RESEARCH ARTICLE

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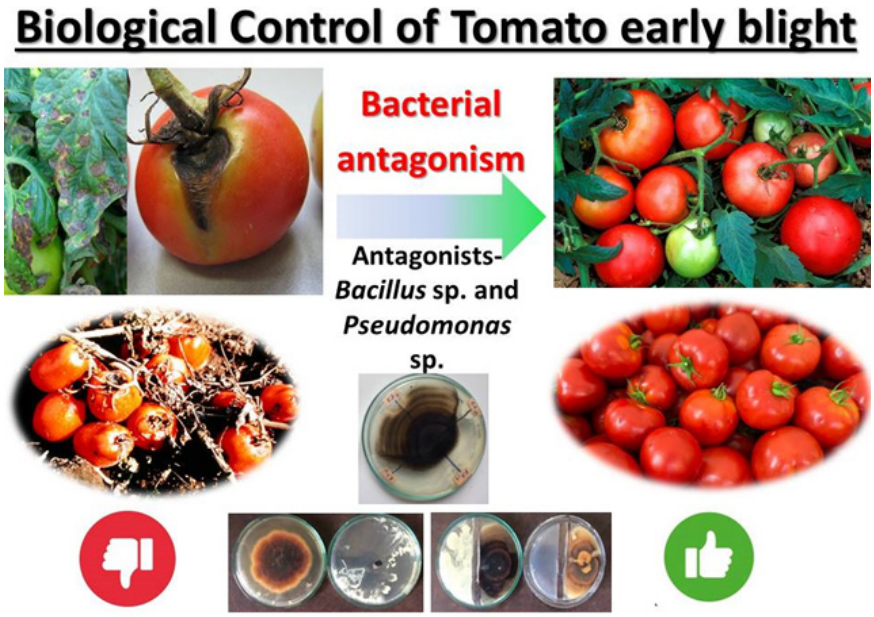

Highlights

- The causal agent of tomato early blight disease can be successfully controlled through bacterial antagonism.
- Seven bacterial isolates have shown promising antagonistic effects with significant impacts on mycelial growth and spore germination of *Alternaria alternata.*
- Antibiosis, hyperparasitism and competition were the antagonistic mechanisms in controlling the fungal pathogen.

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RESEARCH ARTICLE

Biocontrol potential of some selected native bacterial antagonists against tomato (*Solanum lycopersicum* L.) early blight causal agent *Alternaria alternate*

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Abstract: Seven selected bacterial antagonists isolated from healthy tomato rhizosphere and phyllosphere were evaluated for their biocontrol efficacy against the tomato early blight causal agent *Alternaria alternata* under *in vitro* conditions. The causal organism of early blight of tomato was isolated from a tomato leaf sample which showed early blight symptoms and verified it as *Alternaria alternata*. Healthy tomato rhizosphere soil, fructosphere and phyllosphere extracts were used as the potential sources of antagonists. Thirty bacterial isolates from tomato rhizosphere soil and 22 bacterial isolates from tomato phyllosphere were isolated using serial dilution technique. Out of those isolates, 24 were subjected to antagonists screening procedure. It was observed that, all the bacterial isolates except RA 5, RA 21, PA 1, PA 2, inhibited the growth of *A. alternata* significantly ($p<0.05$) in co-cultivation and dual culture showing an average radial growth inhibition of 69 %. Seven different bacterial isolates coded as RA 8, RA 12, RA 17, RA 18, RA 29, PA 4 and PA 12 were selected for further studies. Based on 16 S rRNA analysis, four of these antagonists were identified as *Bacillus thuringiensis*, *Bacillus cereus* and *Pseudomonas* sp. Microscopic observations of *A. alternata* hyphae subjected to antagonism showed deviations from the normal hyphae including thickenings, swellings and vacuolation. Inhibition of *A. alternata* spore germination by selected antagonists was significant at $p<0.05$ level with an average inhibition of 83.7 %. All the selected antagonists produced diffusible antifungal substances and showed hyperparasitism and competition as their mechanisms in antagonizing the fungal pathogen *A. alternata*. These results suggested that these seven bacterial isolates can be further explored as potential biocontrol agents in controlling early blight disease in tomato.

Key words: Antagonism; *Alternaria alternata*; Bacteria; Early blight; Tomato

INTRODUCTION

Tomato is a well-known cash crop grown worldwide. It is one of the most important and highly demanded vegetable crops in the world. Tomato consumption stands second after potato as a vegetable and also as a fruit due to its immense nutritional properties. Tomato is rich in vitamins (K, C and A), minerals (Fe, Ca and P), amino acids, sugars, dietary fibers, antioxidants and about 95.3 % of water (Awan & Shoaib, 2019; Bhanage et al., 2019).

Commercial tomato production is hampered worldwide due to many biotic and abiotic factors. Among these, huge losses are caused by the biotic factors which cause various diseases at different stages of tomato. Various bacterial, viral and fungal infections such as wilt, early blight, damping off, late blight, anthracnose, tomato mosaic virus can be found in tomato (Devi et al., 2017; Verma et al., 2018; Rex et al., 2019; Roy et al., 2019). Among these, early blight is a devastating disease affecting tomato production worldwide.

Early blight is a fungal disease mainly infecting tomato foliage and fruits resulting a great loss both in quality and quantity of tomato yield (Tomazoni et al., 2016; Perveen et al., 2019). It is caused by the fungal species *Alternaria*. It is reported that pre and post-harvest losses in tomato yield caused by this fungal infection vary from 35 % to 78 % (Shamurailatpam & Kumar, 2020). Management of this devastating disease is a great challenge tomato farmers are facing due to the wide host range and prolonged life cycle of the causal agent *Alternaria* sp. The most common control measure practiced by many around the world is the application of synthetic fungicides. It is the only effective way of managing this fungal causal agent as reported all around the world (Gazanfar et al., 2016; Mahanthesh et al., 2017; Rani et al., 2017).

