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BIOLOGICAL CONTROL OF WILT CAUSING *PSEUDOMONAS* SPP. BY THE USE OF LEAF EXTRACTS OF *SCHIMA WALLICHII* (DC.) KORTH. AND *MELASTOMA MALABATHRICUM* L.

Sunrit Basu Sarbadhikary[1] and Narayan C. Mandal[2]*

[1]UGC-JRF, Mycology and Plant Pathology Laboratory, Department of Botany, Visva-Bharati, Santiniketan 731235, West Bengal, India.

[2]Professor, Mycology and Plant Pathology Laboratory, Department of Botany, Visva-Bharati, Santiniketan 731235, West Bengal, India.

***Corresponding Author: Prof. Narayan C. Mandal**

Professor, Mycology and Plant Pathology Laboratory, Department of Botany, Visva-Bharati, Santiniketan 731235, West Bengal, India.

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ABSTRACT

Vascular wilt is a common disease of solanaceous crops caused by many fungal and bacterial species. Two wilt symptoms producing bacterial strains VBLE3 and VBST13 were isolated from infected tomato plants. Both the strains were identified as *Pseudomonas* spp. by 16s rRNA gene sequence homology. The pathogenecity of the bacterial strains were also proved by reproduction of disease in pot experiments. Leaf extracts of two indigenous angiosperm species of Tripura, viz., *Schima wallichii* and *Melastoma malabathricum* showed very good antagonistic effects against both of the wilt pathogens. Prominent zones of inhibition against the pathogens were produced by both leaf extracts when tested by disc diffusion method in the range of 15 to 20 mm. Massive reduction in the numbers of colony forming units (CFU) of VBLE3 and VBST13 were also noticed when treated with these plant extracts. The Minimum Inhibitory Concentration (MIC) value of the crude extracts of *Schima wallichii* and *Melastoma malabathricum* was found to be 100 µg/mL and 150 µg/mL respectively for both the strains. *Schima wallichii* showed bactericidal and *Melastoma malabathricum* showed bacteriostatic mode of action against both the bacterial strains.

KEYWORDS: Vascular wilt, pot experiment, leaf extracts, MIC, bactericidal, bacteriostatic.

INTRODUCTION

Wilt has become a challenging topic in agriculture especially for the farmers in which stunting, wilting, and withering occur in the infected crops. Several soil inhabiting pathogens are responsible for developing vascular wilt in which the vascular system of the host gets affected. As a result the vascular system of the roots and lower portion of the stems turn brown. Several bacteria like *Ralstonia*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, *Xanthomonas* are reported to develop wilt in many cases. Bacterial wilt is very common in solanaceous crops.[1,2,3,4,5,6] Through recent researches, biological control has been established as a modern trend for controlling the plant diseases as it is very much useful as well as safe for the mankind. Biocontrol agents not only suppress the disease but also eliminate the environmental pollution by excluding the use of chemical pesticides.[7] It has been proved that chemicals are very much hazardous for both the crops and the consumers as they cause environmental pollution, which may lead to the death of other organisms by bio magnification and other processes. To avoid these adverse effects, controlling of plant diseases through biological control is being popular nowadays. Biological control of plant diseases through the use of antimicrobial compounds of higher plants has become a common practice in this regard. The vegetation of India is very much diverse and varieties of secondary metabolites produced by the plants were isolated and treated against the phytopathogens in many previous research works.[8,9,10] As the eastern part of India is among the elite producers of solanaceous crops, the ability of the selected species of angiosperm to control the wilt pathogens of these crop plants were assessed.

MATERIALS AND METHODS

Isolation and screening of the pathogens

Pathogens were isolated from the stems and root-stem transition zones of the infected tomato crops collected from three villages of Birbhum, West Bengal, India. After surface sterilization with $HgCl_2$ (0.1%) and washing in water, the infected portions were longitudinally split and placed in sterile water. The bacteria were isolated by dilution plating method on nutrient agar (NA) plates supplied with griseofulvin (200µg/mL). The isolates were primarily screened based

on their colony nature in 2,3,5-Triphenyl Tetrazolium Chloride (TTC) supplied NA plates.[11] Next secondary screening of the isolates was performed by checking their EPS production[12] and pathogenecity in pot experiment.

