EVALUATION OF ANTIFUNGAL POTENTIAL OF SOLANUM LYCOPERSICUM ENDOPHYTIC BACTERIA FOR BIOCONTROL OF PLANT PATHOGENIC FUNGI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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MZU REGN NO.1703974

M.PHIL REGN NO: MZU/M.PHIL./630 OF 12.06.2020

DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCE

SEPTEMBER , 2021

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Submitted
In partial fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl.

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CERTIFICATE

This is to certify that the dissertation entitled "**Evaluation of antifungal potential of *Solanum lycopersicum* endophytic bacteria for bio-control of plant pathogenic fungi"** submitted by **William Carrie** Registration no**. MZU/M.Phil./ 630 of 12.06.2020** to Mizoram University for the award of Master of Philosophy in Biotechnology is a record of research work done by him and his own findings which he performed with sincerity and devotion under my guidance during the year 2019 to 2021.

It is further certified that the scholar fulfilled the requirements as laid down by the University for the purpose of submission of M.Phil dissertation.

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August, 2021

I William Carrie, hereby declare that the subject matter of this dissertation is the record of work done by me, that the contents of this dissertations did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted be me or any researcher degree in any other University/Institute.

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ACNOWLEDGEMENT

First and foremost, I would like to thank my Supervisor **Dr. Esther Lalnunmawii** for giving me the opportunity to work under her and providing me with equipment and necessities. Her motivation and help contributed tremendously to the successful completion of my project.

I would also like to show my gratitude to my Co-Supervisor Dr. **Zothanpuia** and Dr. **Gajanan Mehetre** (Research Associate) who helped me with their invaluable guidance and feedback has helped me in completing this project.

I would like to express my gratitude to **Assoc. Prof**. **Bhim Pratap Singh**, NIFTEM, Sonepat for his kind support and guidance and also **Prof. N. Senthil Kumar** for his support and providing me Biotech-Hub with DNA sequencing facilities to complete my project work. I also give thanks to R.Lalengkimi for her kind contribution in DNA sequencing despite her busy schedule.

I would like to extend my heartfelt thanks to the **Head of Department** and all the **Teachers** and **Research Scholars** of Biotechnology Department, MZU for their support and motivation.

Special thanks to my lab partners Lalrokimi Tochhawng, Purba Jyoti Deka, Lallawmsangi, Farzana Ahmed, Ruth Zomuansangi, Vincent Vineeth Leo and Yogesh Malvi whom I share the journey with ups and downs and who have been with me all the time.

I also thank my parents for their support and motivation. Any attempt at any level can,,t be satisfactorily completed without their support.

At last but not in least, I would like to thank everyone who helped and motivated me to work on this project.

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1. Introduction

Endophytes are organisms that live inside plant tissue without causing any visible disease symptoms (Wilson 1995). Endophytic bacteria are one of the most important plant associated microorganisms that colonize the internal parts of plants (Santoyo et al., 2016). All or most plants are associated with endophytes that can vastly spread through the entire plants tissues. They can colonize the intracellular and intercellular spaces without causing any morphological or observable infection to the host plant. (White et al., 2019; Fouda et al., 2019). They can be found in various environments like aquatic, tropic, temperate, rainforests, deserts, xerophytic and coastal forests and develop a symbiotic association with tissues of many plants (Strobel et al. 2002; Suryanarayanan and Murali 2006). The route through which bacteria enters the host cell started in the seedling stage during seed development. However, majority are suggested to be acquired from the environment, they can be originated from the soil by infecting root junction through the cracks or wounds and spread to the intercellular spaces (Chi et al. 2005 and Hardoim et al., 2012). Establishment of such symbiotic association between beneficial bacteria and plant provide several benefits such as biotic and abiotic stress tolerance and also promote growth of their host plants (Miliute et al., 2015). Various workers have described the importance of plant-associated bacteria which is necessary for stimulation of plant growth and health management (Nihorimbere *et al.*, 2011). There has been an increase interest in the plant microbiome since the past decade due to their ability to protect host plant from biotic and abiotic stress, increase nutrient uptake and promote growth by synthesizing several plant growth promoting compounds (Frank et al., 2017).

Plants are exposed to different biotic and abiotic factors which have diverse effects in their growth and development. Biotic stresses are directed to living components such as pathogenic organisms that lead to low yield and crop losses (Cocq et al., 2017). The Fusarium wilt is considered as one of the most prevalent disease to many important crops including tomato, caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL). The main symptoms include yellowing of the lower leaves, wilting of the plants, browning of vascular tissues and eventually death leading to a great loss in agriculture (Lim et al. 2006). It is estimated that agricultural loss due to fungal infections constitute up to 20% of

crops worldwide and the use of fungicides has become crucial in the last decades for an effective control of plant diseases (Gullino *et al.* 2000). In order to reduce the cost due to plant pathogens, different types of chemical pesticides have been applied since many decades. The application of chemical pesticides can save the plants and also increases their productivity. However, increasing use of chemicals to overcome the problems has raised public concern due to their negative impacts on plant, other non-targeted organisms and the environment. It has been observed that long term use of chemical fertilizer has drastically decreased bacterial diversity in soil and leaching of phosphorus and nitrogen into groundwater cause pollution in the soil and water sources (Sun et al., 2015; Rafi et al., 2019). Therefore, in order to minimize the dependency on chemical fertilizers, an alternate and efficient method is necessary for sustainable development in agriculture.

Biological control of plant diseases is a fast-growing area of research in recent years. Numerous endophytic bacterial species have been identified and reported for their potential in controlling plant pathogens . The control of pests using such biopesticides has become an interest of many researchers due to their advantages over chemicals being used in modern agriculture. Biological control aims in the use of beneficial organisms including endophytes and their products or genetic content to induce positive responses and help plant in fighting against invading pathogens (Tranier et al., 2014). Many endophytic microbes beneficial to plants have been reported from different plants sources including of those economically important plants like Rice, Wheat, Maize, Tomato, Mustard, Soybean, Sugarcane, Strawberry, Chili, Cowpea, Citrus, Pearl millet, and Sunflower (Verma et al., 2017; Yadav et al., 2018). Bacterial endophytes are also well studied for their significant role in the production of bioactive compounds. They produce beneficial secondary active metabolites that are necessary in developing field such as agriculture, biotechnology and medicine, etc. (Begum & Tamilselvi 2016). Several endophytes including species of *Bacillus*, *Pseudomonas* and certain genera of *Actinobacteria* are known potential source of metabolites particularly lipopeptides which are important compounds for antibiosis and biocontrol against invading pathogenic microbes (Brader et al., 2014). Diverse array of bioactive compounds such as peptides, alkaloids, flavonoids, terpenoids, steroids, and phenols besides natural insecticide

azadirachti have been reported from endophytic bacteria for their agricultural and and medical importance (Li et al. 2008; Kusari et al. 2012; Molina et al. 2012).

Antifungal activity of endophytes against plant pathogenic fungi such as *Fusarium* sp., *Rhizoctonia* sp., Verticillium sp., Verticillium sp., and *Phythium* sp. have been reported from many plants (Cho et al., 2007). Tomato plants are also harboring endophytic bacteria that show a great promise in the control of a wide range of phytopathogenic fungi (Kefi et al., 2015). These antifungal compounds produced by endophytic bacteria are mainly lipopeptides like surfactin, iturin and fengycin and other enzymes such as chitinase and proteases that degrade structural polymer of fungi (Katz and Demain 1977). It is demonstrated that the control of certain disease like Fusarium wilt of tomato is even more difficult due to endogenous progress of the pathogen within the host plant‟s vascular tissues. Hence, endophytic microorganisms are considered a better candidate in order to limit the disease (Aydi et al., 2016). Besides their role in plant defense system, benefits of endophytic bacteria to the host plant also includes their ability to induce the growth of plant such as by producing plant growth hormones, nutrient acquisition, phosphate solubilization and nitrogen fixation (Glick 2012).

Although there are diverse endophytic bacteria reported from many plant sources and their potential in controlling plant diseases have also been demonstrated, biological control of plant diseases particularly to fungal diseases is still a challenge. Due to the prevalence of fungal pathogens and the necessity to limit the use of chemical pesticides, the search for new and reliable resource needs to be lengthened. The study of endophytic bacteria from tomato plant has also been increasing in recent years with many more to be explored. Keeping in view, the potential aspects of endophytes as antifungal agents, the present study was designed with the following objectives:

1. Isolation and molecular characterization of endophytic bacteria from healthy and diseased suspected tomato plants.
2. Screening for antifungal activity against selected plant pathogenic fungi.
3. Evaluation of potent strains for plant growth promoting attributes.

2. Literature Review

Endophytes were defined by several scientists as organisms living inside plants without causing any visible disease symptoms (Wilson 1995). Firakova et al. (2007) also defined endophyes as the microbial flora including both bacteria and fungi found in all parts of living plant species and their organs without causing any disease symptoms to host plant. They are treated as endosymbionts and have been reported from many economically and medicinally important plants. For decades, endophytic bacteria have been observed to exist inside monocotyledonous and dicotyledonous plants and are considered as important organisms that can develop beneficial associations with plants (Santoyo et al., 2016, Tervet and Hollis, 1948). Endophytes generally reside inside its host tissue in most of their life cycle without causing any damage and also assist its host plant in nutrients supply and stress response. It is indicated that every single plant is a host to one or more types of endophytes that cause no harm to the host (Strobel and Daisy, 2003). Endophytic colonization of host by the bacteria involves complex communication among them. The process usually initiates through the root exudates from the host plant and recognition of this signal by the endophytic bacteria (Afzal et al., 2019). The colonization includes a multi-step process where the bacterium migrates towards the root surface, attachment and distribution along the root and survival of the population for successful colonization.

Endophytes are also capable of producing several degradative enzymes such as cellulase and pectinases that is responsible for colonizing root tissues and spread actively in the aerial parts of the plants. Utilization of such enzymatic activity have been demonstrated for plant growth promoting bacteria like *Azospirillum irakense*, *Azoarcus* sp. and others (Jha et al., 2013). Although root colonization is usually the main route for endophytes, the aerial parts of plants such as stem, buds, leaves, flowers and seeds are also used (Hallmann et al., 1997). Plant–microbe interactions are mainly through commensalism and mutualism. Endophytes are provided with undisturbed existence and nutrients in the host cell through commensalism, while mutualism benefits both the organism and frequently results in the growth promotion of host plants (Kogel et al.,

2006). These microbial communities play important role in plant development since they are good source of phytohormones,
siderophores, resistance to pathogens, promote biological nitrogen fixation and produce active antibiotics (Magnani et al., 2010, Liarzi et al. 2016).