Yet, extensive application of chemical fungicides is not healthy or eco-friendly. It causes many toxic effects to the environment and thereby consumer demand on fungicide-applied fruits diminishes. Also, continuous application of these chemicals can lead to the development of resistant varieties of the causative agent. Thereby, synthetic fungicides is not a long term viable solution for management of this disease. As a solution, many scientists are trying eco-friendly biological control strategies to mitigate tomato early blight disease (Abdalla et al., 2014; Joseph et al., 2017; Pandey et al., 2014; Roopa et al., 2014; Yadav, 2014).

Among the biological control strategies, microbial antagonism is vital. There are many reports which explain the successful applications of microbial antagonists in plant disease management (Sharma et al., 2009; Zhang et al., 2007; Droby, 2006; Korsten, 2006; Zhang et al, 2005; Janisiewicz & Korsten, 2002; Droby et al., 1992; Wisniewski & Wilson, 1992). Successful attempts of biological control of chilli (Sandani et al., 2019, Intanoo & Chamswarng, 2007 and Boonratkwang et al., 2007),

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strawberries (Freeman et al., 2001) and citrus (Moretto et al., 2001) using microbial antagonists have been reported.

Though development of a biofungicide using individual or a mixture of microbial antagonists is a huge process (Nakkeeran et al., 2005), it is a sustainable, environmentally friendly, green approach for plant disease management. In this study, the most common, destructive *Alternaria* species which cause early blight disease in tomato in Sri Lanka was evaluated for the potential of biological control using antagonistic microorganisms. The objective of this study was to isolate, screen and evaluate potential microbial antagonists against *Alternaria alternata* for biological control of early blight in tomatoes and detection of their antagonistic mechanism.

MATERIALS AND METHODS

Isolation of the early blight causal agent *Alternaria alternata*.

Alternaria alternata, was isolated from infected tomato leaves and it was verified through microscopic observations according to Annon (1993). The isolate was maintained on PDA slants at 4º C.

Isolation of potential antagonistic bacteria

Three different sources were selected based on literature in order to isolate antagonistic microorganisms. They were healthy tomato rhizosphere, healthy tomato fructosphere, healthy tomato phyllosphere.

Isolation of antagonistic bacteria from healthy tomato rhizosphere

One gram of rhizosphere soil sample was suspended in 10 mL of sterilized distilled water and then serially diluted up to 10^{-4} in order to isolate single colonies. Hundred microlitres of each dilution was spread on PDA and incubated at 28 ºC for 24 hours. Resulted contrasting bacterial colonies were transferred to fresh nutrient agar and fungal colonies were transferred to PDA plates to obtain pure cultures of each. They were stored at 4 ºC until screening is completed.

Isolation of antagonistic bacteria from healthy tomato fructosphere

One milliliter of water in which healthy tomato fruits were washed was mixed in 9 ml of sterilized distilled water and then serially diluted up to 10^{-4}. Hundred microlitres of each dilution was spread on PDA and incubated at 28 ºC for 24 hours. Resulted contrasting bacterial colonies were transferred to fresh nutrient agar and fungal colonies were transferred to PDA plates to obtain pure cultures of each. They were stored at 4 ºC until screening is completed.

Isolation of antagonistic bacteria from healthy tomato phyllosphere

One milliliter of water in which healthy tomato leaves were washed was mixed in 9 ml of sterilized distilled water and then serially diluted up to 10^{-4}. Hundred microlitres of each dilution was spread on PDA and incubated at 28 ºC for 24 hours. Resulted contrasting bacterial colonies were transferred to fresh nutrient agar and fungal colonies were transferred to PDA plates to obtain pure cultures of each. They were stored at 4 ºC until screening is completed.

Screening of potential antagonistic candidates against *A. alternata*.

Screening of potential antagonists was done in three steps in order to increase the precision and to ensure easy handling.

Co-cultivation

Isolates which showed inhibition on spore lawn were then subjected to co-cultivation with the test fungus for further screening. Four selected bacterial isolates were co-cultivated with the test fungus *A. alternata* in a single plate.

Radial growth of the fungus towards each bacterial isolate was measured at seven and fourteen days after culturing (DAC). Percent inhibition of radial growth (PIRG) was calculated for each bacterial isolate using the following formula (Sariah, 1994).

$$PIRG = \frac{R1 - R2}{R1} \times 100$$

Where,

R1 – Average radius of *A. alternata* in control plate

R2 – Average radius of *A. alternata* colony facing the bacterial isolate

Bacterial isolates which significantly inhibited the radial growth at $p<0.05$ level were then further confirmed for their antagonistic potential by employing dual culture assay with *A. alternata.*

Dual culture

Each selected bacterial isolate was dual cultured on PDA with the test fungus with a distance of 2 cm apart from each other. Radius of the fungal colony facing the bacterial isolate was measured at seven and fourteen DAC and PIRG value for each bacterium was calculated.

These experiments were repeated once and were arranged in completely randomized design (CRD) with three replicates.

Identification of the antagonists

Morphological identification and grams staining

As a preliminary study, bacterial colony morphologies were recorded and gram staining was performed.

Molecular level characterization

Four screened antagonists were subjected to the analysis of 16 S rRNA gene for the genus and species level identification. Extraction of genomic DNA was carried out with Wizard R Genomic DNA purification kit (Promega-USA) and PCR amplification of the fragments was performed using 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTT GTT ACG ACT T) 3' primers. The resulting PCR products were purified using Wizard SV Gel and PCR Clean Up system (USA) and sequenced by Macrogen (Macrogen Inc., Seoul, South Korea). Obtained sequences were compared with other sequences in the GenBank database using Basic Local Alignment Searching Tool (BLAST) program (National

Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

Investigation on the effect of antagonists on different growth phases of the causal agent, *A. alternata*

Effect of antagonism on hyphal growth and morphology

Microscopic mounts of *A. alternata* mycelial edges, which were subjected to antagonism with each antagonistic bacteria, were prepared using seven days old dual culture assay plates. A control mount was also prepared using an untreated seven days old *A. alternata* culture. The prepared mounts were stained with Lactophenol Cotton Blue and observed under the light microscope (Carl Zeiss- Ax10) for any morphological deviations of the mycelium from the control.

