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Effectiveness of Antagonistic Bacterial Metabolites to Control *Rhizoctonia solani* on Lettuces and *Fusarium oxysporum* on Tomatoes

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Rhizoctonia solani and *Fusarium oxysporum* cause yield losses in numerous economically important crops. To develop a bio-control agent, cell free extracellular compounds (ECs) of 5 bacterial strains *Burkholdria* sp. L1, *Pseudomonas* sp. L4, *Pseudomonas chlororaphis* VN391, *Bacillus subtilis* VN21 and *Enterobacter* sp. VN99 from Vietnamese fields, which reduced levels of *R. solani* root rot in lettuces and *F. oxysporum* root rot in tomatoes, were investigated. In a growth chamber, ECs of all antagonists markedly enhanced the biomass of lettuces (10 to 14.1%) and tomatoes (11.38 to 13.88%). In greenhouses, the disease's severity on both crops treated with ECs of the antagonists was reduced significantly and biomass losses in the plants decreased markedly. The reduction level of *R. solani* root rot in lettuces was 75, 66.7, 50, and 16.7% by ECs of strains L1, L4, VN21 and VN391, respectively. The biomass of lettuces increased markedly by 29.13%, 21.67%, and 23.4% by ECs of strains L1, L4 and VN21, respectively. Similarly, the reduction levels of *F. oxysporum* root rot in tomatoes was 76.3, 75, 41.7 and 25% by ECs of strain L1, L4, VN21 and VN391, respectively, and the biomass was significantly enhanced by 14.42, 12.7 and 13%, respectively. The ECs of strain L1 exhibited the most effective bio-control agents to suppress *R. solani* and *F. oxysporum*.

Keywords: *Rhizoctonia solani* root rot, *Fusarium oxysporum* root rot, extracellular compounds, antagonist, enhanced biomass

Introduction

The use of chemical fungicide has seriously damaged environmental soils, underground water, air and ecological environment, etc. Therefore, the use of biological fungicides originated from microbial antagonists to protect crops from disease has been increasingly interested in proceed to replace chemical fungicides. High doses of chemical fungicides are used to protect crops but makes soils to be barren, severely affecting the ecological environment. Especially, chemical residues on the agricultural products cause serious impact on human health. The development of microbial antagonists for production of plant fungicides is very urgent and meaningful in organic agriculture. So far, several secondary metabolites producing bacteria have been considered as biocontrol agents of plant pathogenic fungi control. For example, *Pseudomonas syringae* synthesize syringomycin, syringostatin and syringotoxin which suppress effectively pathogenic fungi of *Aspergillus flavus* and *Fusarium oxysporum* [4]. Besides, *Pseudomonas* strains produce cepacin A and cepacin B [20], phenazine [25] and pyrrolnitrin [12] which inhibit growth of *Rhizoctonia solani, F. oxysporum* [8]. *Burkholdria cepacia* strains produce several antibiotics including cepacin and pyrrolnitrin which suppress the growth of both *R. solani*- and *F. oxysporum* [2, 13]. In Vietnam, a few papers have presented the use of microbial cells, fungal spores for suppression of plant pathogenic fungi. However, reports of cell free extracellular compounds (ECs) of antagonists against plant fungal diseases are not much. The aim of this study was to

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study the antifungal activity and fungal disease suppression of several ECs of bacterial strains involving *Burkholdria* sp. L1, *Pseudomonas* sp. L4, *P. chlororaphis* VN391, *Bacillus subtilis* VN21 and *Enterobacter* sp. VN99 isolated from Vietnamese fields.