A great diversity of endophytes have been isolated from different parts of the plants such as roots, shoots, buds, leaves, and fruits, they are known to inhabit almost all plants species (Nair et al., 2014). They are found in different region such as temperate, tropical and in boreal forests (Zhang et al., 2006) inhabiting diverse species of plants from woody tree species, such as oak, and pear to herbaceous crop plants, such as sugar beets and maize (Lodewyckx et al., 2002). They can be characterized based on distribution such as obligate endophytes (proliferate only inside host plant), facultative endophytes (usually free living but have the ability to colonize plant under favorable condition), and passive endophytes (does not show active colonization but invasion may occur through wounds or abrasion in the root curls) (Tewari et al., 2019). Various species of cultivable endophytic bacteria including both gram-positive and gram-negative have been reported from a wide range of host such as terrestrial and aquatic plants (Sturz et al., 1996). Among endophytic bacterial communities; Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were known to be dominant and other smaller communities includes Chloroflexi, Armatimonadetes, Planctomycetes, Cyanobacteria and Nitrospira (Sessitsch et al., 2012). It was demonstrated that the endophytes‟ niche can only occupied by those bacteria capable of colonizing intercellular spaces of different part of the plants including seeds. The classical studies of endophytic biodiversity are mainly based on characteristics of isolates from various tissues of surface sterilized plants (Miche et al., 2002, Lodewyckx et al., 2002). The bacteria resides latently or actively colonizing plant tissues and are found in a wide range of plant more than 80 genera and 290 plant species (Jacobs *et al.* 1985).

Endophytes can be characterized into three main groups based on functionality as; biocontrol agents, plant growth promoters, and plant stress homeoregulating microbes (Tewari et al., 2019). The endophytic bacteria can stimulate the growth of host plants by direct and indirect contribution (Santos et al., 2014). They can directly enhance growth of

plants by production phytohormones, biological nitrogen fixation, phosphorus solubilization, acceleration of digestion and production of diverse class of secondary metabolites which confer resistance against invading pathogens. The indirect mechanism involves promotion and improving of nutrients availability and absorption, induces stress tolerance caused by abiotic factors such as exposure to heavy metals, osmotic stress and production of xenobiotic molecules (Xia et al.,2015; Santos et al., 2014; Oliveira et al., 2003). It has been demonstrated that artificial inoculation of endophytic bacteria can potentially act as biocontrol agent by reducing the attack of plant pathogens such as bacteria, fungi and viruses (Chebotar et al., 2015).

Endophytic bacteria also play an important role in the production of biologically active secondary metabolites. Endophytic microorganisms usually produce secondary metabolites of low-molecular weight that include phytohormones, antimicrobial compounds, or their precursors, vitamins like B12 (Ivanova et al. 2006), bioprotectants (Trotsenko and Khmelenina 2002). Secondary metabolites having wide range of applications are synthesized through various pathways and belong to several classes like phenolics, alkaloids, lipids, terpenes, saponins and carbohydrates that are produced through metabolic pathways derived from the primary metabolic pathways (Hussein and Anssary 2019).

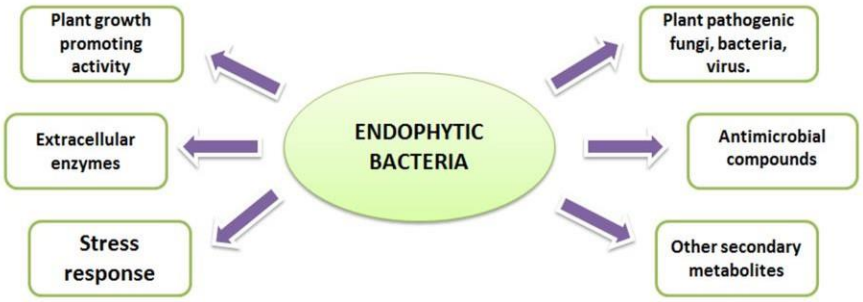


Figure 1. Potential aspect of endophytic bacteria

Plant disease poses humongous biotic stress resulting in huge economic loss due to their infection on economically important plants. Fisher *et al*. (2012) reported that fungal destroy a third of all food crops annually that are enough to feed 8.5% of the seven

billion populations in 2011.Many pathogens can still spoil foods even in post-harvest by toxins production during storage. The deliberate urge to control fungal diseases has resulted in invention of several fungicides, the application of which effects environment and endangering the health of humankind (Latha et al., 2019). Therefore, in order to reduce the use of chemicals, biocontrol of plant diseases have become an important interest of researchers. The proven benefits of endophytes as agent for controlling plant diseases is due to their well adaptation to live inside the plants which provides reliable suppression of many diseases including vascular diseases (Lin et al. 2013). Colonization of plant by endophytes on ecological niche similar to phytopathogens is another advantage which even makes them more suitable as biocontrol agents (Berg et al., 2005).

Many endophytic bacteria such as the genus of Bacillus (Kefi *et al.* 2015), Paenibacillus (Cho *et al.* 2007), Pseudomonas (Bahroun *et al.* 2018), Streptomyces (Taechowisan *et al.* 2003), *Burkholderia* (Paul *et al.* 2013) etc. are reported to be a good source for obtaining active secondary metabolites. Several strains of thesegenera were able to produce secondary metabolites such as iturin, fengycin and surfactin and showed a great promise in the control of a wide range of phytopathogenic fungi (Katz and Demain 1977). Andreolli *et al*. (2019) reported several genes such as *phl*D, *plt*B and *prn*C in endophytic bacteria identified as *Pseudomonas protegens* that were responsible for the synthesis of antifungal compounds. In support, the *in-vitro* antifungal activity screening showed mycelial growth inhibition to *Botrytis cinerea*, *Aspergillus niger*, *Penicillium expansum* and *Neofusicoccum parvum* as well as *in-vivo* antagonistic activity against *B. cinerea* on grapevine leaves respectively. *Bacillus amyloliquefaciens* has also been reported from economically important plant *Solanum lycopersicum* that have high antifungal activity against *Alternaria solani* mainly responsible for early blight in tomato (Yi et al., 2015). Besides antifungal activity, endophytes can also regulate gene expression of host plant in response to pathogens. For example, endophytic *Bacillus* spp. isolated from maize plant can induce the up-regulation of pathogenesis-related genes when seeds are treated with bacterial culture (Gond et al., 2015).

3. MATERIAL AND METHODS

Sample Collection and Processing

Tomato plants were collected from Sihphir, Mizoram tomato farm cultivated by local farmer. The samples were collected for isolation of endophytic bacteria and thoroughly observed for disease symptoms and plant health. Based on observation, different parts of plant tissues; shoot, leaves, flower, and fruits were collected by cutting with a clean scissor from both healthy and disease suspected plant. The samples were then immediately transferred into a plastic bag and sealed properly and brought to the laboratory on the same day. All the samples collected were processed immediately for isolation of endophytic bacteria.

Isolation of Endophytic Bacteria

Endophytic bacteria were isolated from different part of the plant using the method of Sturz *et al.* (1998). Plant tissues were carefully dissected into small pieces with sterilized scalpel and sterilized by chemical treatment as follows. The tissues were first soaked in 70% ethyl alcohol for 30 sec and then transferred to 2% sodium hypochloride for 5 min and finally washed three times with sterilized double distilled water and then dried under laminar air flow. After treatment, the sterilized tissues of 5-10 pieces per plate were transferred into a freshly prepared Luria Bertani agar (LB agar) and Nutrient agar (NA) culture media and incubated at 28 ± 2°C for 3 - 4 days in BOD incubator. The growth of bacteria was observed on daily basis and based on the colony morphology and appearance, different colony from plant tissues was streaked into a new culture media. Pure cultures of bacterial isolates were obtained by repeated sub-culturing into a fresh media until a single colony of same morphology was obtained. Validation of sterilization process was done by pressing the sterilized tissues and spreading the distilled water used for final rinsing onto a newly prepared culture media. The growth of contamination was observed for 3 days. Gram staining was done for selected isolates using gram staining kit.

Molecular Characterization

Genomic DNA isolation

Forty isolates were selected for genomic DNA isolation based on colony morphology and source of plant tissue. DNA isolation was done as per the method of Green and Sambrook (2018). The bacteria were freshly grown in LB media and the overnight culture colonies were suspended to 2 ml microcentrifuge tube containing 0.5 ml of Tris-EDTA, 10% SDS and Proteinase K mixture. The mixture was vortex in Spinix Vortex shaker for 10 min and incubated in water bath at 37ºC for 1 h. The tubes were then treated with 0.5 ml of Phenol: Chloroform: Isoamyl alcohol (25:24:1), mixed well for 10 min and centrifuged at 12000 rpm for 15 min. After centrifuged, the top layer containing DNA were carefully transferred into new tube without disturbing the layer below. This process is repeated twice and add 1/10 volume of sodium acetate. The samples were then treated with Isopropanol and then stored at -20ºC overnight. The next day, overnight incubated samples were centrifuged at 12000 rpm for 10 min and collect the precipitate. Again, 70% alcohol was added and incubated at room temperature for 30 min. Finally the samples were again centrifuged at 12000 rpm for 10 min and then collect the pellet and dried under in laminar air flow. 60µl Tris-EDTA were added to the DNA samples and stored at -20ºC. The DNA samples were run in 0.8% agarose gel electrophoresis and observed the band in under UV light and documented using a Bio-rad Gel Doc XR+ system.

PCR Amplification of 16s rRNA gene.

All DNA isolated were processed for PCR amplification of 16S rRNA gene partial sequence using universal primers:

Forward primer-16F530 (5‟-AGAGTTTGATCCTGGCTCAG-3‟)

Reverse primer-16R1492 (5‟- GGTTACCTTGTTACGACTT-3‟)

The PCR reaction cocktail (50 µl) contained 2 µl (50-100 ng) of genomic DNA, 1X reaction buffer (TrisKCl-$MgCl_2$), 2 mM $MgCl_2$, 0.2 mM dNTP, 1 µM of forward and reverse primer each, and Taq polymerase (5U/µl).

The PCR temperature cycling conditions were:

Initial denaturation at 94°C for 5 min; 35 cycles of *denaturation* at 94°C for 1.5 min, *annealing* at 56°C for 1 min, and *extension* at 72°C for 1.2 min. The final cycle was followed by final *extension* at 72°C for 10 min.

The PCR products were observed and checked in 1.2% Agarose gel electrophoresis by loading 2 µl or PCR product and molecular marker and observed the band in under UV light and documented using a Bio-rad Gel Doc XR+ system.

DNA Sequencing and Phylogenetic Analysis

Amplified 16S rRNA gene products were further sequenced for molecular identification. DNA sequencing of partial 16S rRNA gene was done in Biotech-Hub, Biotechnology Department, Mizoram University. After the sequence results were obtained it was compared with the reference strains of bacteria from National Center for Biotechnology Information (NCBI) genomic database, using BLASTn search for similarity percentage. Type strains with highest similarity percentage were retrieved from NCBI.

For Phylogenetic analysis, multiple sequence alignment was performed using Clustal W packaged in MEGA X software. Phylogenetic tree was constructed by MEGAX software using maximum-likelihood method and Kimura-2-parameter model. The evolutionary models were chosen based on highest Akaike Information Criterion (AIC) values and lowest Bayesian Information Criterion (BIC) scores. The significance of the branching order was determined by bootstrap analysis of 1000 alternative trees.

***In-Vitro* Antifungal screening**

Plant pathogenic fungi; *Fusarium graminearum Schwabe* (MTCC-9064), *Fusarium oxysporum* (MTCC- 1893), *Fusarium oxysporum* f. sp. *lycopersici* (ITCC-3437), *Fusarium udum* (MTCC-2755), *Fusarium proliferatum* (MTCC-286), *Fusarium oxysporum* f.sp. *pisi* (MTCC-2480) were used in this study.