The experiment was repeated once and was arranged in completely randomized design (CRD) with three replicates.

Effect of antagonism on spore germination

A spore suspension (10^6 spores ml^{-1}) of *A. alternata* was prepared using a seven days old *A. alternata* culture. Fifty microlitres of spore suspension and fifty microlitres of 24 h old nutrient broth culture of the antagonists (10^8 CFU/ml) were mixed in a cavity slide. In the control, sterilized distilled water was added instead of the antagonist. These were placed in a humidity chamber for 15 hours and observed for germination under the light microscope. Germination percentage in each treatment was calculated and compared with the control. Spores were considered to have germinated when the germ tube length was half of the length of the spore (Sariah, 1994). The no of germinated spores were counted observing through the high power (x40) of the light microscope and spore germination percentage was calculated for each antagonist treatment.

Detection of the antagonistic mechanism

Production of diffusible antifungal substances

The diffusible nature of antifungal compounds secreted by antagonists was evaluated using the cellophane overlay technique (Nourozian et al., 2006). In this, 12 cm diameter round shaped cellophane membranes were boiled for five minutes and autoclaved in between paper towels. Those sterilized cellophane membranes were then placed on potato dextrose agar (PDA) in petri plates. Three hundred microlitres of 24 hours old bacterial broth culture was spread over the entire cellophane membrane and incubated at 28 ºC for 24 hours. PDA plates with nutrient broth over cellophane membranes were used as the control. After an incubation period of 24 hours, the cellophane membranes with adhering bacterial cultures were carefully removed. Small discs of *A. alternata* were introduced into those treated PDA plates and incubated at 28 ºC for seven days.

The results were expressed as PIRG values compared to control. The experiment was arranged in completely randomized design with three replicates and repeated once for better accuracy.

Production of volatile antifungal substances

The volatile nature of antifungal compounds was evaluated according to agar strip removal technique described by Choi et al. (2006). An agar strip with 1cm width was removed from the middle area of the PDA plate and it was air dried under sterile conditions to remove excess moisture from the cut surfaces. Antagonistic bacteria were introduced into one half of the PDA plate while *A. alternata* was introduced into the other half. A control was done introducing only test fungus into one half of the PDA plate. The plates were sealed and incubated at 28 ºC for seven days. The experiment was repeated once and it was arranged in completely randomized design with three replicates. The results were expressed as PIRG values compared to a control.

Hyperparasitism- Mycelial growth test

To detect whether competition is an antagonistic mechanism extend by the selected antagonists, mycelial growth test was performed. Small agar plugs of seven days old *A. alternata* was dipped in each antagonistic bacterial suspension (10^8 CFU/ml) for overnight period in sterile test tubes. The treated *A. alternata* mycelial plugs were transferred onto fresh PDA media separately and plates were incubated at 28 ˚C for seven days.

Data analysis

Data of each treatment were subjected to analysis of variance (ANOVA) and the means were separated using Dunnet's test and significance of the antagonists was determined by Duncan Multiple Range Test in SAS 9.1.3 software.

RESULTS AND DISCUSSION

Results

Isolation of the pathogen

Alternaria alternata was successfully isolated from infected tomato fruits and leaves collected from Wellawaya area in Monaragala district. The fungus was verified as *A. alternata* based on the morphology of conidia (Figure 1) according to Anon (1993) which are ellipsoidal, ovoid with 9-18 nm width and 20-63 nm length and with a short conical or cylindrical beak of one third or one quarter of the conidial length and 2-5 nm diameter tapering to the apex or blunt where 1-8 transverse septa and 1-2 longitudinal septa in each cell of golden brown to brown colour. *Alternaria alternata* isolate showed an average growth rate of 6.7 mm per day in room temperature on PDA. The isolate was single spored and maintained on PDA at 28 ˚C. Pathogenicity of this isolate was maintained by inoculating and re-isolating the fungus into healthy, ripened tomato fruits and mature leaves once in six months.

Isolation and screening of antagonistic bacteria

Thirty bacterial isolates from healthy tomato rhizosphere and 22 bacterial isolates from healthy tomato phyllosphere were isolated using serial dilution technique. Isolated bacterial colonies were maintained on nutrient agar at 4 ˚C until further screening was processed. Out of the 52 initial bacterial isolates, 24 contrasting bacterial colonies were selected from each source for the screening procedure. As the first screening step, co-cultivation was conducted.

Except three isolates all the other isolates significantly inhibited the mycelial growth of *A. alternata* (Figure 2 and 3).

Isolates which showed significant inhibition at $p<0.05$ level were subjected to the next screening step, dual culture. Five antagonistic bacterial isolates (RA 17, RA 8, RA 12, RA 29, RA 18) from the rhizosphere and two antagonistic bacterial isolates (PA 12, PA 4) from the phyllosphere of tomato showed promising antagonistic effect on *A. alternata* mycelial growth (Table 1) and they were selected for the further biocontrol efficacy testing against the early blight causal agent *A. alternata.*

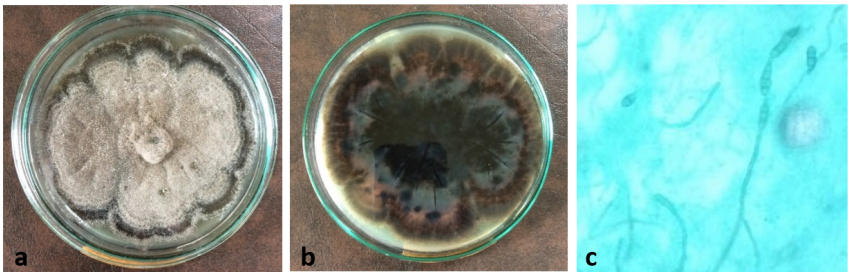


Figure 1: a) and b) *A. alternata* mycelium on Potato Dextrose Agar (a: upside, b: underside of the culture plate, Microscopic view of c) ellipsoidal or ovoid shaped conidia with transverse and longitudinal septa (x 200)

