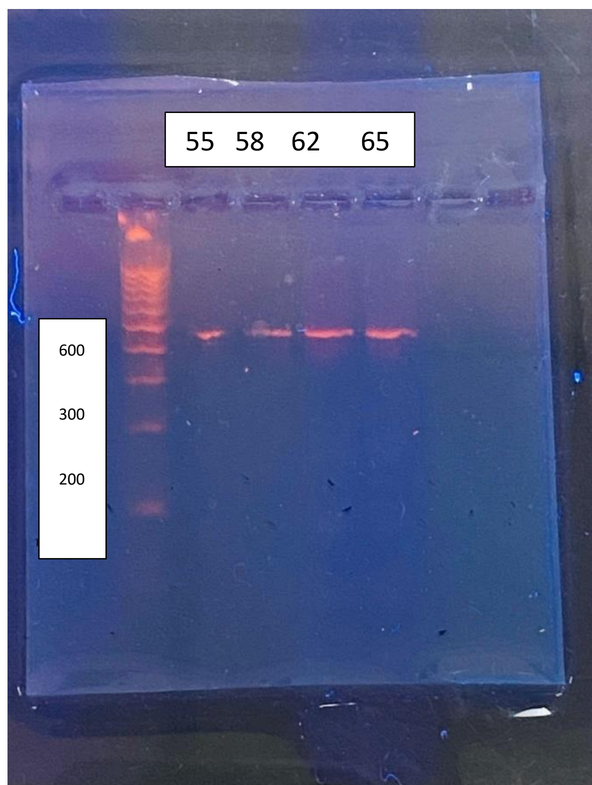


Figure 4.1: Syringomycin specific amplification- Gradient PCR

4.2 Loop Mediated Isothermal amplification (LAMP)

LAMP technique is primarily used for detection and diagnostic purposes as it is advantageous in aspects like it can amplify raw and crude DNA sample to many folds making it a specific amplification. It is simple, efficient, accurate, and a cost alternative process. These qualities of the LAMP technique can be used for early detection of *Pseudomonas syringae* in plants. For Loop Mediated Isothermal Amplification, combination of six primers were used to amplify the target syringomycin gene. The primers were designed using the NEB LAMP primer designing tool where measures considered while choosing the primers were enthalpy threshold of primers, they should be complementary to the target sequence, should not form primer dimers and T_m

should be between 52 to 65ºC. Therefore, temperature gradient used for LAMP reaction was 58 to 65ºC. Table 4.1 depicts the combinations of six primers were designed for LAMP reaction.

Table 4.1: Combination of six primers designed for LAMP reaction

Oligo name	Sequence
Psy/L/F3	GGCAGCAGCTAATGAAAACC
Psy/L/B3	GCGACTGACGCTGAAAGG
Psy/L/FIP	CGTAAAGAACGTCAGCCAGGGAAGCAAGAGAAGAAGGCCAGA
Psy/L/BIP	CTGACAGGGCTCATCAGCGGTCTCCTCGTGAATCGCCT
Psy/L/LF	GCTCCATAACAGACGCACGCATGA
Psy/L/LB	CCGTGGTCAATGTGATCAAC

LAMP product can also be visualized with naked eye since turbidity in the vial increases due to the precipitation of byproduct Magnesium pyrophosphate confirming that amplification has taken place. It was seen that when SYBR green dye was added with the LAMP reaction mixture a distinct visual colour change against the negative control sample (LAMP reaction was carried out with *E.coli* bacteria) was seen with the naked eye (Fig-4.2) making LAMP a cost effective and quantitative process since it cuts down the need to run the product on Agarose gel also.

When Agarose gel was visualized under UV transilluminator and it was observed that large number of concatemers were formed at the temperature range of 58ºC, 62ºC and 65ºC and bands of different lengths were clearly seen (Fig-4.3). The data obtained below confirmed that LAMP is a fast process since visual amplification with naked eye could be seen in matter of one hour, cheap and specific process for early detection of *Pseudomonas syringae* over PCR technique.

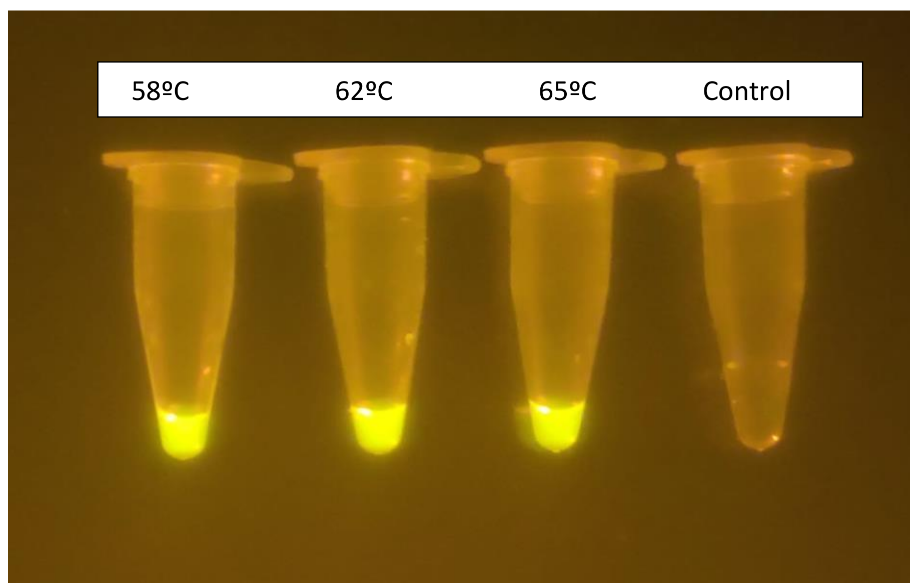


Figure 4.2: Visual detection of LAMP product at different isothermal temperature ranges on addition of SYBR green against the control sample.

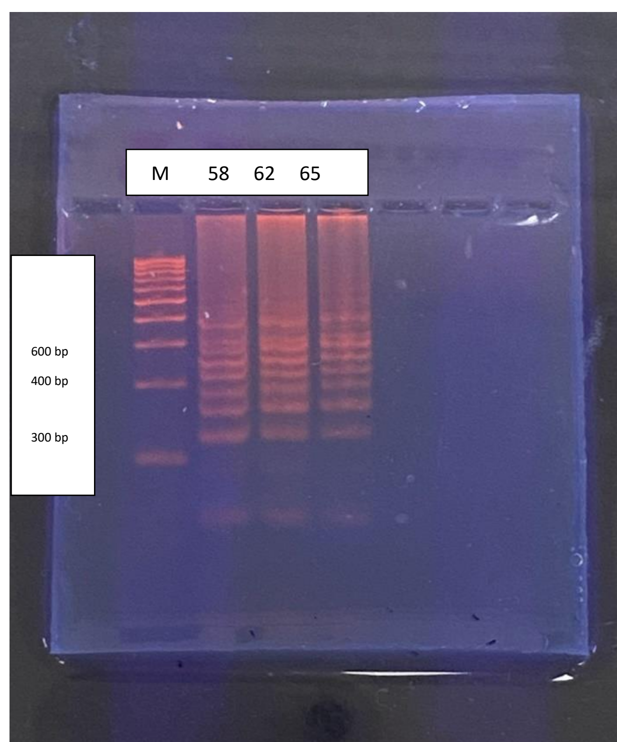


Figure 4.3: Temperature gradient LAMP reaction using Bsm polymerase and combination of six primers