Pathogenecity test of the pathogens

Surface sterilized seeds of tomato were sowed in the respective autoclaved clay pots. Fully grown cultures of each isolates (1.3×10^8 CFU/mL) were sprayed in the soil during the seedling stage of the plants. After two months the results were noted on the basis of the wilt occurrence in each pot. Disease severity of each pathogen was noted by adopting a 0 to 4 rating scale[13] in which, 0=healthy plants, 1= 1 to 33%, 2 = 34 to 66%, 3 = 67 to 97% and 4 = > 97% of plant parts are affected.

Disease severity (%) = {∑ (No. infected plants × their infected degree) / (total examined tested plants × upper infected degree)} × 100.

Characterization and molecular identification

The morphology and Gram nature of the pathogens were observed under light microscope. Carbohydrate utilizations of the isolates were noticed using HiMedia HiCarbo Kit (KB009) and other biochemical tests were performed following standard protocols. Antibiotic sensitivity assay for both the strains were done using Hi-media octadiscs. 16S rRNA gene sequence homologies were carried out for the molecular identification of VBLE3 and VBST13. Amplification of the 16S region was done using forward (5'-TGGAGAGTTTGATCATGGCTC and reverse (5'-ACGGCTACCTTGTTACGACTT-3') primers. ABI 3730xl Genetic Analyzer was used for carrying out forward and reverse DNA sequencing reactions. Using aligner software the consensus sequences of 16S rRNA genes were generated from forward and reverse sequence data and was also used to carry out BLAST with the nrdatabase of NCBI GenBank database. The phylogenetic trees were constructed by neighbor-joining method[14] in MEGA5.[15] The evolutionary distances were computed using Kimura 2 parameter model.[16] *Xanthomonas campestris* was taken as an out group member.

Koch postulate of the pathogens

The two pathogenic strains VBLE3 and VBST13 were inoculated to tomato plants during the seedling stage and appearance of wilt symptoms in the tomato plants were noticed after one month period. Bacteria were re-isolated from root stem transition zones of the infected plants after second month of the assay. Bacteria from VBLE3 treated plants were isolated on Lincomycin and Ampicillin ammended NA plate and from VBST13 treated plants on Sulfamethoxazole and Ampicillin supplied NA plate.

Collection of plant materials and their solvent extractions

In our previous study[17] it was found that among several leaf samples collected from Udaipur, Tripura, the ethanolic leaf extracts of *Schima wallichii* (Theaceae) and *Melastoma malabathricum* (Melastomataceae) showed good antimicrobial activities. Based on those results, in the present endeavour their antibacterial activities against the isolated wilt pathogens were studied.

Antibacterial activity of leaf extracts

The antibacterial activity of the leaf extracts (50 mg/mL) against VBLE3 and VBST13 were checked by the disc diffusion method.[18] Ciprofloxacin (10 µg/mL) and DMSO were used as positive and negative control respectively. Zones of inhibition produced by the leaf extracts were compared with the zone of inhibition produced by the positive and negative control. The test was repeated thrice for ensuring the reliability of the experiment.

Determination of the MIC values

Since leaf extracts of *S wallichii* and *M malabathricum* showed good antibacterial activity against VBLE3 and VBST13, MICs of these two extracts were determined against these two strains. The MICs were determined by counting the CFU. The ethanolic leaf extracts were dissolved in DMSO and were added to NB in different concentrations ranging from 25 to 500 µg/mL. After adding of fixed volume of bacterial culture, culture tubes were incubated at 28ºC for overnight. Then 100 µL of cultures from each tube were spread on NA plates and incubated at 28ºC for overnight. Next day CFU were counted for the determination of the MIC values.