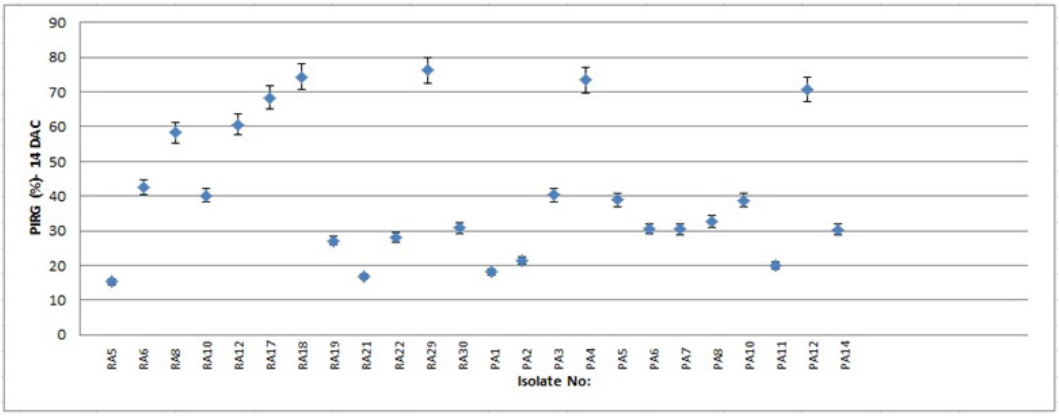


Figure 2: Inhibition of radial mycelia growth of *A. alternata* by 24 antagonistic bacteria in co-cultivation at 14 days after culturing (DAC). (Bars indicate standard error of the means)

Table 1: Confirmation of the antagonism of selected antagonistic bacteria against *A. alternata* in dual culture assay.

Isolate No:	PIRG (%) 7 DAC	PIRG (%) 14 DAC
RA17**	59.45*	66.10*
RA30	40.67*	32.35*
RA8**	53.81*	61.01*
RA12**	51.06*	61.01*
RA6	31.28*	40.42*
RA10	36.74*	39.35*
RA29**	57.85*	74.23*
RA5	20.35*	18.64
RA18**	57.90*	71.18*
RA19	25.01*	26.55*
RA21	11.36	17.24
RA22	18.18*	24.22*
PA10	36.22*	35.21*
PA11	18.24*	21.75*
PA12**	52.29*	69.49*
PA14	25.06*	26.22*
PA5	40.93*	44.21*
PA6	28.29*	31.23*
PA7	29.55*	32.66*
PA8	27.18*	30.75*
PA1	14.84	18.22
PA2	13.69	20.24*
PA3	37.44*	41.25*
PA4**	72.81*	74.57*
Control	0.00	0.00

PIRG-Percent Inhibition of Radial Growth
DAC- Days After Culturing
*Values were significant at $p<0.05$ level
** Isolates selected for the detailed study

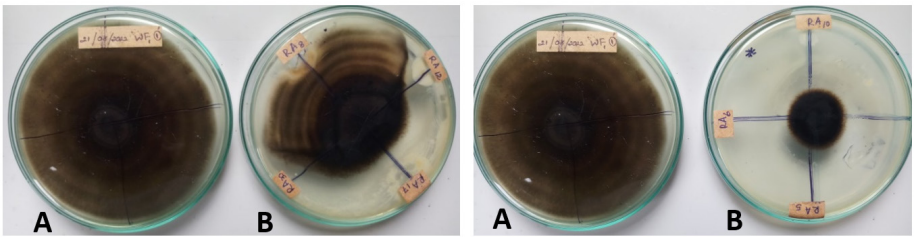


Figure 3: Screening of antagonistic bacteria against *A. alternata* in co-cultivation. A- *A. alternata* in control plate; B- *A. alternata*. subjected to co-cultivation. Note the restricted growth of the fungus in the plate B.

Identification of the antagonists

Morphological characterization

Morphological characterization was carried out as a preliminary study in characterization of the selected bacterial antagonists. Selected promising bacterial antagonists showed many variations in morphology (Figure 4 and Table 2) suggesting they may belong to different bacterial species.

Gram staining

According to the composition of the bacterial cell wall, selected antagonists were characterized as indicated in Table 3. All were gram positive except the antagonist which was coded as RA8.

Molecular characterization

According to 16 S rRNA analysis, the four selected bacterial antagonists were identified at their genus level as mentioned in Table 4.