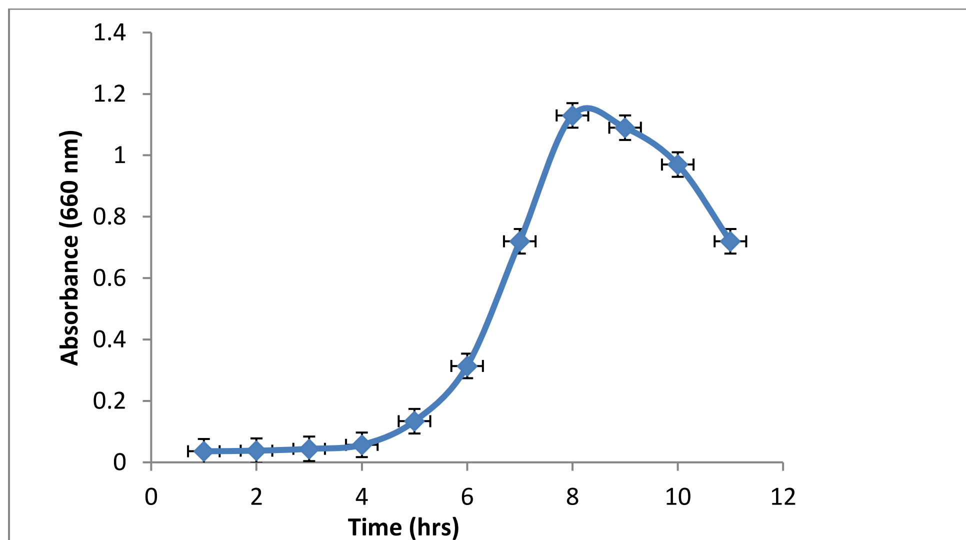
Levan Biopolymer used as a marker for early detection of Phytopathogen *Pseudomonas syringae* in plants.

Pseudomonas syringae causes significant loss in produce and notable proportion of crops like tomatoes, mangoes, apples, almond trees, rice, olives get infected every year, escalating economic concern besides disrupting food security. A systematic study with pure cultures of *P.sy* is important to establish the basic physiological traits and presence of virulence related mechanisms including ability to produce toxins and persistence i.e., fitness as pathogen in the host plant.

4.3 Growth Kinetics of *Pseudomonas syringae* in King's B media

Pseudomonas syringae was grown in King's B media. Its absorbance was checked after every one hour at 660 nm for 10 hours and graph was plotted for absorbance vs. time. It was seen that *Pseudomonas syringae* has a Lag phase of 3 to 4 hours and maximum Abs was achieved at 7th hour.

From the graph drawn for Growth Kinetics of *P.sy* (Graph 4.1), the generation time calculated was 0.81 hours and specific growth rate was 0.177.

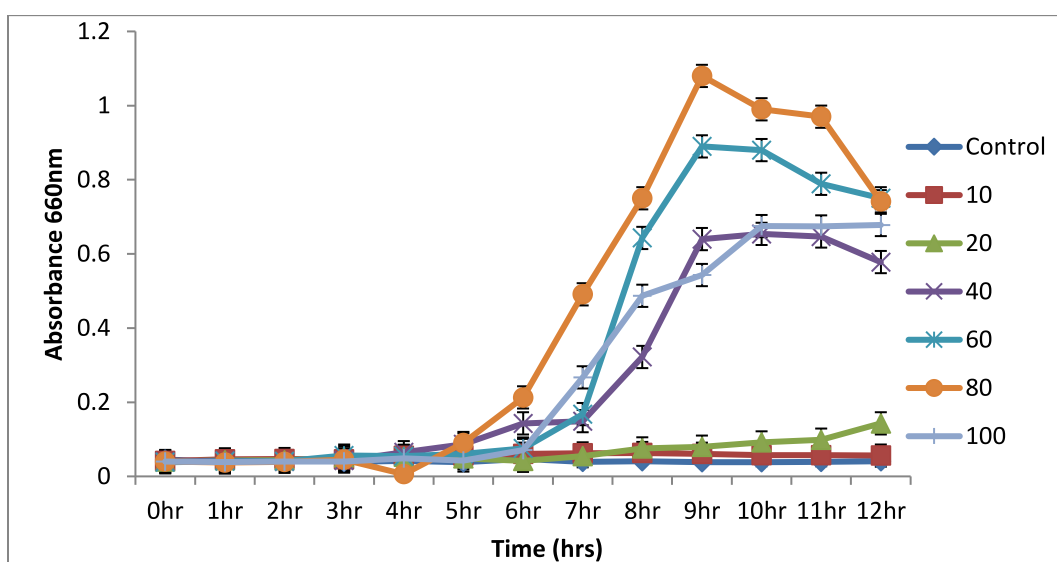


Graph 4.1: Growth Kinetics of *Pseudomonas Syringae* in King's B media

4.4 Carbon utilization patterns of *Pseudomonas syringae*

4.4.1 Growth of *Pseudomonas syringae* in M9 media with sucrose as sole source of carbon.

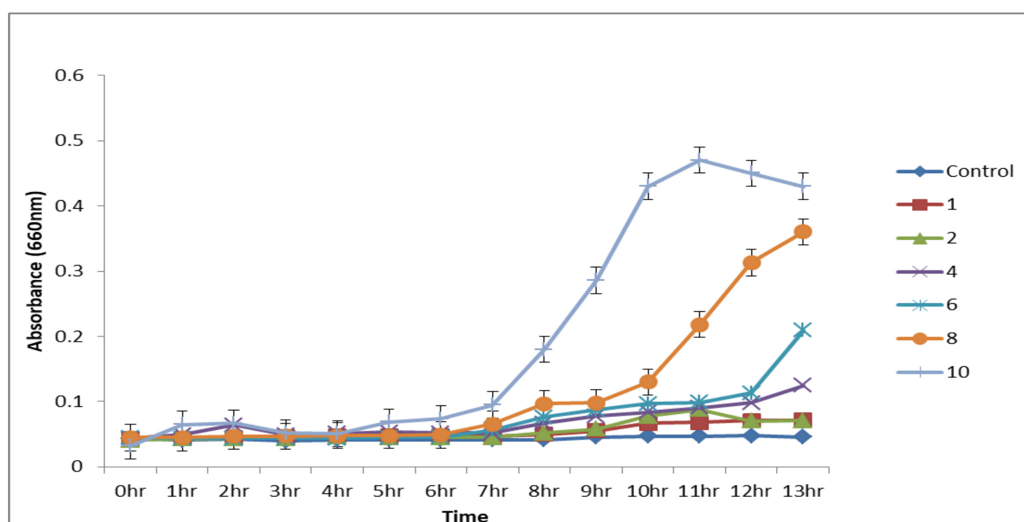
Pseudomonas syringae was grown on six different concentrations of sucrose such as 10 mg/ml, 20 mg/ml, 40 mg/ml, 60 mg/ml, 80 mg/ml, and 100 mg/ml. Control media was also set in which *P.sy* was not inoculated. The growth was observed on all the concentrations for 12 hours. The graph was plotted against value of absorbance vs. concentration. It was found that out of all the concentrations of sucrose 80 mg/ml was the most optimal concentration to grow *Pseudomonas syringae* in M9 media (See graph 4.2).