Material and Methods

Microbial strains

In this study, *Burkholdria* sp. L1, *P.* sp. L4, *B. subtilis* VN21, *P. chlororaphis* VN391 and *Enterobacter* sp. VN99 were isolated from the rhizosphere of different plants in fields surrounding Ha Noi capital and North provinces in Vietnam. Pathogenic fungi of *F. oxysporum* and *R. solani* were purchased from Department of soil microbiology, Institute of Biotechnology, Vietnam Academy of Science and Technology while *F. culmorum* var. *cul, F. Graminearum, F. proliferatum var. proliferatum, F. solani var. solani, F. verticillioides, S. sclerotium* and *V. dahlia* were kindly provided by Department of plant health, Leibniz Institute for Vegetable and Ornamental Crops (IGZ), Groβbeeren, Germany.

Antagonistic strains of L1, L4, VN21, VN99 and VN391 were grown on medium tryptic (T) soy (S) agar (A) (Difco, USA) or King'B agar (1 liter of medium containing 30 g glycerol, 10 g peptone, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar). Inoculated culture plates were incubated at 25°C for 2 days. One loop of active cells of each antagonist from agar culture was cultivated in Tryptone Soya broth (TSB), incubated on an orbital shaker at 200 rpm, at 28°C for 5 days. After fermentation, bacterial cells were removed by centrifugation at 5.700 g/min for 15 min. The extracellular compounds (ECs) was harvested and diluted to 10 folds for seed treatments and seedling treatments.

Preparation of pathogenic fungi

One hundred gram of oat kernels mixed with 120 ml distilled water was deposited in a glass petri dish and autoclaved at 121°C for 60 min. Autoclaved barley kernels inoculated with 3 pieces of 7-day-old *Rhizoctonia* cultures on potato (P) dextrose (D) agar (A) were used for inoculation of lettuces in the chamber and greenhouse. Barley kernels infested *R. solani* were incubated at 20°C for 3 weeks in the dark [22]. *F. oxysporum, F. culmorum* var. *cul, F. graminearum, F. proliferatum var. proliferatum, F. solani var. solani, F. verticillioides, S. sclerotium* and *V. dahlia* were cultivated on PDA plates, at 25°C for 12 days in the dark. Hyphae and conidia were harvested by pouring 12 ml of sterile 0.05% Tween 20 onto the fungal PDA plates of 10 day-olds and scrapped by sterile spreader. Spore number of *F. oxysporum* was adjusted to 10^8 spores/ ml for inoculation of tomatoes seeds and its seedling in the chamber and greenhouse. The rate of the *R. solani* root rot of lettuces, and *F. oxysporum* root rot tomatoes, disease symptoms and disease suppression effect were examined in further studies.

Antifungal activity

In vitro, ECs of antagonists inhibited the growth of pathogenic fungi was examined on dual culture according to the methods [14, 21]. The antifungal activity of ECs of strains L1, L4, VN21, VN391 and VN99 inhibited the growth of *F. graminearum, F. culmorum* var. *cul, F. oxysporum var. oxysporum, F. solani var. solani, F. proliferatum var. proliferatum, F. verticillioides, V. dahliae and S. sclerotium* was determined. A hole of 8 mm in diameter formed on PDA plate was loaded by 50 μl ECs of either strain L1 or L4, N21, N391 and VN99, at a distance of 4 cm from a mycelia disk (8 mm in diameter) of pathogenic fungi, at 25°C in dark. After 3 days of incubation, clear zones and zones of overlapping were assessed. The percentage of clear zone was determined using following formula:

$$\text{Suppression rate (\%)} = (A - B) \times 100/A$$

where, A is mycelial growth away from the ECs of antagonists (the maximum growth of the fungal mycelia) and, B is mycelial growth towards the ECs of antagonists.

Enzyme activity

Each antagonist inoculated in a 250 ml Erlenmeyer flask containing 100 ml TSB was cultivated on an orbital shaking incubator at 200 rpm and 25°C for 4 days. The cell free ECs were obtained by centrifugation at 5.700 *g* for 15 min at room temperature. The activity of chitinases, β-glucanases, cellulases and proteinase in ECs was determined according to the diffusion method using agar plate containing 1.5% agar and 0.1% of either chitin, β-1,4-glucosamine, carboxyl methyl cellulose (CMC) or skim-milk. Each plate containing respective substrate was formed four holes of 8 mm in diameter each. To each hole, 50 μl ECs was loaded and then incubated at 37°C for over night. The enzyme activity in ECs was determined based on clear zone surrounding the loaded holes [1, 3]. Each experiment was

repeated for triplicate.