In-vitro screening of antifungal activity was done for all isolates using dual culture assay. The bacteria isolates were streaked on the center of freshly prepared PDA plate and fungal mycelium disc of 5mm diameter were placed at a distance 2cm away from the bacteria. The inoculated cultures were incubated at 28°C in BOD for 7 days and growth inhibition of fungal pathogens was measured (Korsten et al., 1999). The fungal mycelium inoculated without bacteria was used as control.Growth inhibition percentage were calculated as below:

$$GI(\%) = \frac{R1-R2}{R1} X\ 100$$

where,

R1 - the distance of fungal growth from the point of inoculation on control plate.

R2 - the distance of fungal growth from the point of inoculation to the bacterial colony in dual culture.

Extraction of lipopeptides

Isolates having antagonistic activity were further processed for lipopeptide production and extraction using the method of Kim *et al.* (2004) with minor modification. Loopful colonies of pure cultures were inoculated into freshly prepared LB broth and incubated at Thermo scientific MAXQ shaking incubator at 120 rpm for 3 days. The broth suspension was then centrifuged at 10,000g for 15 min in 4°C and supernatant was collected. The cell free supernatant was acidified with 3 N HCl to pH 2 to precipitate the lipopeptide and left overnight at 4°C. It was then centrifuged again at 10,000 rpm for 20 min at 4°C and the precipitate was collected and neutralized by distilled water. It is further transferred to a separatory funnel and dissolved in Chloroform/Methanol (2:1, v:v) and left overnight. After proper separation, the lower layer were collected and evaporate the solvent at 45°C using Buchi Heating Bath B-100 rotary vacuum evaporator. The crude extract obtained was dissolved in 50% methanol at a concentration of 50 mg/ ml (Figure 2).

Antifungal activity of lipopeptides

To evaluate the antifungal activity of lipopeptide well-diffusion method was used (Kalai-Grami et al., 2013). A well of 4 mm diameter was punched on the center of PDA

plate and 100 µl of 50 mg/ ml lipopeptide sample were loaded on the well. Subsequently, 4mm mycelial disk of fungal pathogens were inoculated at the distance of 2cm away from the well. 50% methanol was also loaded as control. The plates were then incubated in BOD at 25ºC for 7 days and growth inhibition was observed. Growth inhibition percentage was calculated using the same method as described earlier.

Screening For Plant Growth Promoting Activities

Indole Acetic Acid (IAA) production

Production of Indole acetic acid (IAA) was determined using Gordon and Weber (1951) method for the strains showing antifungal activity. Cultures were grown in minimal salts media containing 0.2% L- tryptophan at 28°C for 3 days with continuous shaking at 125 rpm. The culture suspension was then centrifuged at 11000 rpm for 10 min and mixed the supernatant (1ml) with Salkowski‟s reagent (2ml). The mixture was incubated for 30 min in dark at 27ºC. Production of IAA was determined by the development of pink-red colour and the optical density (OD) was measured at 530 nm using a Thermo scientific (Multiskan GO) spectrophotometer. The absorbance was compared with standard curve of IAA for quantitative determination and was expressed in µg/ml.

Ammonia production

Ammonia production was determined as per the method of Cappucino and Sherman 1992. In this method, cultures were grown in 10 ml of peptone water and incubated at 28°C in shaking incubator at 120 rpm for 3 days. The bacterial suspension was centrifuged at 11000 rpm for 10 min and the supernatant was collected in new tube. 0.5 ml of Nesseler‟s reagent was added. to the cell free supernatant and the development of brown to yellow color indicated the production of ammonia.

Phosphate Solubilization

For qualitative estimation of phosphate solubilization single colony of bacterial was streaked onto Pikovskaia‟s medium containing tricalcium phosphate (Pikovskaia, 1948). The culture was incubated at 28°C for 7-10 days. The plates were observed daily for the clear P-zone around the colonies.

Screening for Extracellular Enzymes Production

Chitinase Activity

Chitinase enzyme activity was analyzed in Colloidal Chitin agar medium prepared by adding 10 g/l of colloidal chitin in Minimal salt medium (containing Na2HPO4, 0–65 g/l, KH2PO4, 1.5 g/l, NaCl, 0.25 g/l, MgSO4, 0–12 g/l, CaCl2, 0.005 g/l and NH4Cl, 0–5 g/l) and final pH was adjusted to 6.5.The overnight culture of bacteria were streaked on the surface of the media, incubate at 28°C for 3 days and observe for the formation of clear halo zone (Toharisman et al. 2005).

ACC Deaminase Activity

ACC deaminase activity was screened on sterile minimal DF (Dworkin and Foster, 1958) with the following composition (gram/litre) 4.0 g KH2PO4, 6.0 g Na2HPO4, 2.0 g glucose, 2.0 g gluconic acid, 0.2 g MgSO4*:*7H2O, and 2.0 g citric acid with trace elements: 10 mg H3BO3, 11.19 mg MnSO4*:*H2O, 1 mg FeSO4*:*7H2O, 124.6 mg ZnSO4*:*7H2O, 10 mg MoO3, pH 7.2 and 78.22 mg CuSO4*:*5H2O amended with 3 mM ACC instead of $(NH_4)_2SO_4$ as sole nitrogen source. Cultures were streaked on the DF media and incubate at 28ºC for 3 days. Colonies growing on the plate were considered as producers of ACC deaminase.

Screening of Celullase

The ability of isolates to produce cellulase enzyme was tested on CMC agar containing 1 g/l carboxymethylcellulose (CMC); 0.5 g/l $NaNO_3$; 0.5 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l K_2HPO_4; 0.001 g/l $FeSO_4 \cdot 7H_2O$, 1 g/l yeast extract; 15 g/l agar). The cultures were streaked and incubated for 2 days at 28°C on pH 8.0 and then flooded with Congo red solution for 2 min. The Congo red dye was washed away using 1M NaCl and colonies showing clear zones were considered to be cellulase producers. (Teather and Wood, 1982).

Screening of Xylanase

For xylanase screening pure colonies of potent strains were streaked on xylan induction agar plates (K2HPO4 –0.5%, NaCl-0.5%, Peptone-0.5%, Yeast extract- 0.25%, Agar Agar -1.5% ,milliQ H2O-75% and WSOSX1 (Water Soluble Xylan from Oats for

Screening Xylanase-25%). The plates were incubates for 2 days at 28°C and subjected to Congo red assay as already described.

Detached Leaflets Assay

Tomato leaves showing no disease symptoms were collected and surface sterilized in 2 % sodium hypochlorite for 3 min and then thoroughly rinsed with distilled water thrice and dried under laminar air flow. To each leaflets, three needle-prick wound were applied for treatment and inoculated with mycelium culture of plant pathogenic fungi *Fusarium oxysporum* f.sp *lycopersici*. 2 days bacterial suspensions were sprayed to each leaflets and incubate at 25°C for 7 days in dark (Rajkumar et al.,2005). Leaflet inoculated with suspension of fungal pathogen were indicated as positive control and leaflets without any treatment is indicated as negative control.

***In-vivo* plant growth activity**

Inoculum Preparation

Inoculum was prepared using potent isolates for in-vivo plant growth promoting activity. Bacteria culture were inoculated in LB agar and after 24 h at which the bacteria cells were found in the exponential phase and active, cultures were scraped by adding 20 ml of saline water (0.85% NaCl) and then transferred to sterile tube. The tubes containing bacterial cells were centrifuged at 10000 rpm for 5 min at 4ºC and discard the cell free supernatant and re-suspended the pellet in 20ml sterile tube.

Seed Disinfection

Tomato seeds F1 VEERU 650 were purchased from Aizawl and stored at 4 ºC. The seeds were disinfected by treatment with 70% ethanol for 1 min and 2% sodium hypochloride for 2 min, rinsed with distilled water three times. The seeds were dried in laminar flow cabinet and sterility control was performed by plating 50 seeds per plate on LB agar. The plates were sealed with parafilm and incubated at 25ºC for 2 days. The seeds were considered as surface disinfected if no microbial growth were observed after incubation period and finally used for the experiment.

Endophytes Delivery

Seed Treatment + Soil drench method as described by Algam et. al, (2005) was used for delivering endophytes in this study. Soil was autoclaved before used to remove any microbes present and filled into new polypot. Sterilized seeds were submerged in inoculum prepared for both potent strains and *Fusarium oxysporum* f. sp. *lycopersici* for 24 h before planting and transferred to polypot (6 seed per pot). After planting the soil were also drenched with approximately 100 ml of inoculum after 10 days. The seeds were allowed to germinate and observe seed germination, root length and shoot length. Each seeds were given different treatment. Seeds with *Fusarium oxysporum* f. sp. *lycopersici* were indicated as positive control and seeds without any treatment were indicated as negative control.

4 RESULTS

Isolation of Endophytic Bacteria

A total of 70 endophytic bacteria were isolated from different tissue of *Solanum lycopersicum* L; shoots, leaves, flowers and fruits collected from Sihphir, near Aizawl. Pure cultures of the isolates were obtained by subsequent streaking on LB media and selected based on morphological characteristics after two days of incubation.

All isolates from were given different code based on the source of isolation as HST - Healthy shoot tissue, HLT - Healthy leaf tissue, DST - Diseased shoot tissue, DLT - Diseased leaf tissue, HFRT - Healthy flower tissue , DFR - Diseased fruit tissue and HFT- Healthy flower tissue. Most endophytic bacteria were isolated from Healthy Shoot Tissue - 15 endophytes followed by Healthy Leaf Tissue - 12 endophytes. Number of isolates from different organs is as shown in Table 1.Different colors of bacteria; white, yellow, orange, creamy and different forms of colony; round, irregular, smooth, and rough were obtained.

Eight isolates based on colony morphology and colors were selected as representatives of endophytes for gram staining using gram staining kit. Gram staining shows most of the selected strains as gram positive constituting 62.5% and gram negative 37.5% as observed under microscope respectively (Figure 2).

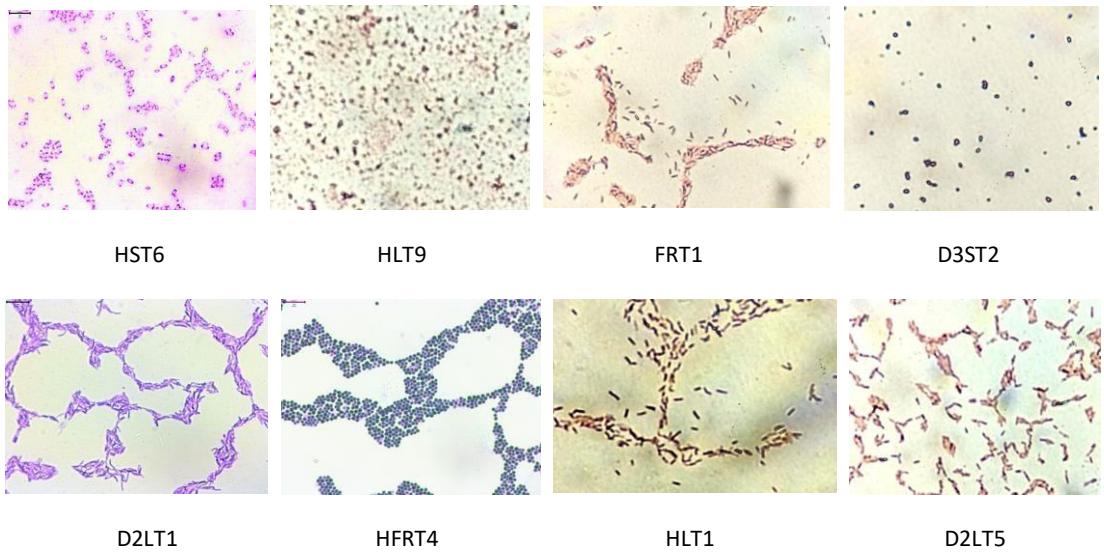


Figure 2. Gram staining of selected isolates showing both gram positive and gram negative

Table 1. Number of endophytic bacteria isolated from different plant tissue.