Graph 4.2: Growth curve of *Pseudomonas syringae* in M9 media with sucrose as sole source of carbon

4.4.2 Growth of *Pseudomonas syringae* in M9 media with Fructose as sole source of carbon.

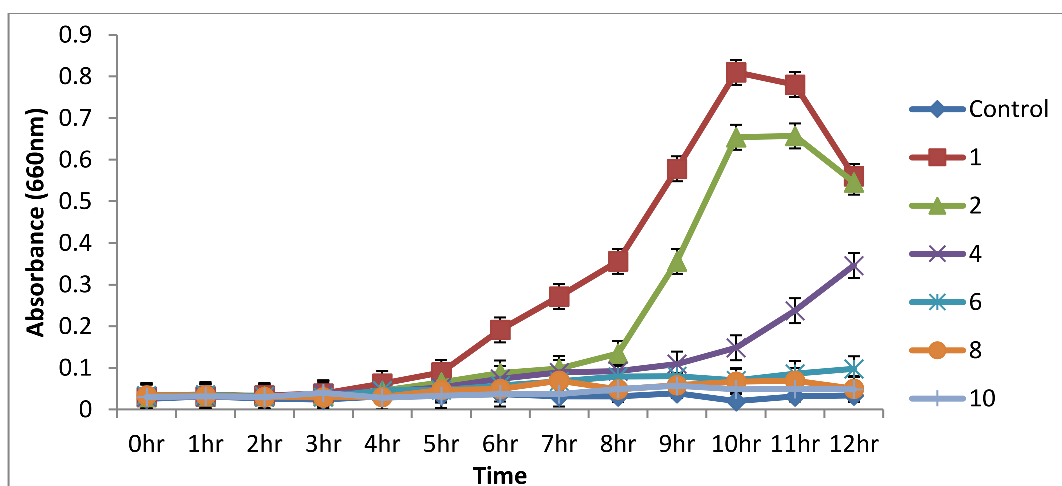
Pseudomonas syringae was grown on six different concentrations of fructose such as 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml. Control media was also set in which *P.sy* was not inoculated. Its growth was observed on all the concentrations for 12 hours. The graph was plotted against value of absorbance vs. concentration. It was found that out of all the concentrations of fructose 10 mg/ml was the most optimal concentration to grow *Pseudomonas syringae* in M9 media (See graph 4.3).



Graph 4.3: Growth curve of *Pseudomonas syringae* in M9 media with fructose as sole source of carbon

4.4.3 Growth of *Pseudomonas syringae* in M9 media with Glucose as sole source of carbon.

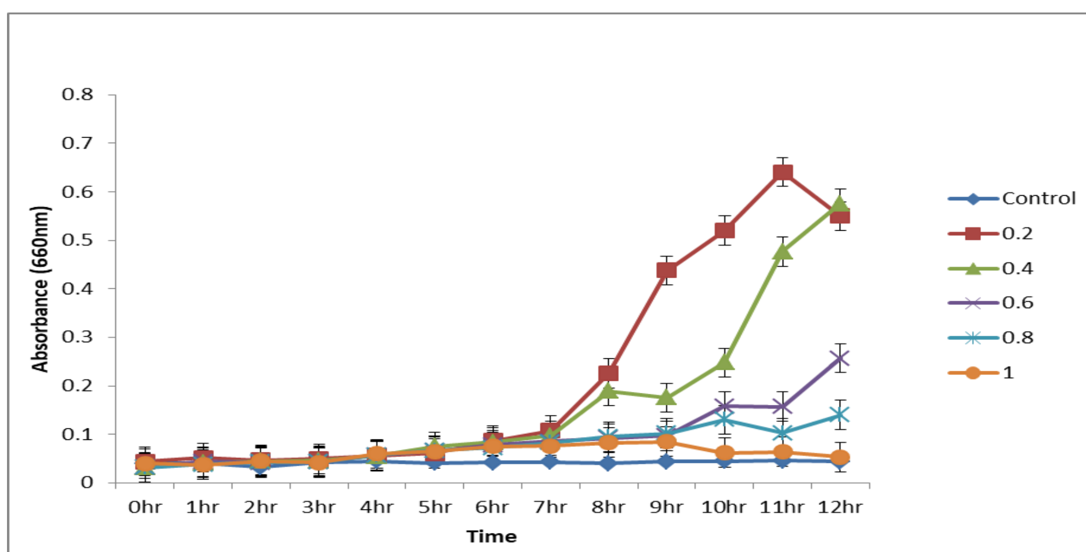
Pseudomonas syringae was grown on six different concentrations of Glucose such as 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml. Control media was also set in which *P.sy* was not inoculated. The growth was observed on all the concentrations for 12 hours. The graph was plotted against value of absorbance vs. concentration. It was found that out of all the concentrations of Glucose 1 mg/ml was the most optimal concentration to grow *Pseudomonas syringae* in M9 media (See graph 4.4)



Graph 4.4: Growth curve of *Pseudomonas syringae* in M9 media with Glucose as sole source of carbon

4.4.4 Growth of *Pseudomonas syringae* in M9 media with Glycerol as sole source of carbon.

Pseudomonas syringae was grown on five different concentrations of Glycerol such as 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, and 1 mg/ml. Control media was also set in which *P.sy* was not inoculated. The growth was observed on all the concentrations for 12 hours. The graph was plotted against value of absorbance vs. concentration. It was found that out of all the concentrations of Glycerol 0.2 mg/ml was the most optimal concentration to grow *Pseudomonas syringae* in M9 media (See graph 4.5).



Graph 4.5: Growth curve of *Pseudomonas syringae* in M9 media with Glycerol as sole source of carbon

4.4.5 Comparison of Growth of *Pseudomonas syringae* in different Carbon sources when grown in M9 media at a particular time.

After studying the Growth Kinetics pattern of *Pseudomonas syringae* in different carbon sources like sucrose, fructose, glucose, and Glycerol in M9 media, it is important to optimize the carbon source in which bacteria grow maximally. This aspect is important for understanding the intensity of infection in the plant host on the basis of preferential utilization of carbon source for expressing its maximal metabolic response. From the pattern of growth observed, it is seen that maximum absorbance of *P.sy* was seen at ninth hour. Fig 4.4 shows the absorbance vs.

optimized concentration of different carbon sources; sucrose accorded the highest growth and was thus adjudged that the best carbon source to grow *P.sy* in M9 media is Sucrose.