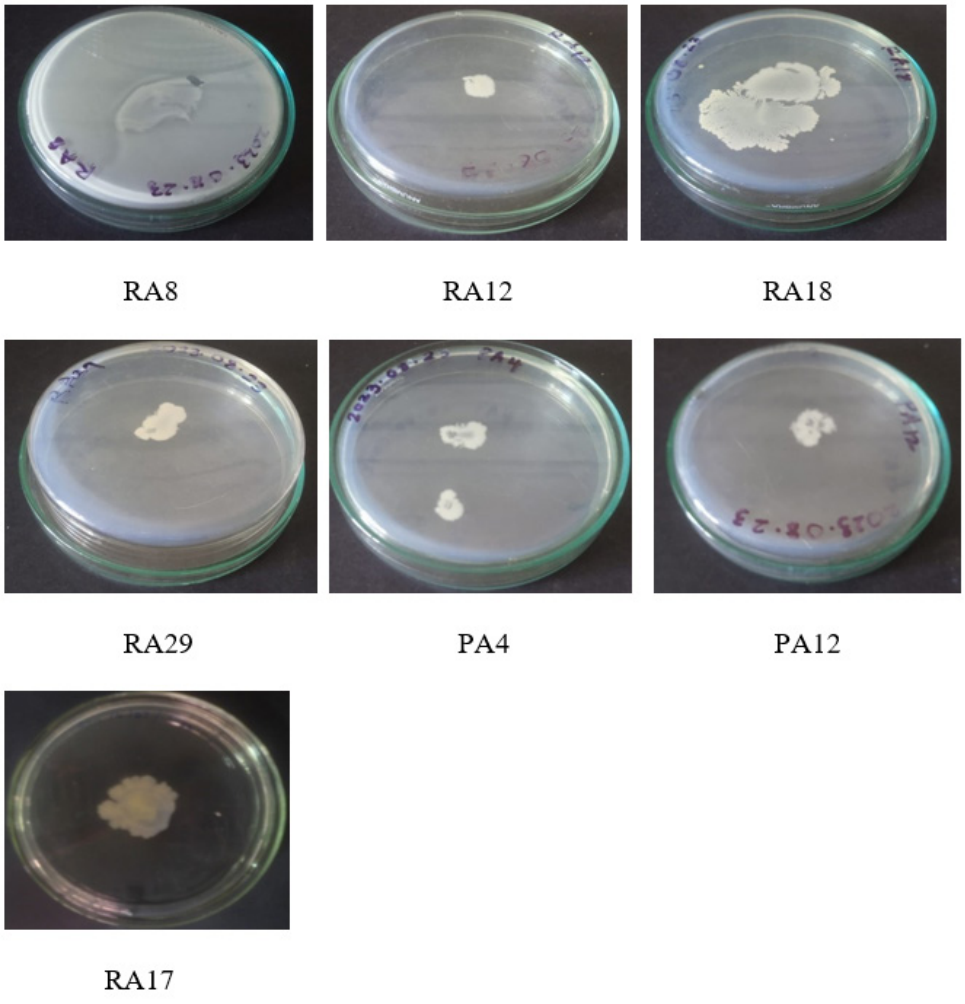


Figure 4: Overnight cultures of selected bacterial antagonists showing their morphological variation on nutrient agar medium.

Table 2: Morphological characterization of selected bacterial antagonists.

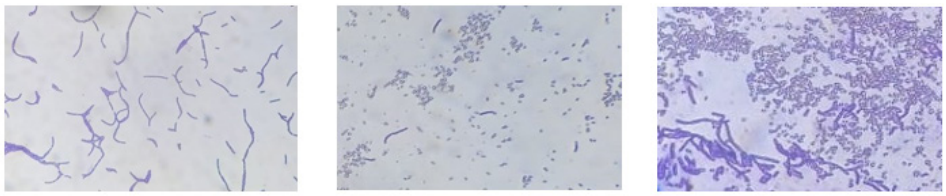
Morphological character	Antagonist						
	RA8	**RA12**	**RA18**	**RA29**	**PA4**	**PA12**	**RA17**
Shape	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Size	8 cm	1 cm	5 cm	2 cm	1.5 cm	1.5 cm	3 cm
Chromogenesis	Whitish colour, Soluble in medium	Light yellow colour, Insoluble in medium	Light yellow, Insoluble in medium	Light yellow, Insoluble in medium	Light yellow, Insoluble in medium	Light yellow, Insoluble in medium	Light yellow, Insoluble in medium
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Elevation	Flat	Raised	Flat	Raised	Raised	Raised	Raised
Surface	Smooth	Smooth	Rough	Smooth	Rough	Rough	Rough
Edge	Lobata	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate
Consistency	Viscid	Butyrous	Viscid	Butyrous	Butyrous	Butyrous	Butyrous
Emulsifiability	Turbid suspension	Turbid suspension	Turbid suspension	Turbid suspension	Turbid suspension	Turbid suspension	Turbid suspension
Odour	Present, Unpleasant	Present, Unpleasant	Present, Unpleasant	Present, Unpleasant	Present, Unpleasant	Present, Unpleasant	Present, Unpleasant

Table 3: Results of the Gram staining for the selected antagonists

Antagonist	Gram(+)ve/ Gram(-)ve
RA8	(-)ve
RA12	(+)ve
RA17	(+)ve
PA4	(+)ve
RA18	(+)ve
RA29	(+)ve
PA12	(+)ve

Table 4: 16 S rRNA analysis and BLAST search sequence alignment results of the selected antagonists and their precision.

Isolate No:	Organism	Precision
RA8	*Pseudomonas* sp.	99 %
RA12	*Bacillus cereus*	99 %
RA17	*Bacillus thuringiensis*	100 %
PA4	*Bacillus thuringiensis*	99 %



PA12 RA29 RA18

Figure 5: Gram positive antagonists (PA12, RA29, RA18) as resulted in gram staining (x 400).

Rest of the bacterial isolates, RA29, RA18 and PA12 also should be belonging to the genus *Bacillus* according to the results of the Gram staining and their cell morphology. Yet, further 16 S rRNA analysis is required for species level confirmation.

Effect of antagonism on hyphal growth and morphology

Mycelial growth of *A. alternata* was significantly inhibited by the selected antagonistic bacterial isolates (Table 1 and Figure 4). Morphology of the *A. alternata* mycelium differed from the normal due to its blackened and diffused nature as seen in Figure 1-B. Microscopic observations of mycelial edges, subjected to antagonism showed many deformations of the hyphae compared to the normal *A. alternata* hyphae. Thickenings and swellings of the *A. alternata* hyphae could be observed as a result of the inhibition inserted by the antagonist in dual culture. Such abnormalities of many other fungal hyphae have been recorded in previous studies as well (Rahman et al., 2007, Sandani et al., 2019).

Effect of antagonism on spore germination

Spore germination of *A. alternata* was inhibited over 50 % by the selected antagonists after 24 hours incubation in a humid chamber. Spore germination percentage during the same incubation period in the control was reported as 80 %. Selected antagonists extend a significant inhibition on spore germination which indicates a strong antagonistic effect on the fungal pathogen.

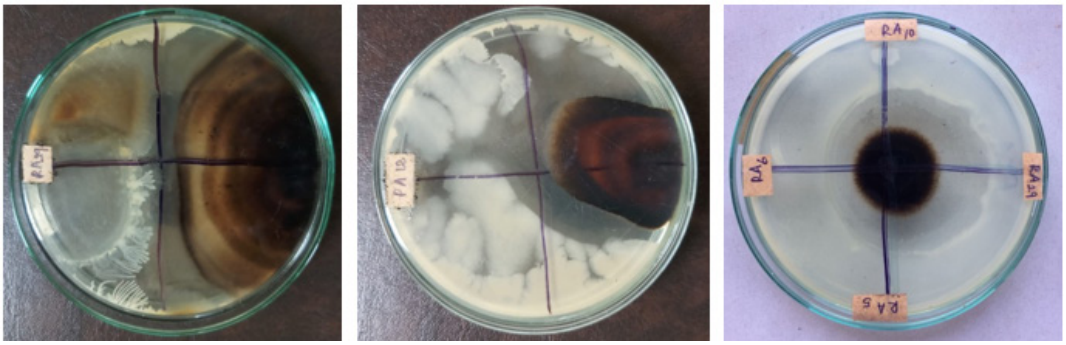


Figure 6: Effect of antagonists on hyphal morphology: Blackening of the mycelium.