Mode of action study

To determine the mode of action of the leaf extracts, time killed study was performed against VBLE3 and VBST13. Leaf extracts were added to the actively growing liquid cultures of VBLE3 and VBST13 at their minimum inhibitory concentration. Activities of the leaf extracts in relation to time were measured by colony counting method.[19]

RESULTS AND DISCUSSION

Isolation and screening of the Pathogens

A total of 94 bacterial strains were isolated from infected solanaceous crops of three different locations of Birbhum, West Bengal. Among these, 17 strains showed the characteristic colony nature on TTC medium. This implies that those 17 strains are capable of producing Extracellular Polysaccharide (EPS) which is indicated by the appearance of white border around the red or pinkish red colony in TTC medium. EPS production is an important virulent factor for the wilt pathogens[20] for wilt development. EPS produced by the wilt pathogens are thought to be responsible for the blockage of water transport in the xylem vessels. Among the 17 strains VBLE3 was found to produce highest EPS both in terms

of fresh and dry weight. Fresh and dry weights of the EPS extracted from the VBLE3 culture were found to be 45.5±1.55 g/L and 0.65±0.07 g/L respectively (Figure 1A and 1B). VBST13 was found to be the second highest EPS producer as it produced 30.85±1.34 g/L fresh weight and 0.45±0.07 g/L dry weight (Figure 1A and 1B).

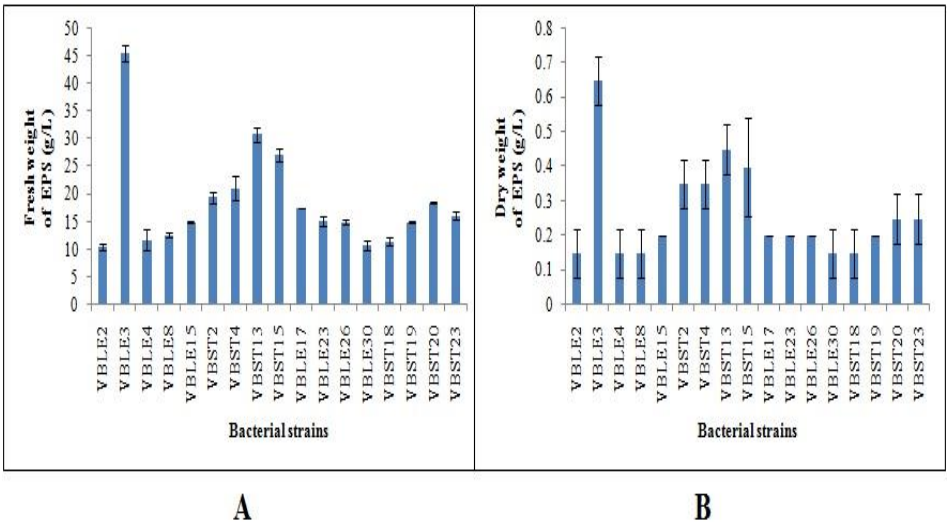


Figure 1: A. Fresh weight (g/L) and B. dry weight (g/L) of EPS extracted from the bacterial isolates

Pathogenecity test of the pathogens

In the pathogenicity test, VBLE3 and VBST13 produced highest wilt symptoms among the other isolates (Figure 2). Figure 3 demonstrates that the disease severity in the VBLE3 treated set was 91.66% and VBST13 treated set was 75%. Moderate wilt occurrences were found in VBST2 and VBST4 treated plants having disease severity of 66.66%. Three other isolates viz., VBST15 (58.33%), VBST20 (8.33%) and VBST23 (16.66%) also developed less amount of wilt symptoms in the used tomato plants. Other 10 isolates did not produce any kind of disease.