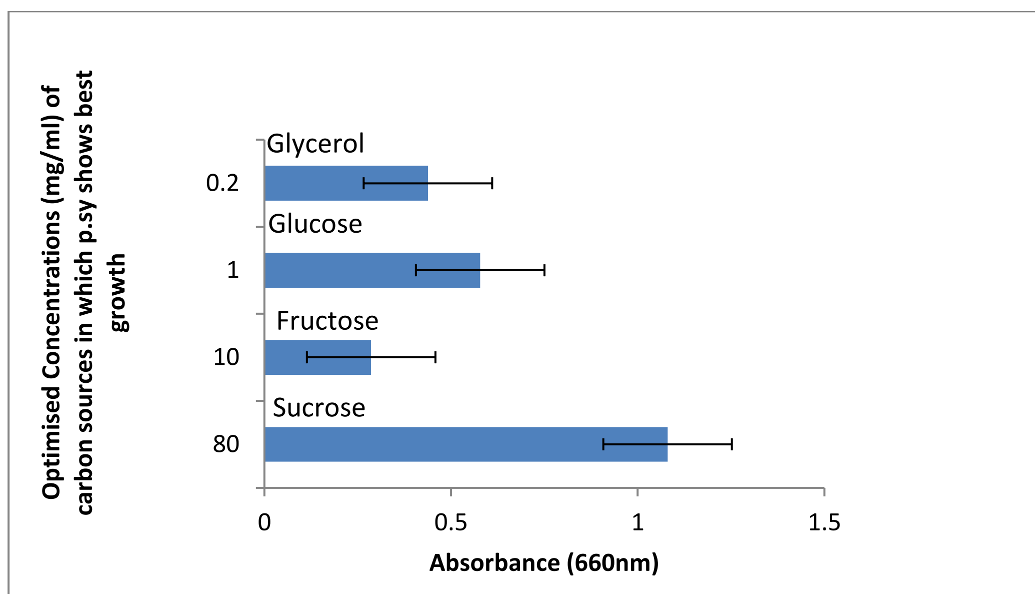


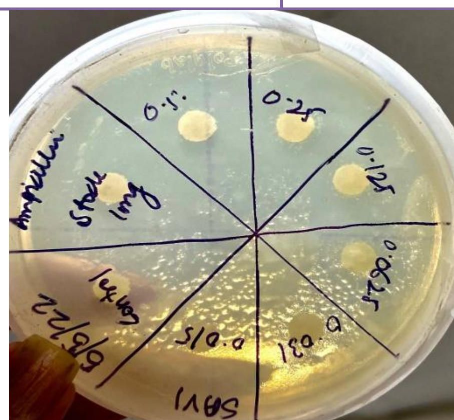
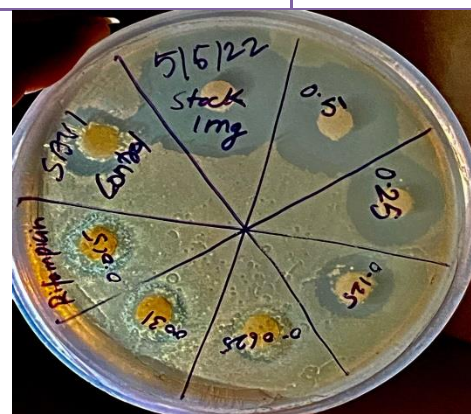
Figure 4.4: Comparative growth of *P.sy* with Sucrose, Fructose, Glucose and Glycerol as sole carbon sources in M9 media

4.5 Antibiotic Susceptibility Test for *Pseudomonas syringae*

Antibiotic Susceptibility test was performed to check the resistance or sensitivity of the bacteria against different antibiotics at seven different concentrations. *Pseudomonas syringae* was incubated on three different antibiotics namely Ampicillin, Chloramphenicol, and Rifampicin and the presence of zone of inhibition was observed.

No zone of inhibition was observed in all the seven different concentration of Ampicillin, while zone of inhibition was seen in Chloramphenicol at higher concentrations like 1mg/ml, 0.5 mg/ml and 0.25 mg/ml, and Zones of inhibition were observed in all the concentrations of Rifampicin. This proves that bacteria *Pseudomonas syringae* is sensitive to antibiotic Rifampicin, while resistant to Ampicillin, and only sensitive of chloramphenicol at higher concentrations.

Table 4.2: Antibiotic susceptibility profile for *Pseudomonas syringae*. Results are mean ± S.D of three observations. NA: Not applicable (detected)

Ampicillin		Chloramphenicol		Rifampicin	
Concentration (mg/ml)	Zone of Inhibition (mm)	Concentration (mg/ml)	Zone of Inhibition (mm)	Concentration (mg/ml)	Zone of Inhibition (mm)
1	N/A	1	180	1	120
0.5	N/A	0.5	160	0.5	100
0.25	N/A	0.25	90	0.25	80
0.125	N/A	0.125	N/A	0.125	70
0.0625	N/A	0.0625	N/A	0.0625	50
0.031	N/A	0.031	N/A	0.031	40
0.015	N/A	0.015	N/A	0.015	40
 **Figure 4.5**: Viability of *Pseudomonas syringae* grown on Ampicillin.		**Figure 4.6**: Viability of *Pseudomonas syringae* grown on Chloramphenicol.		 **Figure 4.7**: Viability of *Pseudomonas syringae* grown on Rifampicin.	

Pseudomonas in general is highly resistant to several antimicrobial compounds and for controlling *P.sy* infections in crop plants antibiotics such as streptomycin have been used for quite a long period of time.

For the phytopathogen *Pseudomonas syringae* releasing toxins (syringomycin) is not the only mode for infection; two other interconnected lifestyles have been noted while interacting with plants. These two lifestyles are responsible for *Pseudomonas syringae* to cause infection in plants. First one is epiphytic phase in which bacteria lives on the surface of the plant and the other is pathogenic phase in which bacteria enters inside the plant tissue and cause infection.

There are many extracellular enzymes present in bacteria which are responsible for initiation of an infection. Amongst many extracellular enzymes Levansucrase (EC 2.4.1.10) is found to be one of the most important enzymes in initiating an infection by biofilm development in *Pseudomonas syringae* by forming exopolysacchiride (EPS). Levansucrases are extracellular enzymes present in bacteria that convert sucrose into β-2,6- linked fructooligosaccharides (FOS). Therefore, Levansucrase enzyme activity to produce Levan biopolymer can be used as a marker to detect the early infection of *Pseudomonas syringae* in plants.

4.6 Detection of Levan biopolymer

Glucose released from the Levan biopolymer produced was visualized. *Pseudomonas syringae* bacterial culture was spotted on sucrose and agar plate, incubated overnight and the whitish slimy spots were formed. These spots confirmed the formation of Levan biopolymer by bacterial culture indicating that by mass production of the bacterial culture, large amount of Levan biopolymer can be extracted from concentrated cell free supernatant.

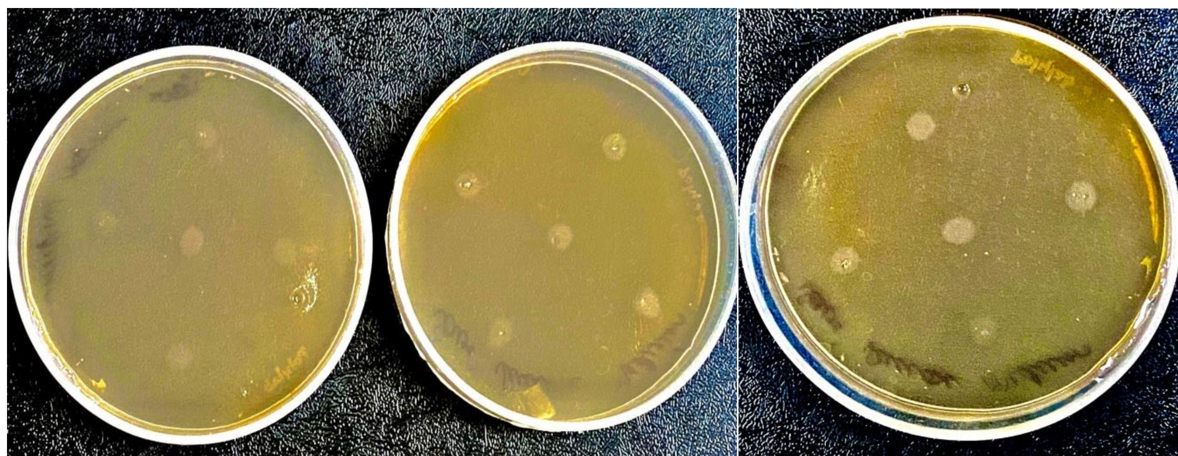


Figure 4.8: The above figure represents whitish slimy spots formed as a result of Levan biopolymer produced

4.7 Levan biolymer yield after Laboratory scale production

After visual confirmation that Levan biopolymer is produced by *Pseudomonas syringae* culture, large amount of cell free supernatant was concentrated to obtain the biopolymer. Cell free supernatant was concentrated by freeze drying (Lyophilization) since subjecting the supernatant to high temperature could hinder the enzyme activity. After freeze drying, supernatant was precipitated to obtain the biopolymer in a form of powder. The powder was subjected to dialysis to remove the salts present in it. From the batch of 200 ml of supernatant, after lyophilization and dialysis 115 mg of yield was obtained (Fig- 4.9).