Plant tissue	Number of isolates
Healthy Shoot Tissue (HST)	15
Healthy leaf tissue (HLT)	12
Diseased shoot tissue (DST)	10
Diseased leaf tissue (DLT)	9
Healthy fruit tissue (HFRT)	6
Diseased fruit tissue (DFT)	8
Healthy flower tissue (HFT)	5

Table 2. Morphological and microscopic characteristics of endophytic bacteria.

Characteristic	HST6	HLT9	D2LT1	HFRT1	D3ST2	HLT1	D2LT5	HFRT4
Colour	Creamy	Creamy	Orange	Creamy	White	Yellow	white	Yellow
Shape	Bacillus	Round	Rod	Rod	Round	Rod	Rod	Round
Gram	+	+	+	-	+	-	+	+

Genomic DNA isolation

Genomic DNA isolation of forty isolates was checked qualitatively by running in 0.8% agarose gel electrophoresis. The product of DNA was visible as a clear band (Figure 3) indicating a good quality of DNA when visualized under UV light and documented using a Bio-rad Gel Doc XR+ system (Hercules, CA, USA). All the DNA concentration (260/280) was also found to be in a range between 20-100 ng/ul.

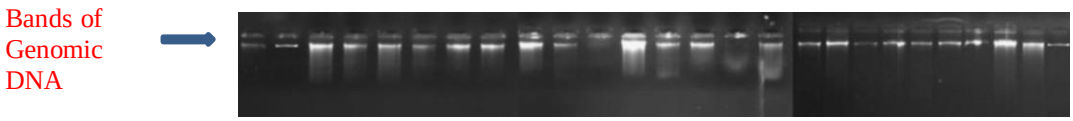


Figure 3. Genomic DNA bands under UV light and documented using Bio-rad Gel Doc XR+ system

PCR amplification of 16S rRNA gene

All DNA isolates were subjected to amplification of 16S rRNA gene commonly used for bacterial identification using Applied Biosystems thermal cycler polymerase chain reaction (PCR). The PCR products were checked in 1.2% agarose gel electrophoresis and the bands obtained were compared with molecular marker of DNA ruler plus (1 kb to 3 kb). The amplified DNA products were observed as a single band within the same distance from well with the 1000 bp marker (Figure 4).

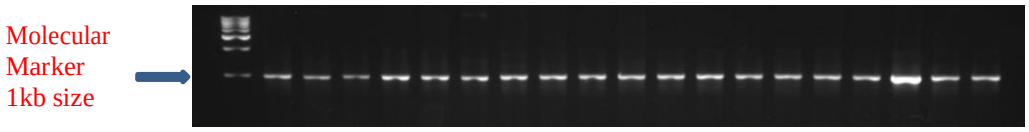


Figure 4. PCR bands under UV light documented using Bio-rad Gel Doc XR+ system

DNA Sequencing and Identification

The DNA sequence obtained was analyzed by Finch TV software and BLAST in NCBI. All selected isolates were molecularly confirmed with similarity percentage ranging from 99% - 100%. The isolates belong to nine different genera of endophytic bacteria from all selected sample in which *Bacillus* sp. is observed to be the dominant genus (Figure 5). The dominant genus *Bacillus* constitute 62.5% followed by *Ochrobactrum*, *Brevundimonas*, and *Lysinibacillus* each 7.5% and others rare genus including *Alcaligenes* (5%), *Enterobacter* (5%), *Pseudomonas* (5%), *Staphylococcus* (2.5%) and *Cytobacillus* (2.5%) as shown in table 3.

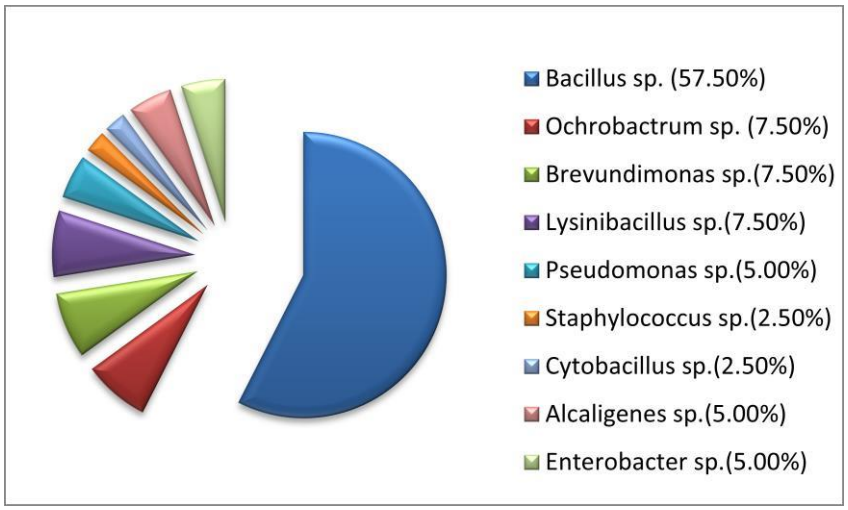


Figure 5. Percentage frequency of endophytic bacteria genera isolated from tomato plant

Table 3. Isolates and closest relative species in NCBI.

Sl.No	Isolate Name and Code	Closest relative species in BLAST and accession no	% Similarity
1	*Lysinibacillus fusiformis* (HST1)	*Lysinibacillus fusiformis* (MK431518)	99.00
2	*Ochrobactrum* sp. (HST2*)*	*Ochrobactrum* sp. (MK139875)	99.00
3	*Brevundimonas* sp. (HST4)	*Brevundimonas* sp. (KT630838)	99.00
4	*Bacillus licheniformis* (HST5)	*Bacillus licheniformis* (KY922738*)*	99.00
5	***Bacillus siamensis*** (HST6)	*Bacillus siamensis* (MN17648*)*	99.00
6	*Bacillus tequilensis* (HST7)	*Bacillus tequilensis* (JQ9905071)	99.00
7	*Alcaligenes faecalis* (HST8*)*	*Alcaligenes faecalis* (KM502543)	99.00
8	*Brevundimonas diminuta* (HST9)	*Brevundimonas diminuta* (KF929392)	99.00
9	*Enterobacter* sp. (HST10)	*Enterobacter* sp. (KX914535)	100
10	*Lysinibacillus fusiformis* (HLT1)	*Lysinibacillus fusiformis* (MK431518)	99.00
11	*Bacillus subtilis* (HLT2)	*Bacillus subtilis* (MG662180)	99.00
12	*Bacillus cereus* (HLT4)	*Bacillus cereus* (MG065717)	99.00
13	*Lysinibacillus fusiformis* (HLT5)	*Lysinibacillus fusiformis* (MG733576)	100
14	*Bacillus subtilis* (HLT6)	*Bacillus subtilis* (KF053069)	99.00
15	*Bacillus tropicus* (HLT7)	*Bacillus tropicus* (MW478751)	99.00
16	*Bacillus australimaris* (HLT8)	*Bacillus australimaris* (MH169000)	99.00
17	***Bacillus velezensis*** (HLT9)	*Bacillus velezensis* (MT649755)	100
18	*Bacillus cereus.* (HLT10)	*Bacillus cereus* (KR078256)	99.00
19	*Bacillus megaterium* (*HFR1*)	*Bacillus megaterium* (MH071287)	99.00
20	*Bacillus* sp. (HFR3)	*Bacillus* sp. (MN826392)	99.00

21	*Bacillus* sp. (HFR4)	*Bacillus* sp. (KJ944112)	99.00
22	*Bacillus tropicus* (HFT1)	*Bacillus tropicus* (CP053955)	99.00
23	*Bacillus cereus* (HFT2)	*Bacillus cereus* (MN595060)	99.00
24	*Enterobacter hormaechei* (HFT3)	*Enterobacter hormaechei* (MT386317)	99.00
25	*Ochrobactrum tritici* (HFRT3)	*Ochrobactrum tritici* (MG459004)	99.00
26	*Staphylococcus saprophyticus* (HFRT4)	*Staphylococcus saprophyticus* (MG892848)	100
27	*Bacillus amyloliquefaciens* (DFR1)	*Bacillus amyloliquefaciens* (MN844066)	99.00
28	*Cytobacillus kochii* (DFR3)	*Cytobacillus kochii* (MW287222)	99.00
29	*Ochrobactrum anthropi* (DFR4)	*Ochrobactrum anthropi* (MK503652)	99.00
30	*Pseudomonas putida* (DFR5)	*Pseudomonas putida* (MN709258)	99.00
31	*Bacillus tequilensis (*DST6)	*Bacillus tequilensis* (MZ066821)	99.00
32	*Bacillus methylotrophicus* (DST10)	*Bacillus methylotrophicus* (KP851958*)*	100
33	*Pseudomonas* sp. (DST11)	*Pseudomonas* sp. (LN885503)	100
34	***Bacillus licheniformis*** (D2LT1)	*Bacillus licheniformis* (MN396732)	99.00
35	*Bacillus cereus* (D2LT3)	*Bacillus cereus* (MW148500)	100
36	*Bacillus altitudinis* (D2LT4)	*Bacillus altitudinis* (KJ716447)	99.00
37	*Brevibacterium* sp. (D2LT5)	*Brevibacterium* sp. (MT433875)	99
38	*Alcaligenes faecalis* (D2ST1)	*Alcaligenes faecalis* (AB967983)	100
39	*Bacillus nealsonii* (D2ST2)	*Bacillus nealsonii* (KT965174)	99
40	*Bacillus* sp. *(*HFR3)	*Bacillus* sp. (MN826392)	99.00

Phylogenetic Tree Construction

Phylogenetic tree was constructed by MEGA software using Maximum Likelihood method and Kimura 2 parameter with lowest Bayesian Information Criterion (BIC) values and highest Akaike Information Criterion (AICs) value.

The topology of the tree generated differentiated the isolates into three major clades as shown in Figure 6.

All the genus of *Bacillus* formed a major clade I with a bootstrap support value of 95%. The rare genus *Ochrobactrum* and *Brevundimonas* form another clade with bootstrap value of 99% and *Alcaligenes, Enterobacter,* and *Pseudomonas* form another clade with 88% of bootstrap value.