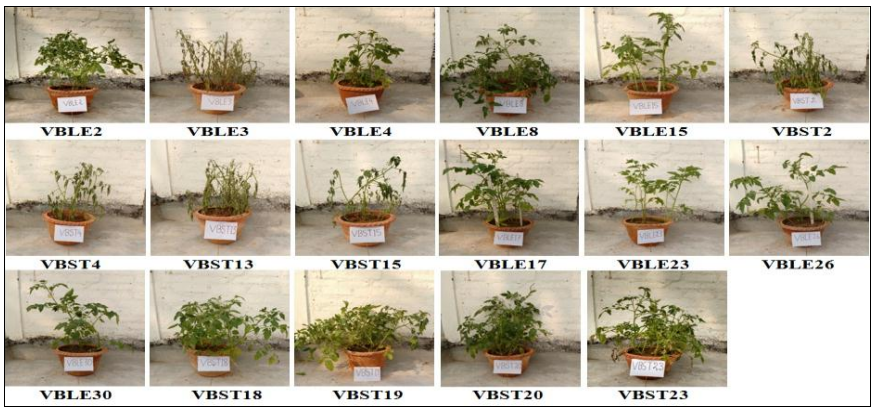


Figure 2: Pathogenecity test of the bacterial isolates in tomato in pot experiment

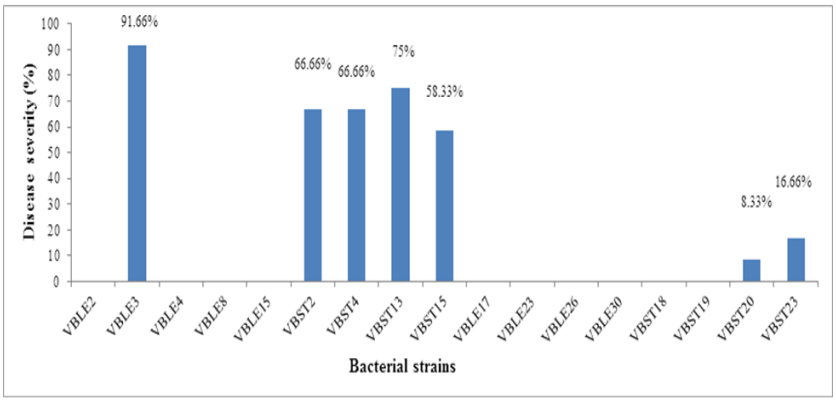


Figure 3: Disease severity of the bacterial isolates in tomato in pot experiment

Characterization and molecular identification

Both VBLE3 and VBST13 were found to be Gram negative, rod shaped bacteria. The strains were found to be Catalase, VP and Nitrate Reductase positive and negative in MR and Indole test. In the Sugar utilization test both strains utilized Lactose, Maltose and Cellobiose but did not utilize Sorbitol, Inositol and Dulcitol (Table 1). The above results showed a clear analogy between the isolated bacterial pathogens with *Ralstonia solanacearum* biovar II which is also a destructive wilt causing bacterial pathogen. *Ralstonia solanacearum* biovar II is characterized by their ability to oxidize the sugars only. It fails to oxidize the sugar alcohols in sugar fermentation assay.[21,22,23] In the antibiotic sensitivity test VBLE3 and VBST13 showed similar type of results almost for every antibiotic used. Some differences in their antibiotic resistant pattern were also noted as VBLE3 was resistant to Lincomycin but sensitive to Sulfamethoxazole but the results were opposite in case of VBST13 (Table 2). The 16S rDNA sequence of the strain was compared to sequences from type *Pseudomonas* strains. The topologies of the phylogenetic trees were evaluated by bootstrap analysis of the sequence data using CLUSTAL W software based on 1000 random re-samplings[24] (Figure 4). The strains VBLE3 and VBST13 showed maximum sequence similarity with *Pseudomonas koreensis* and *Pseudomonas lundensis* respectively.