Figure 4.9: Crude enzyme extract after freeze drying of cell free supernatant. This was used as source for the exopolysaccharide (Levan) and enzyme (Levan sucrase).

4.8 Solubility Test for Levan biopolymer

It was observed that stock solution and different dilutions of Levan biopolymer was completely soluble when subjected to process of sonication while stock solution of biolymer was partially soluble when dissolved in water, but all its other dilutions were completely soluble (as shown in table 4.3)

Table 4.3: Solubility Test for Levan biopolymer (+ represents partially soluble, ++ represents completely soluble)

	Stock	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Sonication	++	++	++	++	++
Dissolving in water	+	++	++	++	++

4.9 Biochemical tests of Levan biopolymer

Table 4.4: Biochemical tests for Levan biopolymer

Sugar Estimation		Protein Estimation	
Sugar Estimation with Glucose as standard	Sugar Estimation with Sucrose as standard	Folin Lowry method	Bradford method
0.0041 g/L	0.0059 g/L	0.05 mg/ml	0.042 mg/ml

4.10 Detection of Fructose present in Levan biolymer by Seliwanoff's test

Levan biopolymer produced is a homopolymer of fructose sugar. *P.sy* converts the sucrose to β-2,6- linked fructooligosaccharides which leads to the formation of Levan biopolymer. Therefore, by checking the presence of fructose in the isolated extract, the presence of Levan biopolymer can be confirmed. Seliwanoff test was used to check the presence of fructose in the sample. It uses mixture of HCl and Resorcinol. HCl reacts with ketose sugar to form 5- hydroxyl methyl furfural which on reaction with resorcinol forms red coloured complex. This assay was performed using control (no biopolymer was added) and stock solution (1 mg/ml) of Levan biopolymer. The colour change in tube containing Levan biopolymer from transparent to cherry red confirmed the presence of fructose sugar (Gerwig et al. 2021)

Figure 4.10: The above figure represents colour change in test tube containing levansucrase from transparent to cherry red due to presence of fructose.

4.11 Determination of Levansucrase activity

From the equation y = 48.559x + 0.1201 from standard curve of sucrose obtained during Phenol sulphuric acid test, concentration in test tube A was calculated as 0.07 mg/ml and concentration in Test tube B was calculated as 0.0059 mg/ml.

Table 4.5: Table showing Absorbance of test tube A and test tube B during determination of Levansucrase activity

Test tubes	Absorbance (490 nm)
A	3.527
B	0.41

Therefore, from the equation $[EPS]_L = [EPS]_A - [EPS]_B$, $[EPS]_L$ calculated is 0.0641 mg/ml, where $[EPS]_L$ represents the concentration of EPS produced during the Levansucrase activity.

4.12 Zymography

A native gel was conducted to check the presence and to visualize the Levansucrase protein in the isolated biopolymer extract. This is also known as Zymography. The purpose of native gel is to separate the protein in its native state on the basis of shape, charge and size. After running the gel, it was kept in buffered sucrose solution and white colored bands were then seen in visual light. These white bands indicate the *in situ* Levansucrase activity (Frederiks et al. 2004)

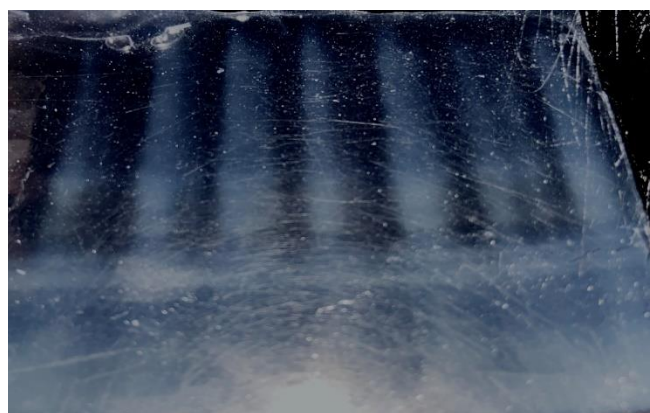


Figure 4.11: *In situ* detection of Levan sucrase activity; concentrated supernatant in native gel were incubated in buffered sucrose.

Epiphytic fitness of *Pseudomonas syringae*

As deliberated above *Pseudomonas syringae* interacts with plants in two phases. Epiphytic phase is in which bacteria lives on the surface of host plant without actually causing infection to the plant but causing infestation and second is pathogenic phase when bacteria start to colonize in the plant tissue due to its flagellar motility and finally infect the plant. Mango leaf adhesion assay and Swarming motility tests were performed to check the epiphytic fitness of *Pseudomonas syringae*.

4.13 Mango leaf adhesion assay

The figure below shows the results of Mango leaf adhesion assay. The confluent growth clearly suggests the Epiphytic fitness of *Pseudomonas syringae* on Mango leaf. The leaves (5 cm^2) were homogenized from the infected mango leaf from *Pseudomonas syringae*, diluted appropriately and plated the solution on King's B agar plate. The total cfu is mentioned in table below. (Table-4.6)

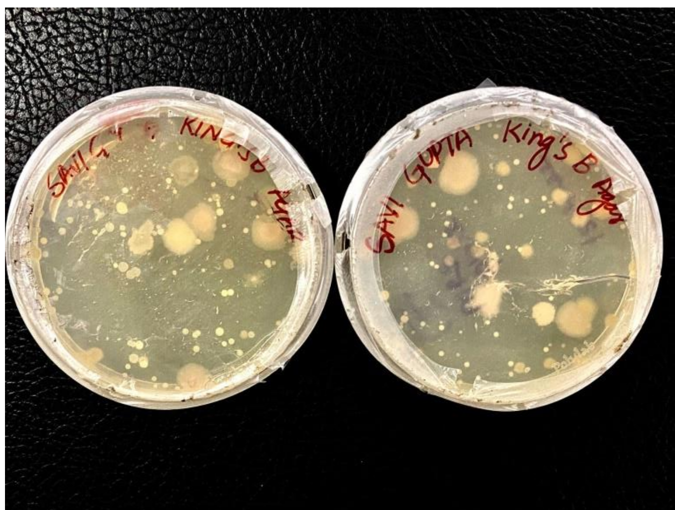


Figure 4.12: The viability of *P.sy* on mango leaves. *P.sy* was inoculated on mango leaves (surfaces) and incubated at ambient temperature, The homogenized leaf solution was spread on Kings B media to confirm visibility after 2, 4 days.

Table 4.6: P.sy count in King'B agar plate during Mango leaf adhesion assay. Results are mean ± S.D of three observations

	Plate 1	Plate 2
Day 2	32 cfu/cm2±2.3	33cfu/cm2±1.6
Day 4	97 cfu/cm2±1.8	99 cfu/cm2±2.1

4.14 Swarming motility assay

To understand the fitness of infection, swarming motility of *Pseudomonas syringae* in semisolid agar was carried out. Swarming motility assay is also an important feature of EPS and biofilm formation all of which enable colonization and persistence of the pathogen. Motility is a function of flagellar movement in *P.sy*; the swarming motility of the bacterial population was clearly observed in the semi solid agar, evident from concentric rings expanding and progressing outwards from inoculum spot.