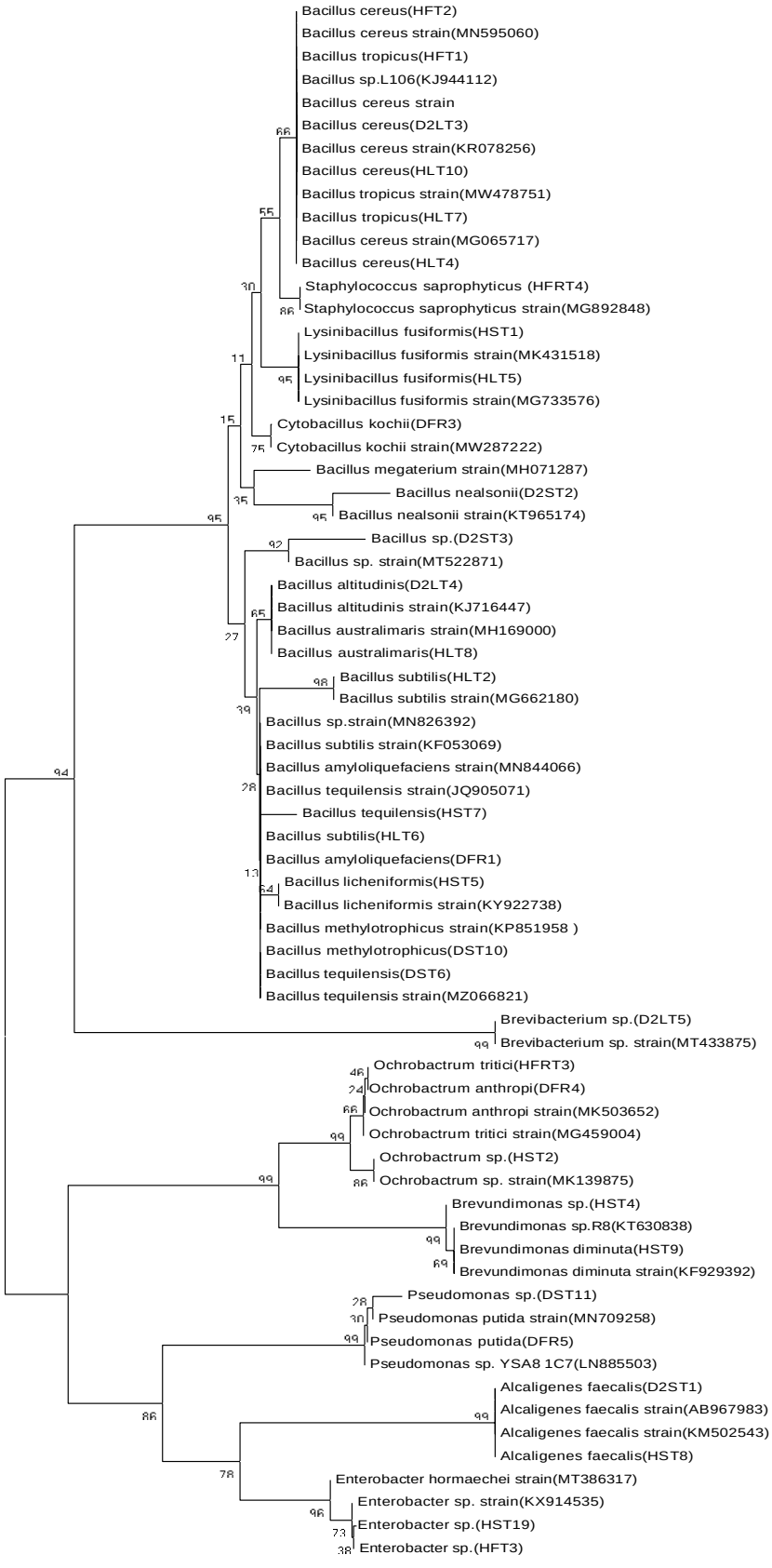


Figure 6 . Phylogenetic tree of isolates and relative strain

In-Vitro Antifungal screening

All the isolates were screened for their antagonistic activity against selected fungal pathogens and were evaluated by measuring inhibition percentage. Three endophytic bacteria HLT9, HST6 and D2LT1 were found to inhibit fungal mycelia growth of all pathogens over control. Growth inhibition was measured on 7th day and inhibition percentage was calculated. Highest growth inhibition was observed from HLT9 against *Fusarium oxysporum* by 41.6% followed by HST6 against *Fusarium oxysporum* f. sp. *lycopersici* by 39.1% and lowest inhibition from D2LT1 against *Fusarium udum* (MTCC-2755) by 26.8% (Table 4)**.**

HLT9 HST6 D2LT1

Figure 7. Pure culture of isolates having antagonistic activity identified as *Bacillus velezensis* (HLT9), *Bacillus siamensis* (HST6), and *Bacillus licheniformis* (D2LT1)

Antifungal activity of Lipopeptides

The culture extract (lipopeptides) of HST6,HLT9 and D2LT1 obtained from Chloroform : Methanol (2:1) were used for screening using disk diffusion method as stated earlier. In extract screening **Highest** growth inhibition was observed from extract of **HLT9** against *Fusarium oxysporum* f. sp. *lycopersici* (ITCC-3437) by **43.4%** and *Fusarium graminearum Schwabe* (MTCC-9064 by 40.9% which is higher than the dual culture assay while lowest inhibition was observed from extract of **HST6** against *Fusarium oxysporum* (MTCC- 1893) by **20.8%** inhibition (Figure 9)**.** Similar results have also been reported by Kefi et al., 2015 with growth inhibitory activity ranging from 27 to 53 % against *Botrytis cinerea*.

Table 4. Antagonistic activity of three strains by dual culture and their extract against plant pathogenic fungi.

Plant Pathogenic Fungi	**Fungal growth inhibition by bacteria (%)**			**Fungal growth inhibition by bacterial extract (%)**		
	HST6	**HLT9**	**D2LT1**	**HST6**	**HLT9**	**D2LT1**
Fusarium graminearum Schwabe (MTCC-9064)	31.8	27.0	36.3	36.4	40.9	36.0
Fusarium oxysporum f. sp. lycopersici (ITCC-3437)	39.1	34.0	34.0	39.0	**43.4**	39.0
Fusarium oxysporum (MTCC- 1893)	37.5	**41.6**	37.0	20.8	29.1	37.0
Fusarium oxysporum f.sp. pisi (MTCC-2480)	30.4	34.5	34.0	34.0	40.0	30.0
Fusarium udum (MTCC-2755)	34.6	34.0	26.8	39.0	34.0	30.0
Fusarium proliferatum (MTCC-286)	33.3	33.0	28.0	33.0	33.0	28.0

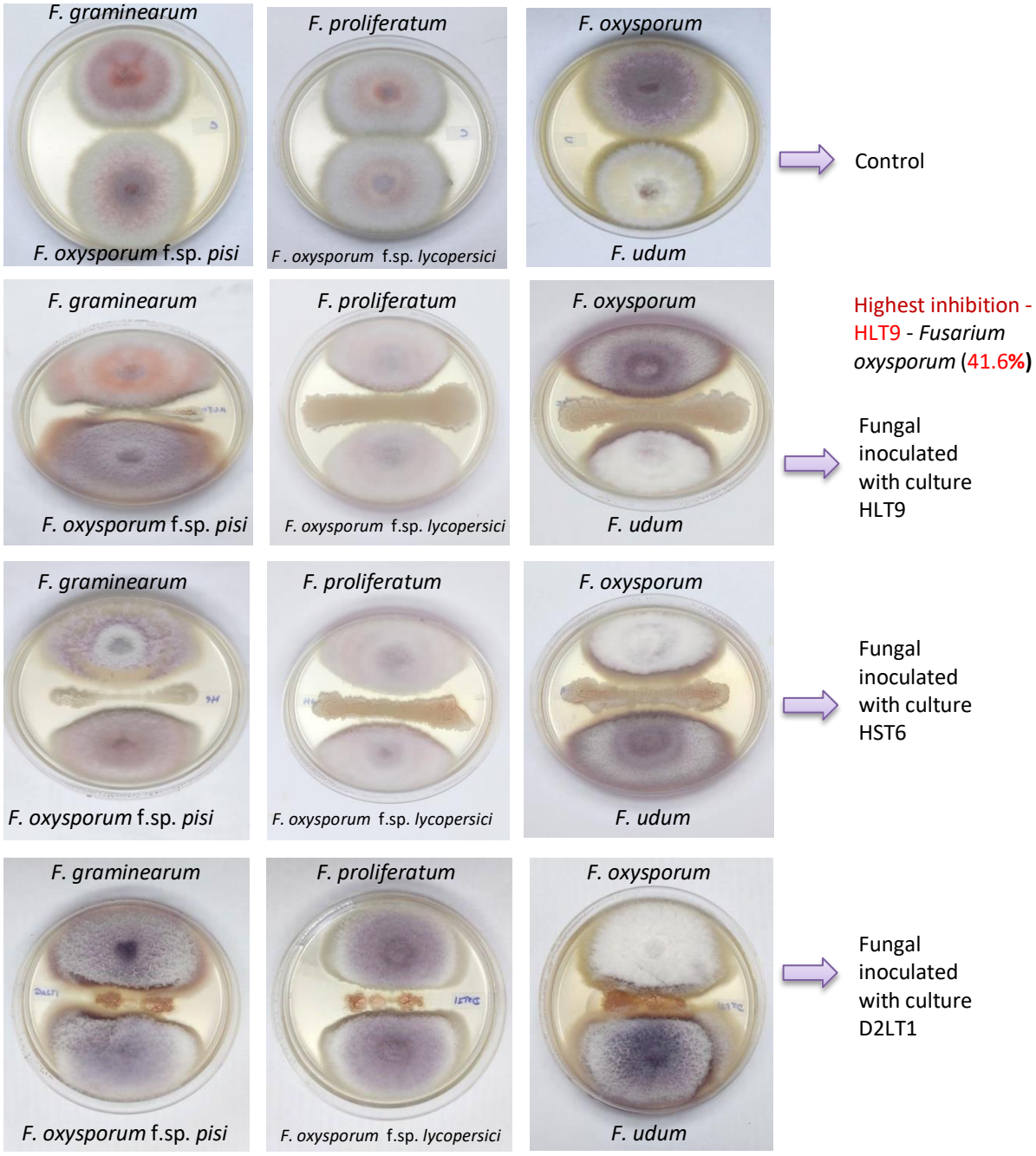


Figure 8. Antagonistic activity of bacterial strains HLT9, HST6 and D2LT1 against fungal pathogens