Table 1: Biochemical and morphological characterization of VBLE3 and VBST13

Bacterial strain	Morphological Characters			Biochemical Tests				
	Colony nature	Gram staining	Cellular structure	MR Test	VP Tests	Nitrate Reductase Tests	Indole Tests	Catalase Tests
VBLE3	Small, cream colour, smooth	Gram Negative	Rod shaped	-	+	+	-	+
VBST13	Small, cream colour, smooth	Gram Negative	Rod shaped	-	+	+	-	+

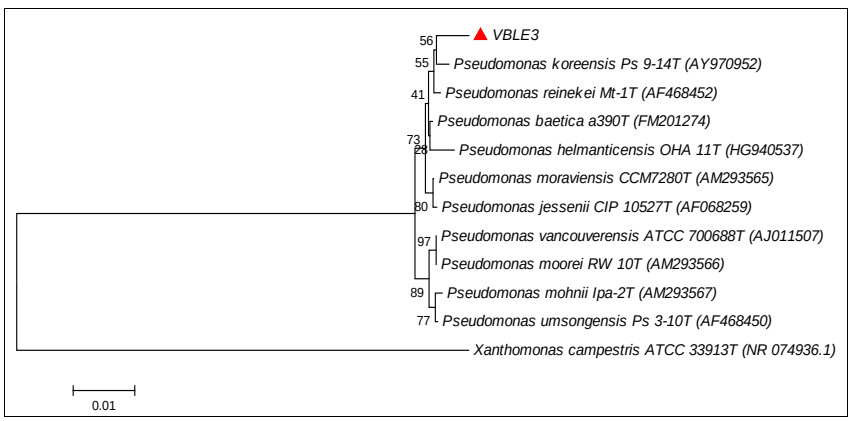
Sugar utilization						
Bacterial strains	Lactose	Maltose	Cellobiose	Inositol	Sorbitol	Dulcitol
VBLE3	+	+	+	-	-	-
VBST13	+	+	+	-	-	-

+: able to; -: unable to perform the functions.

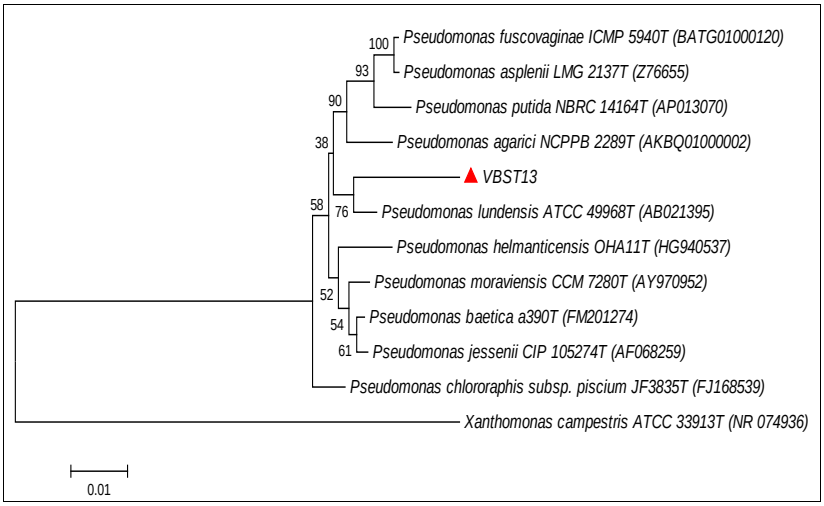
Table 2: Antibiotic sensitivity profiles of VBLE3 and VBST13

Antimicrobial agent	VBLE3	VBST13	Antimicrobial agent	VBLE3	VBST13
Tetracycline	+	+	Chloramphenicol	-	-
Amikacin	+	+	Norfloxacin	+	+
Carbenicillin	+	+	Tobramycin	+	+
Ciprofloxacin	+	+	Ticarcillin	-	-
Co –Trimazine	+	+	Gentamycin	+	+
Kanamycin	+	+	Oleandomycin	-	+
Nitrofurantoin	-	-	Trimethoprim	-	-
Streptomycin	+	+	Sulfamethoxazole	+	-
Ciprofloxacin	+	+	Ampicillin	-	-
Oxacillin	+	-	Penicillin G	-	-
Methicillin	-	-	Lincomycin	-	+

+: Sensitive; -: resistant.