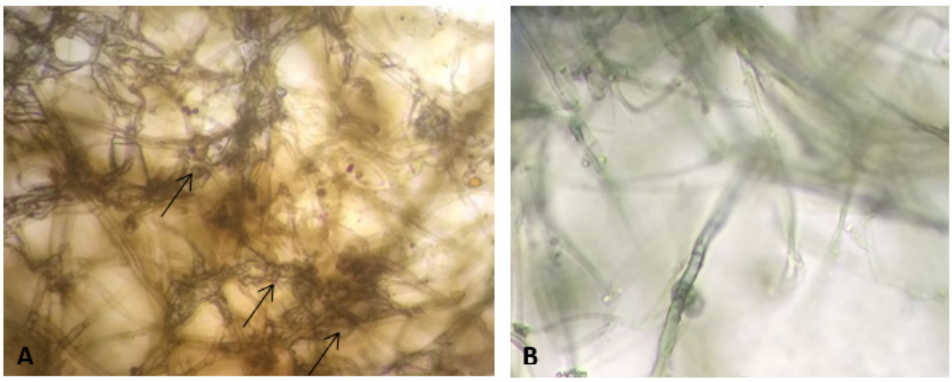


Figure 7: Microscopic view of *A. alternata* mycelia A) Subjected to antagonism; B) Normal hyphae – Arrow heads show coiling, swelling and vacuolation of hyphae (x 400).

Table 5: Average spore germination percentages of *A. alternata* in the presence of antagonists

Antagonist	Spore germination percentage (%)
Bacillus thuringiensis strain 1 (RA17)	12.5
Pseudomonas sp. **(**RA8)	20.0
Bacillus cereus (RA12)	21.3
Bacillus thuringiensis strain 2 (PA4)	0.00
RA29	15.0
RA18	31.0
PA12	18.5
Control	80.0

Detection of the antagonistic mechanism

Production of diffusible and volatile antifungal substances

None of the selected antagonists is producing volatile antifungal substances according to the results of the agar strip removal technique. Percent inhibition of radial growth of *A. alternata* was 0 % for all the treatments. All the antagonists produced diffusible antifungal substances and showed an inhibition over 50 % in the mycelial growth of *A. alternata* in cellophane overlay technique. RA 8 and RA 18 antagonistic bacterial isolates could completely inhibit the growth of *A. alternata* by the diffusible antifungal substances they produce, while RA 17, RA 29, RA 12, PA 12 and PA 4 showed percent inhibition of *A. alternata* radial growth in 57.77 %, 83.11 %, 77.55 %, 66.66 % and 80.55 % in order. These results reveal that production of diffusible antifungal substances is an antagonistic mechanism extended by the selected bacterial antagonists.

Mycelial growth test

All the selected bacterial antagonists significantly inhibited the growth of *A. alternata* in mycelial growth test at $p<0.05$ level. This indicates that hyperparasitism and competition are also antagonistic mechanisms extend by the selected antagonists in suppressing the tomato early blight causal agent *A. alternata* (Figure 10).

Table 6: Growth inhibition of *A. alternata* by diffusible and volatile antibiotics produced by antagonistic bacteria at fourteen days after incubation.

Antagonist	Inhibition of radial growth (%)	
	Diffusible antibiotics	Volatile antibiotics
Bacillus thuringiensis strain 1 (RA17)	57.8	0
Pseudomonas sp. (RA8)	100.0	0
Bacillus cereus (RA12	77.6	0
Bacillus thuringiensis strain 2 (PA4)	80.6	0
RA29	83.1	0
RA18	100.0	0
PA12	66.7	0

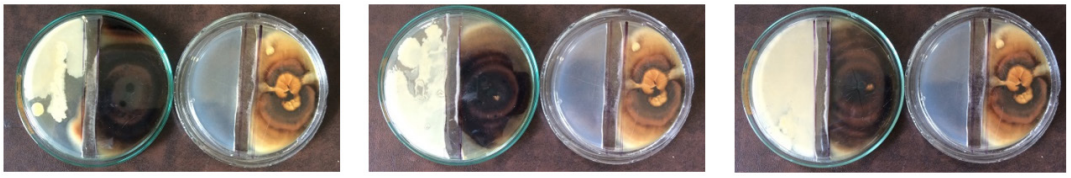


Figure 8: *In vitro* screening of volatile antifungal substances produced by selected antagonistic bacteria against *A. alternata*; T- Treated; C- Control.

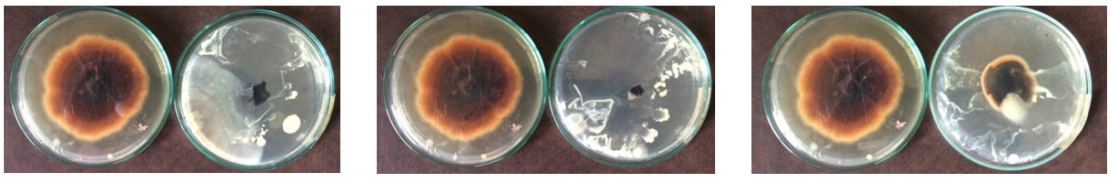


Figure 9: *In vitro* screening of diffusible antifungal substances produced by selected antagonistic bacteria against *A. alternata*; T- Treated; C- Control.