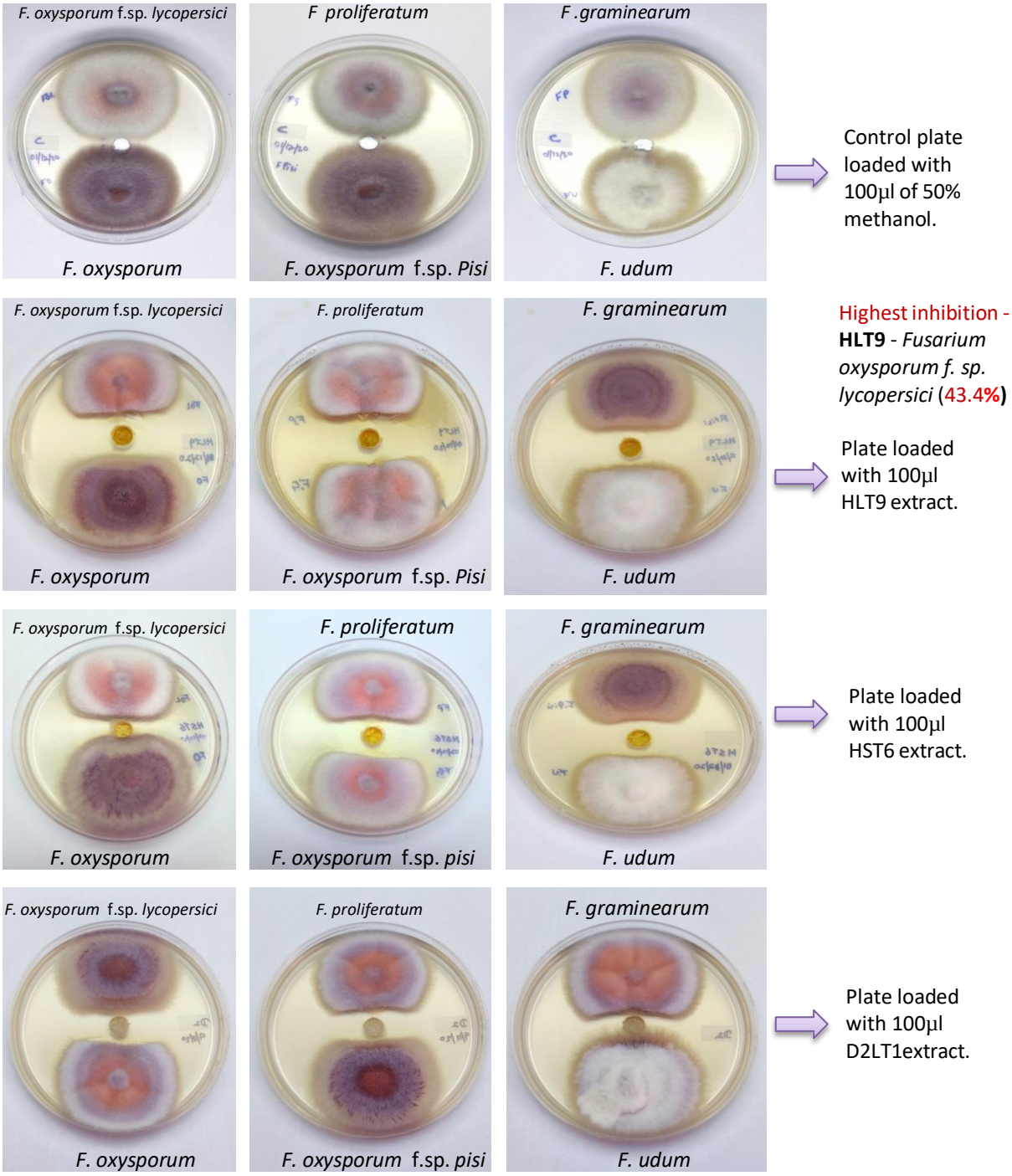
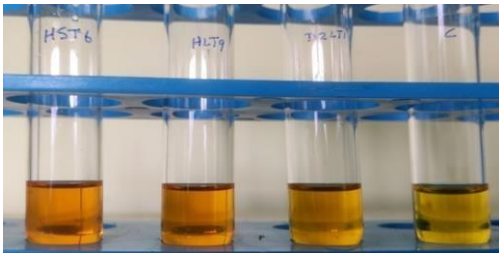


Figure 9. Growth inhibition of fungal mycelium by HLT9, HST6 and D2LT1 extracts

Screening For Plant Growth Promoting Activities

IAA Production

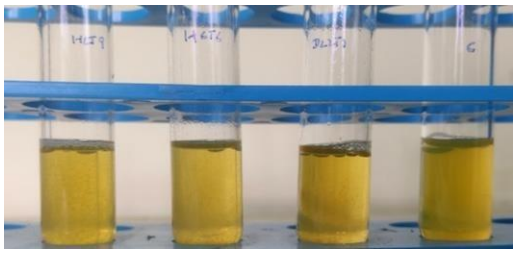
All the selected cultures were positive for IAA production showing development of pink colour upon adding Salkowski‟s reagent. Quantification of IAA by measuring the OD at 530 nm using a Thermo scientific (Multiskan GO) spectrophotometer shows that **HST6** produce highest amount of IAA at a concentration of **8 µg/ml** and the other two isolate HLT9 and D2LTI produce a concentration less than 5µg/ml when compared with standard curve.



(a)

Ammonia Production

Qualitative assay for production of ammonia shows all the potent cultures as producers of ammonia. The development of brown to yellow color was observed with addition of Nessler‟s reagent which indicates the production of ammonia.



(b)

Figure 10. (a) IAA production by HST6,HLT9, and D2LT1 with formation of pink colour.
(b) Ammonia production by HST6,HLT9, and D2LT1

Phosphate Solubilization

For phosphate solubilization test, all the selected three isolates cultures were found negative as no clear zone was observed after incubation period in PKV agar media.

Extracellular enzymes production

Screening for ACC deaminase and Chitinase activity.

The three potential cultures were streaked on sterile minimal DF (Dworkin and Foster) salts media amended with 3 mM ACC for ACC deaminase activity. All the potent cultures were found to be producing ACC deaminase as growth is observed after incubation of 3 days. On the other hand they were found negative for chitinase activity due to no formation of clear zone around the culture colony.

Screening of Cellulase and Xylanase

The cultures were grown on screening media and showed a zone around the colony when stained with Congo red and are considered to be producing cellulase. The highest zone was observed from HLT9 (0.5cm), HST6 (0.5cm) and D2LT1 (0.4cm).

For xylanase activity screening, all the cultures also showed a zone around the colony and considered to be producing xylanase with a highest zone from HLT9 (1.5cm), HST6 (1.5cm) and D2LT1 (1.4cm).

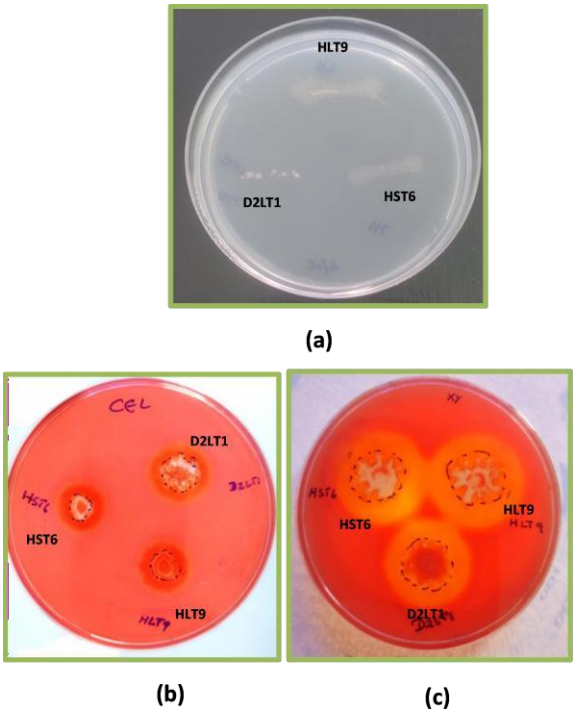


Figure 11. Visualization of (a) ACC deaminase activity (b) Cellulase and (c) xylanase activity with iodine solution

Detached Leaflet Assay

When overnight cultures were sprayed on detached leaflets and incubate, all three endophyte strains were able to inhibit the lesions induced by fungal infection. HLT9 was the most protective isolate as there is no symptoms of infection were observed from fungal pathogen when compared to positive control which induces disease severity. The other two strain HST6 and D2LT1 also showed a great protective with much less infection from the pathogen over positive control (Figure 12).

Leaflets infected with FOL (Positive control)

Untreated leaflets (Negative Control)

Leaflets treated with culture HLT9

Leaflets treated with culture HST6

Leaflets treated with culture D2LT1

Figure 12. Fungal growth inhibition on plant leaves treated with endophytic bacteria

In-vivo plant growth activity

The most potent isolates HLT9, HST6 and D2LT1 identified by 16S rRNA gene sequencing as strains of *Bacillus velezensis, Bacillus siamensis* and *Bacillus licheniformis* were used for in vivo pot experiments on tomato seedlings.

Combined inoculation of seedling with *Bacillus velezensis, Bacillus siamensis, Bacillus licheniformis* and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) showed a greater seed germination and significant increase in root and shoot height in comparison to fungal only treated and control. Highest seed germination was observed HLT9-FOL and D2LTI-FOL inoculated seeds with 77% seed germination. Lowest seed germination is observed in FOL only treated seedlings with 46 % seed germination.

Root length and shoot length were also measured after 20 d of seed inoculation. Longest root length was observed from HLT9-FOL treated with an average root length of 6.64 cm. Highest shoot length were also observed from HLT9-FOL treated with an average shoot length of 9.18 cm. All the parameters used for in-vivo plant growth activity; seed germination, root length and shoot length shows a higher activity than the control. This indicates that endophytic bacteria ***Bacillus velezensis*** (HLT9) has the most plant growth promoting activity and as well as response against pathogenic fungi (Table 5).

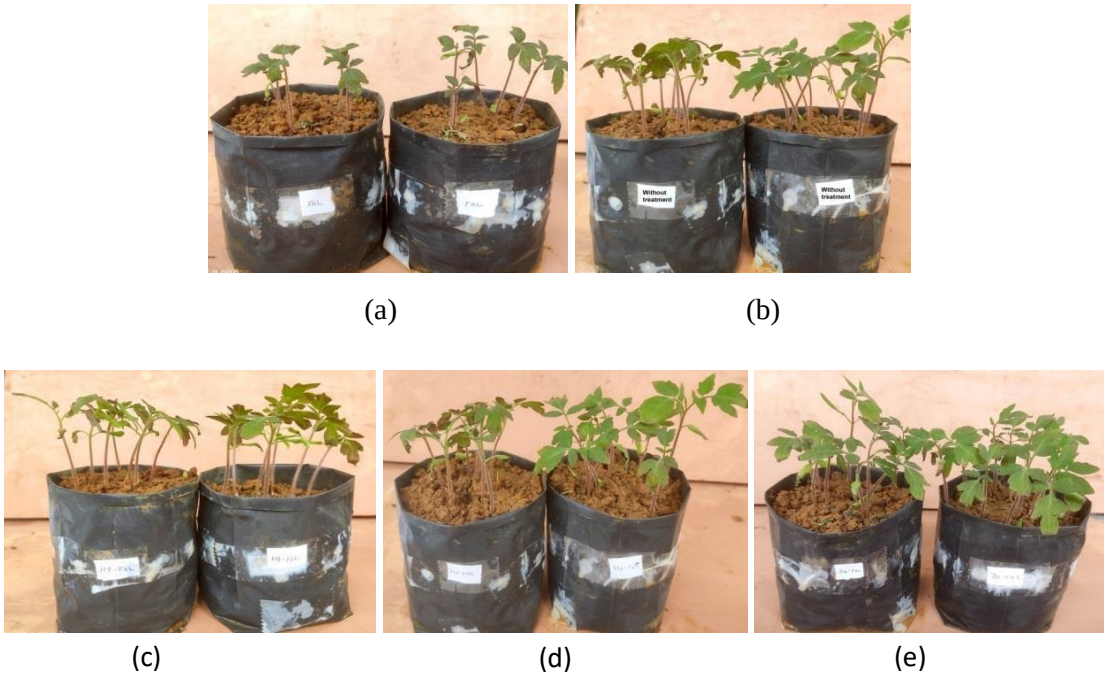


Figure 13. Higher number of seed germination observed in endophytes treated seeds.