A



B

Figure 4: Phylogenetic tree showing the relative positions of the strains A. VBLE3 and B. VBST13 as inferred by the neighbour-joining method

Koch Postulate of the pathogens

Presences of the screened strains in the infected tissues were confirmed by the colony morphology TTC supplemented NA plates, Gram nature, antibiotic sensitivity profile and 16S rDNA sequence homology of re-isolated strains.

Antibacterial activity of leaf extracts

Both the leaf extracts showed good antibacterial activity against the two pathogenic strains producing hollow zone around the paper discs. The crude extracts of *S wallichii* produce zone of inhibition ranging from 14 to 17 mm where as crude of *M malabathricum* produced 13 to 16 mm against these two plant pathogenic strains. Ciprofloxacin produced zone of inhibition of 20 mm or more than it. Both the strains were resistant to the DMSO (Figure 5).

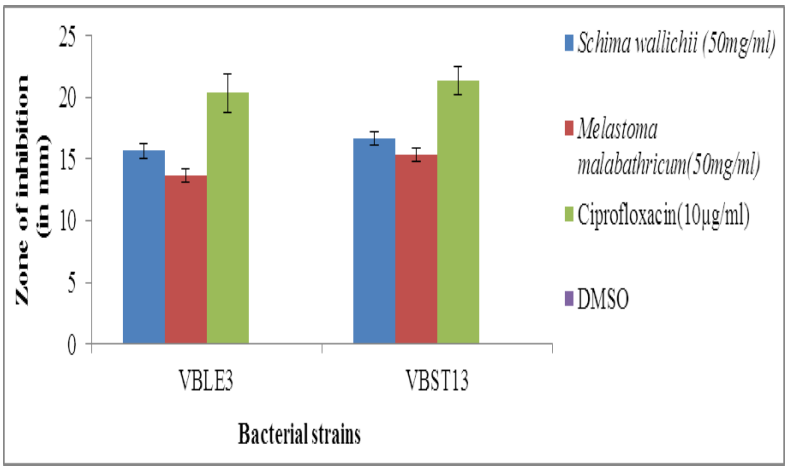


Figure 5: Antibacterial activities of the leaf extracts against VBLE3 and VBST13.

Determination of the MIC values

The MICs of the leaf extracts against the two strains were determined by counting the Colony Forming Units (CFU). The MIC values of *S wallichii* against both the strains were found to be 100 µg/mL. For *M malabathricum* the value was 150 µg/mL against both the strains (Table 3). These results reflect that the leaf extracts of *S wallichii* is a better option than the other for killing both the pathogens.

Table 3: MICs of the leaf extracts against VBLE3 and VBST13

Concentration (µg/mL)	Leaf extract of *S wallichii*		Leaf extract of *M malabathricum*	
	VBLE3	VBST13	VBLE3	VBST13
Control	43×10^9	28×10^9	43×10^9	28×10^9
25	32×10^9	18×10^9	34×10^9	21×10^9

50	25×10^{9}	16×10^{9}	24×10^{9}	15×10^{9}
100	$\mathbf{16\times 10^{7}}$	$\mathbf{12\times 10^{7}}$	17×10^{9}	11×10^{9}
150	38×10^{6}	66×10^{6}	$\mathbf{12\times 10^{8}}$	$\mathbf{13\times 10^{7}}$
200	20×10^{4}	22×10^{4}	45×10^{6}	34×10^{6}
250	12×10^{3}	15×10^{3}	32×10^{4}	16×10^{4}
500	14×10^{2}	18×10^{2}	14×10^{3}	42×10^{3}

Mode of action

Mode of action of the leaf extracts were determined by counting the Colony Forming Units (CFU) at every hour. *S wallichii* showed bactericidal and *M malabathricum* showed bacteriostatic mode of action against both the bacterial strains (Figure 6). These results indicate that the leaf extract of *S wallichii* due to having bactericidal mode of action is more potent than *M malabathricum* in killing the pathogenic bacteria.