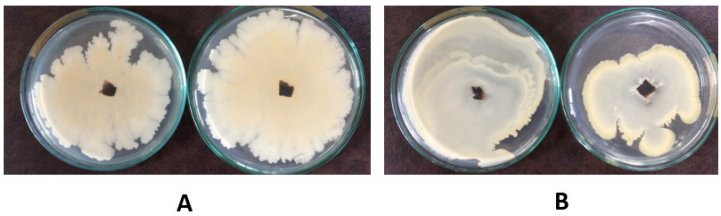


Figure 10: Mycelial growth test: A - *B. thuringiensis* strain 2 (PA4) treated culture plates; B - *Pseudomonas* sp. (RA8) treated culture plates.

DISCUSSION

Biological control is a reliable, eco-friendly alternative for harmful synthetic fungicides and pesticides, which is highly practiced worldwide. Bio-fungicides are very attractive user friendly solutions for crop disease management caused by fungal pathogens (Zhou et al., 2001; Freeman et al., 2004; Vagelas, et al., 2009). In developed countries, such bio-fungicides are commercially available and used by farmers in their cultivations (Nakkeeran et al., 2005). Development of a bio-fungicide is a lengthy, huge process which includes many crucial steps such as isolation and screening of potential antagonistic candidates, detection of the antagonistic mechanism of selected antagonists, optimization of culture conditions for the selected antagonists for mass production, evaluation of *in vivo* efficacy in managing the disease, development of a suitable formulation and field efficacy evaluation. Isolation and screening of potential antagonistic candidates and detection of the antagonistic mechanism employed by the selected antagonists are very important, essential, initial steps in the long process of bio-fungicide development.

Since tomato early blight is a devastating threat to commercial tomato production in Sri Lanka, and yet it is not successfully managed by synthetic fungicides, there is a high need of a reliable alternative for the management of this fungal disease. Identification of the tomato early blight causal agent in Sri Lanka and detection of potential antagonistic candidates against that fungal pathogen is of greater importance as it is a timely requirement. The possibility of utilizing native bacteria isolated from healthy tomato plants itself for bio control activities on early blight causal agent *A. alternata* was proved according to the results of above described *in vitro* assays of this study. It has been suggested that isolation of antagonistic microorganisms

indigenous to an environment similar to that in which they are supposed to function, may ensure greater efficacy in plant protection as they can persist and aggressively colonize the new environment without any problem (Cook, 1993). In this study also, potential antagonistic candidates isolated from healthy tomato rhizosphere, phyllosphere and fructosphere were screened against the early blight causal agent in two steps. Antagonistic candidates selected against *A. alternata* for evaluation of biocontrol efficacy of tomato early blight were from healthy tomato rhizosphere and phyllosphere.

Out of the seven selected bacterial antagonists, six belonged to the closely related bacterial genus, *Bacillus* and one belonged to the genus *Pseudomomnas.* As revealed by the BLAST search sequence alignments, the four bacterial isolates which were antagonistic against *A. alternata* were *Bacillus thuringiensis* strain 1, *Bacillus cereus*, *Bacillus thuringiensis* strain 2 and *Pseudomonas* sp. As revealed in the gram staining and their cell morphology which were rods often arranged in pairs or chains with rounded or square ends, the rest of the three antagonistic bacterial isolates also should belong to the genus *Bacillus* (Public Health, England, NHS, 2018). In the scientific literature related to biological control through bacterial antagonism, these bacterial genera possess prominent roles being strong antagonists in many disease controlling biological strategies (Janisiewicz & Roitman, 1988; Upadhyay et al., 1991; David & O'Gara, 1994; Haas & Keel, 2003; Kloepper et al., 2004; Jacobsen et al., 2004). *Bacillus* sp. is a prominent biocontrol agent due to their wide range of beneficial characteristics, metabolic functions and ecological behaviours. *Bacillus* strains are of remarkable importance as biocontrol agents against a wide range of phytopathogenic fungi due to their potential in biosynthesizing a wide variety of metabolites with antimicrobial activity (Zhang et al., 2009; Waites et al., 2008; Arrebola et al., 2010; Hossain et al., 2016; Kim et al., 2015; Nam et al., 2016). In addition, as *Bacillus* strains are endospore forming, chemoheterotrophic, aerobic or facultative anaerobic and motile with peritrichous flagella, they are known to colonize plant root surface, compete with other organisms, facilitate mineral nutrient uptake and thereby increase the plant growth in any environment (Waites et al., 2008; Turner & Backman, 1991; Takayanagi et al., 1991). Their capability in forming extremely resistant spores is of greater importance in their function as biocontrol agents in crop disease management compared to other antagonistic agents. Moreover, *Bacillus* sp. is also identified as plant growth promoting rhizobacteria, which can lead to an indirect biocontrol mechanism since it could enhance plant growth (Gardener, 2004). *Pseudomonas* sp. is known to produce broad spectrum antibiotics which can play an important role in the suppression of multiple plant pathogenic fungi (De Souza & Raaijmakers, 2003). Also strains of *Pseudomonas* species are identified as aggressive colonizers of the rhizosphere of various crops and have a broad spectrum of antagonistic activity against plant pathogens through their antibiosis, siderophore production (iron-sequestering compounds) and nutrition or site competition (Upadhyay et al., 1991; Winkelmann & Drechsel, 1997; Bull et al., 1991). Some species of *Pseudomonas* can also produce HCN that are toxic to certain pathogenic fungi as revealed by David & O'Gara in 1994.

Effect of the selected antagonists on mycelial growth of *A. alternata* was prominent in both screening steps, co-cultivation and dual culture assays. Isolates which showed significant inhibition on mycelial growth were selected for further studies and their effect on different growth phases of the causal agent was evaluated. Impact on the germination of spores, which ensures the survival of the pathogen, and the impact on the morphology of the hyphae also was significant as in the mycelial growth. PA4 antagonist, the *Pseudomonas* sp. completely inhibited the germination of *A. alternata* spores while all the other selected antagonists showed a significant inhibition greater than 50 %. These suggest the high potential of these bacterial strains being successful biological control agents on *A. alternata* agreeing with many scientific findings (Sandani et al., 2019; Rahman et al., 2007). Further studies must be focused on this aspect as seed borne infection, fruit rot and tomato leaf colonization of *A. alternata* could be conveniently controlled by such antagonistic agents or their products at the initial stage. As the present study has discovered the high efficacy of antagonistic bacteria in controlling spore germination and subsequent growth of the fungus, the possibility of practical utilization of these antagonists or their products in managing initial colonization on plant parts is required to be investigated.