(a) Seeds treated with FOL **(b)** Seeds without treatment **(c)** Seeds treated with HLT9-FOL

(d) Seeds treated with HST6- FOL **(e)** Seeds treated with D2LT1-FOL

(a) Seeds treated with Fungal pathogen (FOL)

(b) Seeds without treatment

c) Seeds treated with HLT9 and FOL

(d) Seeds treated with HST6 and FOL

(e) Seeds treated with D2LT1 and FOL

Figure 14. Effect of *Bacillus* spp. HLT9, HST6 and D2LTI on seed germination, plant root and plant shoot length on combined inoculation with *Fusarium oxysporum f. sp. lycopersici* (FOL)

Table 5. Effect of *Bacillus* spp. HLT9, HST6 and D2LTI on seed germination, plant root and plant shoot length on combined inoculation with *Fusarium oxysporum f. sp. lycopersici.*

Seed Treatment	**Seed Germination (%)**	**Average Root length (cm)**	**Average Shoot length (cm)**
No treated	72	5.74± 0.88	7.10 ± 0.51
FOL treated	46	5.18 ± 0.85	7.08 ± 0.46
HLT9 - FOL treated	**77**	**6.64** ± 0.47	**9.18** ± 0.69
HST6 - FOL treated	73	5.96 ± 0.36	8.90 ± 0.98
D2LT1 - FOL treated	**77**	5.82 ± 0.79	9.02±0.92

Discussion

Endophytic bacteria have various properties that can be exploited for agricultural applications. In recent years, endophytes bacteria are gaining more research attention as biocontrol agents that help in controlling various plant diseases including the phytopathogenic fungi (Zheng et al., 2016). In addition, endophytic bacteria also help their host plants by various ways like growth promotion by increasing nutrient acquisition, protection against various pathogenic attacks, biotic and abiotic stress tolerance etc. (Khare *et al*., 2018). Various studies have showed the usefulness of various bacterial species as the biocontrol agents than rhizospheric bacteria because of endophytic bacteria can defend themselves in better way from environmental stressors (Rosenblueth and Martínez-Romero, 2006). However, among the different bacterial species, the genus Bacillus was reported to be highly effective against several phytopathogens including various fungal pathogens (Meena and Kanwar, 2015). Masum et al., 2018 reported that the endophytic *Bacillus velezensis* species with higher growth inhibition potential against several phytopathogens. Therefore, it is interesting to study endophytic populations and evaluating their metabolic potential from various crop plants. In this work, a number of endophytic bacteria were isolated from healthy as well diseased tomato plants (*Solanum lycopersicum* L.) tissues. In total seventy endophytic bacteria were isolated from different tissue *of Solanum lycopersicum* L; shoots, leaves flowers and fruits samples. The dominant genus Bacillus constitute 62.5% followed by Ochrobactrum, Brevundimonas, and Lysinibacillus each 7.5%, rare genus including Alcaligenes (5%), Enterobacter (5%) and others. Various previous studies also reported Bacillus species with variable strain (Keita et al., 2013; Rocha et al., 2017) from various crop plants. The biocontrol potential of the endophytic strains was evaluated using both *in-vitro* and *in-vivo* experiments against six different phytopathogenic fungi of *Fusarium* spp. We also attempted for characterizing the antifungal metabolites produced by the potent strains. In total, three endophytic bacteria HLT9, HST6 and D2LT1 were found to inhibit the fungal mycelial growth of all pathogens over control. Highest growth inhibition was observed from HLT9 against *Fusarium oxysporum* by 41.6% followed by HST6 against *Fusarium oxysporum* f. sp. *lycopersici* by 39.1% and lowest inhibition

from D2LT1 against *Fusarium udum* (MTCC-2755) by 26.8%. The pathogenic fungal growth inhibition directly by the plant leaves was also tested using the detached leaflets assay. Interestingly, all three endophytic strains were found to be effective in inhibiting the lesions induced by fungal infection on the leaves. HLT9 was the most protective isolate as there is no symptom of infection observed from fungal pathogen when compared to positive control which induces disease severely. The other two strains HST6 and D2LT1 also showed a great protective with much less infection from the pathogen over positive control. These potent strains were identified as *Bacillus* sp. based on the morphological and molecular data. Phylogenetic analysis of the 16S rRNA gene indicated the close relationship of isolate HLT9 with *Bacillus velezensis* (HLT9), HST6with *Bacillus siamensis* (HST6), and D2LT1 with *Bacillus licheniformis* as the reference strains. Previously, several strains of Bacillus species were often reported as the endophytes of the internal tissues of plants (Han et al. 2015). In addition, the Bacillus species are also known to improve plant growth and development. Various Bacillus species like *B. subtilis* strains have been previously used for control of plant diseases including take-all in wheat, dumping-off of tomato, chestnut blight, Fusarium root infection and many others (Hazarika et al 2019). In our study, the potent strains also exhibited other PGPR properties such as found positive for IAA production. The strain HST6 showed the highest amount of IAA production (8 µg/ml) and the other two isolates HLT9 and D2LTI (less than 5µg/ml) when. In analyzing their potential, when used combined as biocontrol against fungal pathogens and also PGPR attributes, a seedling growth assay in pot experiments showed very promising results. Inoculation of tomato seedling with potent strains such as *Bacillus velezensis, Bacillus siamensis*, and *Bacillus licheniformis* showed a greater seed germination and significant increase in root and shoot height in comparison to control. Highest seed germination was observed in HLT9-FOL and D2LTI-FOL inoculated seeds with 77% seed germination. Overall, this study has revealed the endophytic population of bacteria associated with the tomato plants and a few of the endophytic bacteria belonging to Bacillus species were found as potent biocontrol agents with various other plant growth promotion properties.

Conclusion

Phytopathogenic fungi cause major diseases responsible for massive loss in different crop production. Endophytic bacteria are known to be active against such pathogenic fungi. In this study, we explore the endophytic bacterial population associated with the tomato plants (*Solanum lycopersicum* L.) using culturable approach. The isolated strains belong to different bacterial species including both Gram positive and Gram-negative bacteria. In tomato plants, *Bacillus*, *Ochrobactrum*, *Brevundimonas*, and *Lysinibacillus*. were found as the major bacterial genera with the highest dominance of *Bacillus* sp. These strains were evaluated for their antifungal potential and other plant growth promotion activities. The results established the antifungal potential of the endophytic isolates of three *Bacillus* spp. (HLT9, HST6 and D2LT9) along with promising plant growth promotion properties. The study has opened up chances for applicability of these strain as promising biocontrol agent in near future with further optimization and formulation studies.

Abbreviations

%	Percentage
±	Plus or Minus
°C	Degree Celsius
16S rRNA	16S-Ribosomal Ribonucleic Acid
AIC	Akaike Information Criterion
BIC	Bayesian Information Criterion
BOD	Biological Oxygen Demand
BlAST	Basic Local Alignment Search Tool
bp	Base Pair
d	Days
dNTPs	Deoxynucleotides
e.g.	Exempli gratia: For example
et al.	*et alii*: and others
etc.	et cetera: and other things
h	Hour
kb	Kilobyte
M	Molar
mM	Milli Molar
mm	Millimeter
MTCC	Microbial Type Culture Collection
NCBI	National Center of Biotechnology Information
n	Number
NA	Nutrient Agar
PCR	Polymerase Chain Reaction
pH	Negative Ion Of Hydrogen Ion Concentration
sp.	Species
TAE	Tris Base, Acetic Acid and EDTA
µM	Micromolar

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DEGREE : MASTER OF PHILOSOPHY

DEPARTMENT : BIOTECHNOLOGY

TITLE OF DISSERTATION : Evaluation of Antifungal Potential of *Solanum lycopersicum* Endophytic Bacteria for Biocontrol of Plant Pathogenic Fungi

DATE OF PAYMENTS OF ADMISSION : 21.08.2019

COMMENCENENT OF SECOND SEM/DISSERTATION :
(From conclusion of end semester exam)

APPROVAL OF RESEARCH PROPOSAL

BOS : 29.05.2020

SCHOOL BOARD : 12.06.2020

REGISTRATION NO. & DATE : Reg No. MZU/M.Phil./630 of 12.06.2020

DUE DATE OF SUBMISSION :

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Sl.No	QUALIFICATION	SUBJECT	BOARD/ UNIVERSITY	PERCENTAGE
1	Master (M.Sc)	Biotechnology	Mizoram University	71%
2	Bachelor (B.Sc)	Biotechnology	University of Science and Technology Meghalaya	66%
3	HSSLC	Biology, Chemistry, Physics, English, Alt.English	MBSE	55%
4	HSLC	Science,Maths, Social Science, English, Mizo	MBSE	67%

Present Position

Doing Master of Philosophy (M.Phil) entitled "**Evaluation of Antifungal Potential of *Solanum lycopersicum* Endophytic Bacteria For Biocontrol of Plant Pathogenic Fungi**" under the supervision of Dr.Esther Lalnunmawii in Department of Biotechnology, Mizoram University.

Publications : 03

- Zothanpuia, **W. Carrie**, V.V. Leo, A.K. Passari R. Lalmuanpuii and B.P. Singh (2019). *In-vitro* Evaluation of Actinobacteria for its Potential in Bio-control of Fungal Plant Pathogens. Science and Technology Journal,7 (1).
- Leo, V. V., Viswanath, V., Deka, P., Ramji, D. R., Pachuau, L., **Carrie, W**., & Singh, B. P. (2021). Saccharomyces and Their Potential Applications in Food and Food Processing Industries. In *Industrially Important Fungi for Sustainable Development* (pp. 393-427). Springer, Cham.
- Gajanan T. Mehetre, Zothanpuia, Purbajyoti Deka, **William Carrie**, Lalrokimi, Bhim Pratap Singh (2021). Thermophilic and Thermo-tolerant Cyanobacteria: Environmental and Biotechnological Perspectives.

Presentations

- Presented a paper entitled "**Solanum lycopersicum derived endophytic bacteria as bio-control arainst fungal plant pathogens**" in three days International webinar organized by Dept. of Biotechnology, Pachhunga University Colleg, and Mizoram University from 24th – 26th June 2020.
- Delivered and Invited talk entitled "**In-Vitro technique for cultivation of mushroom and preservation**" in "*Three days skill development programme on Mushroom cultivation for NER*" organized jointly by the Rajiv Gandhi National Institute of Youth Development under its Youth Led Sustainable Development Programme (YLSDP) in Higher Institution at Pachhunga University.