Figure 4.13: Swarming motility of *P.sy* in semi solid media

5. DISCUSSION

5. Discussion

Pseudomonas syringae infection was reported for the first time in United states (Bryan 1933). From last 10 years, bacterial speck disease in plants due to infection from *Pseudomonas syringae* have increased tremendously where 20-70% disease incidences are there causing 30-80% loss of the yield (Chen et al. 2020). There has been serious economic loss worldwide due to decrease in tomato production (Yunis et al. 1980) as well as other produce. *P.sy* outbreaks of bacterial canker of Kiwifruits in New Zealand (Vanneste et al. 2017) have been reported, outbreak and spread of Kiwi fruits canker in Korea (Kim et al. 2016), outbreak of tomato bacterial speck disease in China (Chen et al. 2020), etc. has posed severe concerns and urged immediate measures to avoid epidemic in field indicating the prevalence and impact of *P.sy* as a plant pathogen of concern. A deliberation of the *P.sy* epidemiology has urged researchers for developing immediate solutions for intervention. These include rapid, affordable, and accurate detection and effective timely intervention. Thus far, several approaches have been suggested in this regard. The virulence mechanisms of Pseudomonas are highly complex and a single step solution for this. However, biochemical entities elaborated during *P.sy* infection can be helpful for initiating and confirming the infection . In view of this it was envisaged that both Levan, Levan sucrase followed by identification of the predominant toxin producing gene may provide a better and feasible approach. It is imperative that prior to initiation of such a study, the physiology especially the basic traits need to be evaluated. These include a growth profile, carbon utilization pattern and mechanisms of virulence (motility, epiphytic fitness, production of Levan and Levansucrase) as well as the susceptibility to antibiotics.

The Pseudomonads is generally known for being highly resistant to compounds which are antimicrobial in nature and it is expected that *P.sy* will be no exception. In fact, antimicrobials such as streptomycin and copper have been used from a long time to reduce down *P.sy* infections from crop plants. Besides, Since, *P.sy* also come into contact with antibiotics which are medically used, and their resistant genes, therefore, these can spread in the immediate natural environment and also through infected plant products which are consumed in contact with other produce (Hwang et al. 2005). Therefore, it was relevant to evaluate the antimicrobial profile of *P.sy*. In the current study, *P.sy* was sensitive to Rifampicin and higher concentrations

of Chloramphenicol while resistant to Ampicillin. It is known that bacteria have efflux pumps in them which are multidrug resistant and protect the bacterial cells against a wide spectrum of antimicrobial compounds; recently multidrug transporter from the plant bacterium *P.sy pv. Tomato* have been reported (Santamaria -Hernando et al. 2019). It has been suggested that equivalent genes of this protein are conserved within many *Pseudomonas* species that interact with plants. This ability enables sustenance of the pathogen in plants treated with antimicrobials. Growth kinetics of the bacteria in a non-selective media is a crucial step to ascertain its generation time and specific growth rate which is required to optimally grow the bacteria for further studies. Ecological problems nowadays rely on the mathematical expressions and quantitative analysis like growth kinetics (Panikov et al. 1995). *Pseudomonas syringae* is a chemohetrotroph (Plessis et al. 2002). Chemohetrotrophs require organic chemical substances as source of carbon (Lee et al. 2007) therefore *P.sy* was grown on different carbon sources in M9 media to optimize the best carbon source for its growth. M9 is a chemically defined media in which different carbon sources were used to check the growth of bacteria. Growth of the bacteria depends on the medium in which it is grown (Ehrenberg et al. 2012). It was observed that sucrose followed by glucose was the best carbon source in M9 media to grow *Pseudomonas syringae*. This was therefore used for further studies and in obtaining crude cell free supernatant. The latter, was used as a source both for the biopolymer Levan and enzyme Levansucrase(Lsc).

Since phytopathogen *Pseudomonas syringae* causes profound economic loss in agriculture worldwide, therefore there is an urgent need to develop detection tool(s) that can detect the early infection of bacteria in the plants. Detection of such phytopathogen at early stage is of continual interest for plant pathologists (Goudarzi et al. 2020). There are many methods by which detection of *P.sy* can be done like biochemical tests (LOPAT and GATTA), serological tests as well as culturing the bacteria on King’s B medium, but all these methods are time consuming and are not specific or accurate. This can be a problem for early detection of bacteria and best method for early detection with high sensitivity and accuracy is LAMP (Goudarzi et al. 2020). LAMP rapidly detects and amplifies presence of even minute amount of bacteria in infected tissues without the need to follow complex mechanisms and is easy to perform, cost effective, less time consuming and an accurate method which gives quantitative

visual results and is suitable for quarantine and field detection also (Chen et al. 2020). Due to these supporting advantages of LAMP, molecular markers specific for *Pseudomonas syringae* can be designed which can be amplified using LAMP in developing Point of Care Biosensors.

The SyrD gene is required for Syringomycin production in *P.sy* (Quigley et al. 1994). Detection of toxin producing SyrD gene by PCR was reported (Kaluzna et al.). However, LAMP is clearly advantageous over PCR when it comes to diagnostics and detection. It is more specific than PCR since it requires set of 4-6 primers unlike two primers in PCR and works at a single temperature therefore there is no need of thermal cycler unlike PCR. LAMP technology not only cuts out the need of using expensive equipment like thermal cycler but also the need of electrical power since it works at a single constant temperature therefore depending on the incubation temperature and heating. LAMP product can also be seen with the naked eye since there is increased turbidity or colour change. This is not possible by PCR hence, making LAMP a quantitative technique as well (Goudarzi et al. 2020). Therefore, SyrD gene was used as a marker which was amplified by LAMP technique and gave successful visual results as well as after running it on the Agarose gel. Isothermal temperature range used for LAMP technique was 58ºC, 62ºC, and 65ºC.

Pseudomonas syringae is a model phytopathogen which is used to study biofilm production as well as plant bacterial interaction worldwide. This is because *P.sy* has two phases in its lifecycle. First is the epiphytic phase in which plant lives on the surface of the plant and second is pathogenic phase in which bacteria enters inside the plant and initiates infection (Ponce et al. 2020). Late blight of tomato is one of the major causes of disease in tomato fruit including septoria leaf spot, anthracnose, fusarium and verticillium wilt, early blight etc. caused by *Pseudomonas syringae* (Wyenandt et al. 2020). *Pseudomonas syringae pv*. Tomato causes necrotic lesions on the leaves, stems, and fruit of tomato plants (Goode et al. 1980). *P.sy* causes disease symptoms ranging from stem cankers to leaf spot (Melloto et al. 2008). All these diseases are possible because bacteria release certain exopolysaccharides which helps in the attachment of bacteria to the plant and starts Biofilm formation (Gross et al. 1992). These exopolysaccharides are formed by certain extracellular enzymes. Levansucrase being a principal

enzyme. The latter leads to the formation of biopolymer needed for attachment to plant surface and to initiate an infection. Therefore, presence of Levan biopolymer can be used as another marker for the early detection of *P.sy* in plants. Detection of Levan biopolymer is important for confirming the catalytic activity of the extracellular Levansucrase enzyme.