Light microscopic studies revealed that the selected antagonists cause several structural deformations of the hyphal morphology of *A. alternata*. Many abnormalities such as swellings, thickenings, vacuolation, coiling of the hyphae were prominent. Antagonistic nature such as toxicity etc. of these antifungal compounds released by antagonists may result in such deformations in the mycelia. Similar results in the microscopic observations of abnormal mycelia have been achieved with antagonists against phytopathogenic fungi as described by Sandani et al., (2019), Upadhyay & Jayaswal (1992), Sariah (1994), Rahman et al. (2007) and Svetlana, et al. (2010). Vacuolation and coiling of the hyphae can result in as the hyphal tips can't protrude or extend further due to the effect of the antagonist on PDA. Vacuoles are responsible for the cell expansion and in driving the protoplasm forwards as hyphae elongate at tips (Deacon, 2013). Therefore, as there is a great protest for the hyphal tip elongation in the presence of an antagonist, vacuolation can result due to the effort of the fungus to overcome the barrier in its attempt of survival.

The strong impact of the selected antagonists on *A. alternata* mycelial growth, its morphology and germination of spores showcase the ability of these bacterial antagonists in biologically controlling the pathogenic fungus interrupting its vegetative growth and the reproduction stages. Based on these results and observations on antagonism of the pathogenic fungus, a cascade of studies is lined up to perform in development of a successful biofungicide. The initiative of such a huge, important process depends on a reliable, accurate finding of a strong antagonistic effect as

described above.

Understanding of the underlying mechanism of a particular biological interaction is also essential in a biological control study. In a strive of understanding the mechanism of a detected biological control strategy, all the types of interactions that can prevail in any two particular organisms have to be considered. Types of interactions prevailing between plants and microorganisms have been referred to as mutualism, antagonism, commensalism, neutralism, competition, amensalism, parasitism and predation (Bankhead et al., 2004; Bull et al., 2002; Katska, 1994; Chisholm et al., 2006; Fitter & Garbaye, 1994; Hoitink & Boehm, 1999). Here also the mechanism shown by these antagonists against *A. alternata* can be antagonism, competition or parasitism. Also antagonists follow certain mechanisms in antagonizing the pathogen. These mechanisms can be antibiosis, hyperparasitism, host defense mechanisms induction etc. Identification of these mechanisms between the antagonists and pathogen and also antagonists and the host plant are very important in development of a high efficacy biocontrol agent against a particular plant pathogen.

In understanding of the antagonistc mechanisms employed by the selected bacterial antagonists, according to the results of the antifungal compounds production detection assays, they are using antibiosis while some shown to apply some other mechanisms like hyperparasitism and competition. All the selected antagonists are producing diffusible antifungal compounds which have a greater impact on the mycelial growth of *A. alternata.* These diffusible antifungal compounds can be antibiotics, hydrolytic enzymes, bacteriocins, biosurfactants or some other secondary metabolites according to a review done by Beneduzi et al. (2012). Different antibiotics such as 2, 4- diacetylphloroglucinol, pyoluteorin (Subagio & Foster, 2003), pyrrolnitrin, phenazines (Kirner, et al., 1998), cepaciamide A (Jiao et al., 1996), cepacidine A (Lee et al., 1994) and xylocandin complex (Meyers et al., 1987) are produced by many *Pseudomonas* and *Burkholderia* species for their antifungal activities while antagonistic *Bacillus* strains are known to biosynthesize antibiotics such as zwittermicin, bacillomycin, fengycin, bacilysin, difficidin and many cyclolipopeptides (Athukorala et al., 2009; Chen & Nelson, 2008; Arrebola et al., 2010; Almenar et al., 2007; Hossain et al., 2016). Among these pyrrolnitrin, a chlorine-containing phenylpyrrole derivative with antifungal activity, which is produced by a number of *Pseudomonas* strains (Arima et al., 1964) has exhibited a broad spectrum of activity against many fungi and bacteria, even at relatively low concentrations (El-Banna & Winkelmann, 1998). Identification of the specific antifungal substances produced by the identified bacterial antagonists against *A. alternata* is very important and it is a milestone of this study which would open up new arenas for the biological control of this early blight disease in tomato.

Competition for nutrients and substrates is also an effective biocontrol mechanism employed by many biocontrol agents as revealed in many research studies (Elad & Baker, 1985; Keel et al., 1989; Loper & Buyer, 1991). The nutrient sources present in the soil and the rhizosphere are frequently not sufficient for the occupied microorganisms. Microbes have to compete for nutrients on plant surface, which include exudates, leachates or senesced tissues. An interesting finding is that plant-associated non-pathogenic microorganisms are said to protect the plant by rapid colonization, thereby utilizing the limited available substrates. Because of this, there is a very poor chance for pathogens to survive in that niche. This is also a viable mechanism detected in the selected antagonists against *A. alternata* and it emphasizes the efficacy of these antagonists in managing the tomato early blight pathogen.

Hyperparasitism is also one of the possible antagonistic mechanisms of the selected antagonists. In hyperparasitism, the pathogen is directly attacked by a specific biocontrol agent such as hypoviruses, facultative parasites, obligate bacterial pathogens or predators that kills it or its propagules. Certain studies have illustrated how this hyperparasitism has been involved in the biological control of phytopathogens (Milgroom & Cortesi, 2004).

This study provides the essential direction in moving towards the biological control strategy which is highly recognized and accepted in the global as a reliable alternative for synthetic fungicides in managing the early blight of tomato in Sri Lanka. Based on the results of this study further required steps in development of a biofungicide can be designed and implemented in search for an user friendly, greener tomato early blight management in Sri Lanka.

CONCLUSION

Selected seven bacterial antagonists are promising in suppressing the tomato early blight causal agent, *A. alternata* in all of its growth phases and they are potential bio control agents for successful management of tomato early blight disease.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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