Workshop and Trainings Attended

- Participated in the training on " **Analysis of Bioactive Compounds using HPLC**" held on 13th -15th October 2020.
- Attended Orientation programme on **National Intellectual Property Rights (IPR) Policy** held at Gujarat Council on Science and Technology (GUJCOST), Gandinagar during 3rd to 5th October,2019.
- Participated a programme organized by Dept. of Biotechnology, Mizoram University and Dept. of Horticulture, Aromatic & Medicinal Plant (HAMP), Mizoram University. Co-organized and funded by Indian Council of Agricultural Research – National Bureau of Agriculturally Important Microorganisms (ICAR-NBAIM), Mau, Uttar Pradesh.

Declaration

I hereby declared that all the information mentioned above is true to my knowledge and belief. I will be solely responsible if any of the information is found incorrect.

Place : Aizawl,Mizoram **WILLIAM CARRIE**

Date :

ABSTRACT

EVALUATION OF ANTIFUNGAL POTENTIAL OF SOLANUM LYCOPERSICUM ENDOPHYTIC BACTERIA FOR BIOCONTROL OF PLANT PATHOGENIC FUNGI

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

WILLIAM CARRIE

MZU REGN NO.1703974

M.PHIL REGN NO: MZU/M.PHIL./630 OF 12.06.2020

DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF LIFE SCIENCE

SEPTEMBER 2021

EVALUATION OF ANTIFUNGAL POTENTIAL OF SOLANUM LYCOPERSICUM ENDOPHYTIC BACTERIA FOR BIOCONTROL OF PLANT PATHOGENIC FUNGI

By

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Submitted

In partial fulfillment of the requirements for the degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl.

BACKGROUND AND AIM

Plants are exposed to different biotic and abiotic factors which have diverse effects in their growth and development. Biotic stresses are directed to living components such as pathogenic organisms that lead to low yield and crop losses (Cocq et al., 2017). The Fusarium wilt is considered as one of the most prevalent disease to many important crops and it is estimated that agricultural loss due to fungal infections constitute up to 20% of crops worldwide (Gullino *et al.* 2000). The deliberate urge to control fungal diseases has resulted in invention of several fungicides, the application of which effects environment and endangering the health of humankind (Latha et al., 2019). Therefore, in order to reduce the use of chemicals, biocontrol of plant diseases have become an important interest of researchers. Antifungal activity of endophytes against plant pathogenic fungi such as *Fusarium* sp., *Rhizoctonia* sp., Verticillium sp., Verticillium sp., and *Phythium* sp. have been reported from many plants (Cho et al., 2007). Tomato plants are also harboring endophytic bacteria that show a great promise in the control of a wide range of phytopathogenic fungi (Kefi et al., 2015). The proven benefits of endophytes as agent for controlling plant diseases is due to their well adaptation to live inside the plants which provides reliable suppression of many diseases including vascular diseases (Lin et al. 2013). Therefore, this study aims in exploration of endophytic bacteria from *Solanum lycopersicum* L and evaluation of their antifungal activity against selected phytopathogenic fungi including their plant growth promoting activity.

MATERIALS AND METHODS

Tomato plants were collected from Sihphir tomato farm cultivated by local farmer near Aizawl, Mizoram. Endophytic bacteria were isolated from different part of the plant using the method of Sturz *et al.* (1998). Forty isolates were selected for genomic DNA isolation based on colony morphology and source of plant tissue. DNA isolation was done as per the method of Green and Sambrook (2018). All DNA isolated were processed for PCR amplification of 16S rRNA gene partial sequence using universal primers and Amplified 16S rRNA gene products were sequenced for molecular identification. DNA sequencing of partial 16S rRNA gene was done in Biotech-Hub, Biotechnology Department, Mizoram University.

In-vitro screening of antifungal activity was done for all isolates using dual culture assay (Korsten et al., 1999) using six phytopathogenic fungi of *Fusarium* spp. Isolates having antagonistic activity in antifungal screening was further processed for lipopeptide production and extraction using the method of Kim *et al.* (2004). To evaluate the antifungal activity of lipopeptide well-diffusion method was used (Kalai-Grami et al., 2013) and growth inhibition was observed and measured.

All the isolates having antifungal activity were further screened fort their plant growth promoting activity and production of extracellular enzymes. Production of Indole acetic acid (IAA) was determined using Gordon and Weber (1951) method for the strains showing antifungal activity. Qualitative assay for ammonia production was done as per the method of Cappucino and Sherman 1992. For qualitative estimation of phosphate solubilization single colony of bacterial was streaked onto Pikovskaia's medium containing tricalcium phosphate (Pikovskaia, 1948) and incubated for 7 days. Screening for extracellular enzyme production such as chitinases, ACC deaminase, cellulase and xylanase were done for all the potent isolates.

Detached leaflet assay was performed for the potent isolates by spraying 2 days bacterial suspensions to tomato leaflets and incubated in dark 7 days (Rajkumar et al.,2005).Growth inhibition was observed and compared with control. *In-vivo* plant growth activity was also done by inoculating tomato seeds with suspension of potent isolates seed germination, root length and shoot length were measured and compared with control.

RESULTS

A total of 70 endophytic bacteria were isolated from different tissue of *Solanum lycopersicum* L; shoots, leaves, flowers and fruits. Most endophytic bacteria were isolated from Healthy Shoot Tissue - 15 endophytes followed by Healthy Leaf Tissue - 12 endophytes. Number of isolates from different organs. Eight isolates based on colony morphology and colors were selected as representatives of endophytes for gram staining using gram staining kit. Gram staining shows most of the selected strains as gram positive constituting 62.5% and gram negative 37.5% as observed under microscope respectively.

All selected isolates were molecularly confirmed and the isolates belong to nine different genera of endophytic bacteria from all selected sample in which *Bacillus* sp. is observed to be the dominant genus (Figure 5). The dominant genus *Bacillus* constitute 62.5% followed by *Ochrobactrum*, *Brevundimonas*, and *Lysinibacillus* each 7.5% and others rare genus including *Alcaligenes* (5%), *Enterobacter* (5%), *Pseudomonas* (5%), *Staphylococcus*(2.5%) and *Cytobacillus* (2.5%). In phylogenetic tree construction, all the genus of *Bacillus* formed a major clade I with a bootstrap support value of 95%. The rare genus *Ochrobactrum* and *Brevundimonas* form another clade with bootstrap value of 99% and *Alcaligenes, Enterobacter,* and *Pseudomonas* form another clade with 88% of bootstrap value.

Antifungal screening shows three endophytic bacteria identified as ***Bacillus velezensis*** (HLT9), ***Bacillus siamensis*** (HST6) and ***Bacillus licheniformis*** (D2LT1) having antifungal activity by inhibiting fungal mycelia growth. Highest growth inhibition was observed from HLT9 against *Fusarium oxysporum* by 41.6% followed by HST6 against *Fusarium oxysporum* f. sp. *lycopersici* by 39.1% and lowest inhibition from D2LT1 against *Fusarium udum* (MTCC-2755) by 26.8%.

In extract screening, highest growth inhibition was observed from HLT9 extract against *Fusarium oxysporum* f. sp. *lycopersici* (ITCC-3437) by 43.4**%** and *Fusarium graminearum Schwabe* (MTCC-9064 by 40.9% which is higher than the dual culture assay while lowest inhibition was observed from HST6 extract against *Fusarium oxysporum* (MTCC- 1893) by 20.8% inhibition**.**

Quantification of IAA by measuring the OD at 530 nm using a Thermo scientific (Multiskan GO) spectrophotometer shows that HST6 produce highest amount of IAA at a concentration of 8 µg/ml and the other two isolate HLT9 and D2LTI produce a concentration less than 5µg/ml when compared with standard curve. Qualitative assay for production of ammonia also shows all the potent cultures as producers of ammonia while they are negative for phosphate solubilization test.

Production of extracellular enzyme assay shows that the potent cultures were able to produce ACC deaminse as growth is observed after incubation of 3 days while they are found negative for chitinase activity due to no formation of clear zone around the colony. All the cultures showed a zone on the media and are considered to be producing cellulase

with a highest zone by HLT9 (0.5cm), HST6 (0.5cm) and D2LT1 (0.4cm) . In xylanase activity screening, all the cultures showed a zone on the media and are considered to be producing xylanase with a highest zone from HLT9 (1.4cm), HST6 (1.5cm) and D2LT1 (1.4cm).

In detached leaflet assay, HLT9 was observed to be the most protective strain as there was no symptoms of infection from fungal pathogen when compared to positive control which induce disease severity. The other two strain HST6 and D2LT1 also shows protective with little infection from the pathogen. The *In-vivo* plant growth activity from potent isolates shows highest seed germination in HLT9-FOL and D2LTI-FOL inoculated seeds with 77% seed germination, longest root length from HLT9-FOL treated with an average root length of 6.64 cm and highest shoot length were also observed from HLT9 - FOL treated with an average shoot length of 9.18 cm.

DISCUSSION

Endophytic bacteria have various properties that can be exploited for agricultural applications. In recent years, endophytes bacteria are gaining more research attention as biocontrol agents that help in controlling various plant diseases including the phytopathogenic fungi (Zheng et al., 2016). Among the different bacterial species, the genus Bacillus was reported to be highly effective against several phytopathogens including various fungal pathogens (Meena and Kanwar, 2015). In this work, a number of endophytic bacteria were isolated from healthy as well diseased tomato plants *Solanum lycopersicum* L. tissues. In total seventy endophytic bacteria were isolated from different tissue *of Solanum lycopersicum* L; shoots, leaves flowers and fruits samples. The dominant genus was observed as Bacillus which constitute 62.5% followed by Ochrobactrum, Brevundimonas, and Lysinibacillus each 7.5%, rare genus including Alcaligenes (5%), Enterobacter (5%) and others. Various previous studies also reported Bacillus species with variable strain (Keita et al., 2013; Rocha et al., 2017) from various crop plants The biocontrol potential of the endophytic strains was evaluated using both *in-vitro* and *in-vivo* experiments against six different phytopathogenic fungi of *Fusarium* spp. The pathogenic fungal growth inhibition directly by the plant leaves was also tested using the detached leaflets assay. Interestingly, all three endophytic strains were found to

be effective in inhibiting the lesions induced by fungal infection on the leaves. In our study, the potent strains also exhibited other PGPR properties such as found positive for IAA production, ammonia and other extracellular enzymes. Both the *in-vitro* and *in-vivo* studies shows the three isolates as potential isolates for bio-control of plant pathogenic fungi particularly to HLT9 that shows a promising outcomes such as highest in seed germination, root length and shoot length. Overall, this study has revealed the endophytic population of bacteria associated with the tomato plants and a few of the endophytic bacteria belonging to Bacillus species were found as potent biocontrol agents with various other plant growth promotion properties.

CONCLUSION

Phytopathogenic fungi cause major diseases responsible for massive loss in different crop production. Endophytic bacteria are known to be active against such pathogenic fungi. In this study, we explore the endophytic bacterial population associated with the tomato plants (*Solanum lycopersicum* L.) using culturable approach. *Bacillus* spp. is the most dominant genus isolated with other genus of *Ochrobactrum*, *Brevundimonas*, and *Lysinibacillus.* These strains were evaluated for their antifungal potential and other plant growth promotion activities. The results established the antifungal potential of the endophytic isolates of three *Bacillus* spp. (HLT9, HST6 and D2LT9) along with promising plant growth promotion properties. The study has opened up chances for applicability of these strain as promising biocontrol agent in near future with further optimization and formulation studies.

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