Laboratory scale production of Levan biopolymer was accomplished after analysing the results from growth Kinetics of *Pseudomonas syringae*. In the present study, 115 mg of extracellular metabolites was obtained by concentrating 200 ml of cell free supernatant. The crude powder produced was used for the determination of Levansucrase activity and was found that 0.0641 mg/ml of biopolymer was produced during the Levansucrase activity. Biochemical tests confirmed that 0.0059 g/L of sugar was present in the powder (sucrose was used as a standard) and 0.05 mg/ml of protein was present (when BSA was used as a standard) by Folin-Lowry method. Further Zymography was performed to check the catalytic enzyme activity of Levansucrase. Zymography is a simple and effective technique for detecting potential hydrolytic activities of enzymes *in situ* and thus infer *in vivo* (Vandooren et al. 2013) functionality. Results of Zymography were clearly evident as white coloured bands indicating strong Levansucrase activity moreover the results inferred that stability of the extracellular enzyme is not affected in the external milieu thus its presence in infected plant exudates or area of infection can be attempted (Li et al. 2006).

Pseudomonas syringae is found in a huge range of epiphytic community inducing diverse symptoms in plants. *Pseudomonas syringae pv. syringae* has large variety of hosts than any other of its pathovars both in epiphytic phase and pathogenic phase (Arrebola et al. 2009). This makes it important to study the epiphytic fitness of *P.sy* in plants. Mango leaf adhesion assay was used to study Epiphytic fitness (Ponce et al. 2020). On an average, a 4-log increase of *P.s*y were found on King's B agar plate when homogenised solution of mango leaf infected with *Pseudomonas syringae* was plated. This confirmed the epiphytic fitness of the bacteria. Another method to confirm the epiphytic fitness was motility - a prime requisite of *P.sy* for initiating infection; the swarming motility assay elegantly demonstrates this. Overnight grown cultures of *P.sy* culture inoculated at the centre of semi solid King's B agar (Berti et al. 2007) produced

concentric rings moving outwards in succession (from the centre) confirming flagellar motility of *Pseudomonas syringae* due to which it enters the pathogenic phase. These results confirmed that the *P.sy* was capable of causing infections (fitness as pathogen). These observations also reveal the capability of *P.sy* for toxin production, the extracellular enzyme levansucrase and exhibit swarming motility, a characteristic directly related to flagellar involvement. Moreover, the survival and proliferation observed in the mango leaf adhesion assay confirmed its robustness as a pathogen.

The gram-negative phytopathogen *P.sy* is the infection causing agent of a variety of blight, bacterial spot and speck diseases on a variety of crops, including beans, apples, cabbage, oats, cucumbers, flowers, peas, tobacco, beets, tomato, rice and olives but diseases are not limited to only these crops. *P.sy* are subdivided into varieties known as pathovars, based on the host to which it infects (Hwang et al. 2005). Based on cumulative reports, there is wide variety of hosts and disease symptoms found in this species. The latter, in fact opens up unique opportunities for investigating the intrinsic features which are responsible for host specificity. The gamut of *P.sy* virulence associated mechanisms include antimicrobial resistance, ice nucleation proteins, toxins, and others. In view of these, *P.sy* represents a popular model for investigating plant pathogen pathogenesis and subsequently seek avenues where rapid detection using metabolites can be developed as point of site tools for timely detection and intervention of diseases. Overall, the results of the present study provide encouraging leads that can be used sequentially for developing a fail-safe and rapid detection process. This involves an initial detection of Levansucrase from the infected area. Followed by LAMP PCR to establish the pathogen identity. Both these methods require relatively less time, offer cost effective solutions, and offer simplicity in terms of operation. While further studies will be required for validation and improvisations of this method, the findings are encouraging and offer prospects for application.

6. CONCLUSION

6. Conclusion

SyrD gene present in *Pseudomonas syringae* was successfully used as a molecular marker for early detection of the bacteria in infected plants by amplification through LAMP technology owing to its more benefits and being specific than PCR. The presence of the exopolysaccharide biopolymer Levan and Levansucrase enzyme elaborated by *Pseudomonas syringae* for initiation of infection in plants was also evaluated as markers to detect early presence of bacteria. Therefore, the latter can be used in conjunction with SyrD for detecting *P.sy* infection with certainty.

A scope of further studies includes developing a process in which complex DNA extraction is not required and amplification can be done directly from plant samples with suspected infection. Further procedures can be designed to make field detection and validation of the combined method however due to paucity of time the latter could not be carried out (limitations of the current study). Nevertheless, an encouraging prospect of applying the approach under real time studies (artificially infected plant or actual samples) should yield convincing insights.

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Appendix

A.1 Composition of King's B media

The medium used for the growth of *Pseudomonas syringae* is King's B media. *Pseudomonas syringae* shows best growth in King's B media.

Composition of King's B Agar media (For 1L):

- Proteose peptone - 20g (carbon and nitrogen source)
- Glycerol CP - 10 ml (source of energy)
- K_2HPO_4 - 1.5g (enhances pigment production)
- $MgSO_4$ - 1.5g (enhances pigment production)
- Agar - 15g (helps in solidification)
- Distilled water- 1L (1000ml)

Composition of King's B Broth media (For 1L):

- Proteose peptone - 20g (carbon and nitrogen source)
- Glycerol CP - 10 ml (source of energy)
- K_2HPO_4 - 1.5g (enhances pigment production)
- $MgSO_4$ - 1.5g (enhances pigment production)
- Distilled water- 1L (1000ml)

A.2 Composition of M9 minimal media

1. 5X M9 salt solution (g/L)
    - Na_2PO_4 - 33.90 g/L
    - KH_2PO_4 - 15.00 g/L
    - NaCl - 2.50 g/L
    - NH_4Cl - 5.00 g/L

2. 1M $MgSO_4$

   24.65 g $MgSO_4.7H_2O$ in 100ml distilled water

3. 1M $CaCl_2$

   147.014 g of $CaCl_2.2\ H_2O$ in 100ml distilled water

A.3 Preparation of 1L M9 minimal media

1. 5X M9 salt solution - 200 ml
2. 1M $MgSO_4$ - 2 ml
3. 1M $CaCl_2$ - 0.1 ml
4. Carbon source - 20 ml
5. dd H_2O - 778 ml

A.4 Graph for Logarithmic Stage of Growth kinetics of *Pseudomonas syringae* in King's B media.

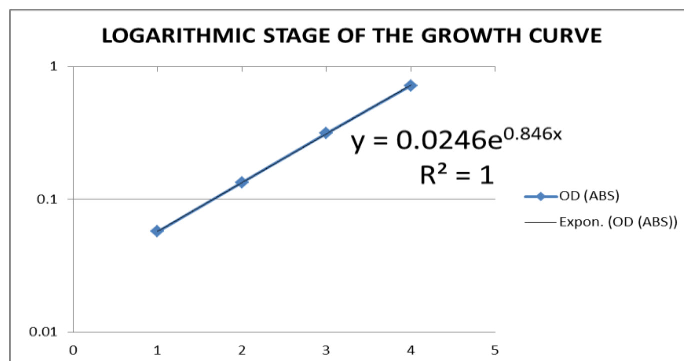

Figure A: Logarithmic stage of Growth Kinetics of *Pseudomonas Syringae* in King's B media

A.5 Primer Designing for PCR technique

SyrD gene (Genbank ID: M97223.1) responsible for Syringomycin production is used as a target gene. Below mentioned is the sequence needed to be amplified. Region in bold includes against which forward and reverse primers were designed.