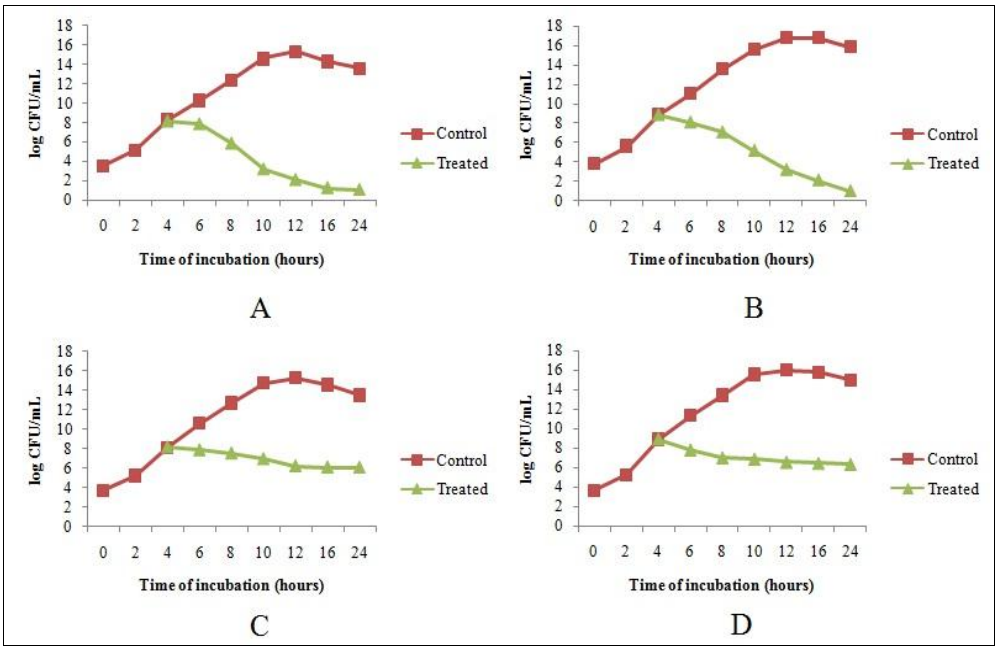


Figure 6: Mode of action of *Schima wallichii* against A. VBLE3, B. VBST13 and *Melastoma malabathricum* against C. VBLE3, D. VBST13

Among 94 bacterial strains isolated from the infected portions of the diseased plants, VBLE3 and VBST13 were found to produce highest EPS both in terms of fresh and dry weight which is a key and necessary factor for wilt disease development. VBLE3 and VBST13 also showed highest wilt symptoms in tomato in pathogenecity test in pot experiment. VBLE3 and VBST13 showed maximum similarity with *Pseudomonas koreensis* and *Pseudomonas lundensis* respectively in 16S rDNA sequence homology. The pathogenecity of VBLE3 and VBST13 was also established through Koch postulate by comparing the Gram nature, colony morphology in NA and TZC supplied NA plates, antibiotic sensitivity profiles and 16SrDNA sequence of the re-isolated bacteria. Ethanolic leaf extracts of *Schima wallichii* and *Melastoma malabathricum* showed excellent antibacterial potentials against VBLE3 and VBST13. The MIC of leaf extract of *Schima wallichii* and *Melastoma malabathricum* were found to be 100 µg/mL and 150 µg/mL respectively against both the wilt pathogens. Leaf extract of *S. wallichii* showed bactericidal mode of action against VBLE3 and VBST13 where as the extract of *M. malabathricum* exhibited bacteriostatic mode of action.

CONCLUSION

The two *Pseudomonas* spp. isolated from the wilt infested tomato plants were found to posses enough pathogenic potentials as evidenced by their colony nature in TTC supplied medium, EPS production and wilt development abilities in tomato. The two leaf extract used for controlling the pathogens were also found to show good antibacterial efficiencies against the pathogenic isolates. Leaf extract of *Schima wallichii* was found more efficient than *Melastoma malabathricum* in controlling the pathogens as it showed lower MIC values and bactericidal mode of action against VBLE3 and VBST13. So, the proper formulated leaf extracts can be used as potent biocontrol agents for controlling wilt disease.

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