AAACCAAGCAAGAGAAGAAGGCCAGACCAGGCTCGATCATGCGTCTGTTATGGAGCAGCCATCCCTGGCTGACGTTCTTTAC
GCTGCTGACAGGG CTCATCAGCGGCGTTGCGTCCATTGCCGTGGTCAATGTGATCAACCAGGCGATTCACGAGGAGACCTTTC
AGCGTCAGTCGCTGTTCTGGTTTGTCGGCCTGAGCGTGGTGGCGCTCCTGTTCCGCAACGGTGCGTCGCT
GTTTCCGGCTTACGCCAGCATGCGCATCATGACCCGTCTGCGCATTGCCCTGTGCCGCAAGATCCTCGGC
ACGCCGCTTGAGGAAGTCGACCGCCGCGGTGCGCCGAATGTGCTGACCCTGCTGACCAGCGATATTCCGC
AACTCAACGCCACGCTGTTGATCATGCCGACGATCCTGGTGGAGTCGGCG**GTGTTCCTGTTCGGTATTGCC**

A.6 Primer Designing for LAMP technique

Table A: Designed primers for LAMP

	5' pos	3'pos	length	Tm	Tm according to sigma	oligos
F3	421	470	20	59.20	63.6	GGCAGCAGCTAATGAAAACC
B3	625	642	18	60.08	64.9	GCGACTGACGCTGAAAGG
FIP			42		84.2	CGTAAAGAACGTCAGCCAGGGAAGCAAGAGAAGAAGGCCAGA
BIP			38		87.3	CTGACAGGGCTCATCAGCGGTCTCCTCGTGAATCGCCT
LF	502	521	20	61.74	63.9	GCTCCATAACAGACGCACGCATGA
LB	586	605	20	60.65	64.0	CCGTGGTCAATGTGATCAAC
F2	472	491	20	60.56		AGCAAGAGAAGAAGGCCAGA
F1C	527	548	22	64.20		CGTAAAGAACGTCAGCCAGGA
B2	607	624	18	59.37		TCTCCTCGTGAATCGCCT
B1C	552	571	20	64.55		CTGACAGGGCTCATCAGCGG

A.7 Standard curve of Glucose for Phenol sulphuric Test

Table B: Table for glucose standard of concentration vs. Absorbance

Sr. no	Concentration (g/L)	OD (490nm)
1	0.001	0.186
2	0.002	0.211
3	0.004	0.333
4	0.006	0.524
5	0.008	0.765
6	0.01	0.846

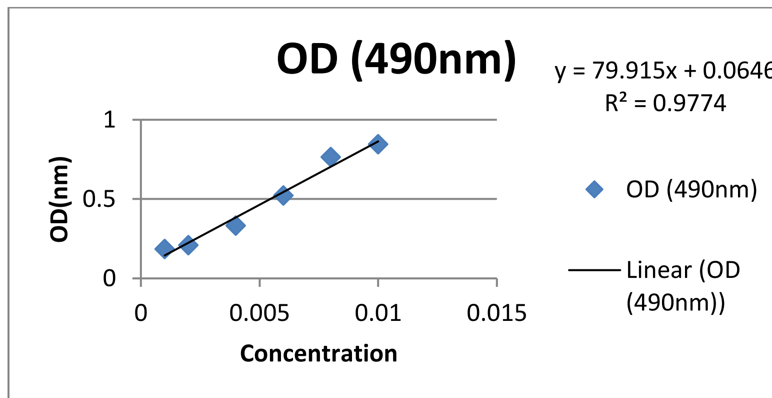

Figure B: Graph of glucose standard of concentration vs OD

A.8 Standard curve of Sucrose for Phenol sulphuric Test

Table C: Table for sucrose standard of concentration vs Absorbance

Sr. no	Concentration (g/L)	OD (490nm)
1	0.001	0.207

2	0.002	0.236
3	0.004	0.301
4	0.006	0.337
5	0.008	0.45
6	0.01	0.695

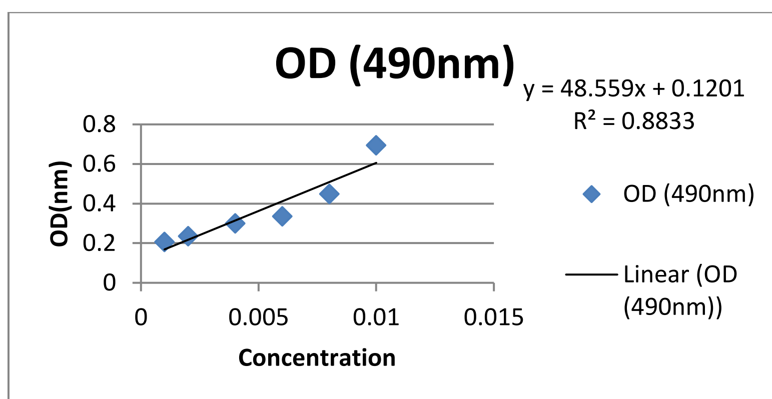

Figure C: Graph of sucrose standard of concentration vs OD

A.9 Standard curve of BSA for Folin- Lowry Method of Protein Estimation

Table D: BSA standard of concentration Vs OD for Folin- Lowry method

S. no	Concentration(mg/ml)	OD (660nm)
1	0.05	0.356
2	0.1	0.688
3	0.15	0.825
4	0.2	1.007
5	0.25	1.137

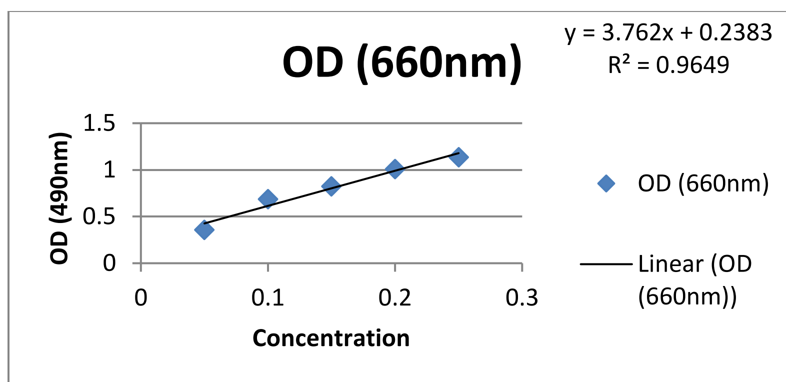

Figure D: Graph of BSA standard of concentration Vs OD for Folin Lowry method

A.10 Standard curve of BSA for Bradford Method of protein Estimation

Table E: BSA standard of concentration Vs OD for Bradford method

S. no	Concentration(mg/ml)	OD (595nm)
1	0.025	0.813
2	0.05	1.353
3	0.1	1.517
4	0.2	1.573
5	0.3	1.609
6	0.4	1.655
7	0.5	1.712

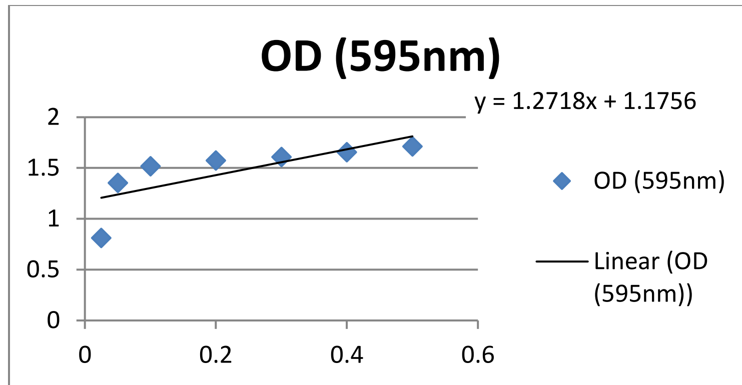

Figure E: Graph of BSA standard of concentration Vs Absorbance for Bradford method

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