Chamber experiments

Effect of ECs on growth of lettuces and tomatoes: The seeds of lettuces and tomatoes were sown in plant container of 92 seedlings filled with quarz sand and organic substrate (Fruhsdorfer Einheitsede type P; chemical analysis in 100 g containing 75 mg N, 75 mg P and 125 mg K, pH 5.9) in a ratio of (1:3, v/v), watered, cultivated in the growth chambers with 16 h light (L) /8 h dark (D), light input 500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with 70 and 90% relative humidity, at 20°C and 25°C, respectively. At one-leaf stage, each plant was treated with 10 ml of ECs of either L1 or L4, VN21, VN391 and VN99. In control, plants were not treated with ECs. Each treatment included 3 replications with 6 plants each in a randomized design. Fresh and dry weight of trunk-leaves and roots were measured after two weeks of ECs treatment.

Greenhouse experiments

Seeds and plants treated with ECs: The seeds of tomatoes and lettuces were submerged in ECs of antagonists of either L1 or L4, VN21, VN391 and VN99. After treatments, seeds were sown in plant container of 92 seedlings filled with quarz sand and organic substrate (Fruhsdorfer Einheitsede' type P). The lettuces and tomatoes were cultivated at 20°C and 25°C, respectively, with 16 h /8 h (L/D) photoperiod. Plants were maintained at constant temperature with lighting at IGZeV, Berlin, Germany, for 2-3 real leaves and transferred to pots contained 500 g mixture of sands and organic substrate ('Fruhsdorfer Einheitsede' type P) in greenhouses at the same conditions. Each treatment included 3 replications with 6 plants each in a randomized design.

Lettuces inoculated with R. solani and ECs of antagonists: The second treatment was performed after transplanting to potted plant 1 day. Each potted seedling was treated with 20 ml ECs of either L1 or L4, VN21, VN391 and VN99. Five barley kernels were used for inoculation of each seedling, which placed 1-cm deep, at a distance of 2 cm from each; in the control-1: lettuces were not treated with both *R. solani* and ECs; in the control-2: lettuces were only inoculated with *R. solani*; at 20°C, 16 h/8 h (L/D) photoperiod. Each treatment included six replicates with 3 plants each arranged in a randomized design. Daily, potted plants were watered lightly to maintain the substrate moisture. Weekly, plants with symptoms of root rot were assessed. The symptoms of root rot, biomass of lettuces were measured after 4 weeks treatment with *R. solani* and ECs.

Tomatoes inoculated with F. oxysporum and ECs of antagonists: Twenty ml spore suspension (10^8/ml) of *F. oxysoprum* was inoculated for each plant, after 1 day of transplanting. The second treatment of ECs was performed after transplanting to pots 1 day. The soils surrounding each tomato was drenched with 20 ml ECs of either strain L1 or L4, VN21, VN391 and VN99. In the control-1: tomatoes were not treated with neither *F. oxysoprum* nor ECs; in control -2: tomatoes were only inoculated with pathogenic *F. oxysoprum*. The plants were cultivated in a greenhouse at 25°C with 16 h/8 h (L/D) photoperiod. Each treatment included six replicates with 3 plants each arranged in a randomized design. Daily, the pots were watered lightly to maintain the substrate moisture. Weekly, the number of plants with symptoms of bottom rot were assessed. The symptoms of root rot, and biomass were measured after 4 weeks treated with both *F. oxysoprum* and ECs.

Characterization of ECs of strain L1

Preparation of ECs: The active cells of strain L1 was cultivated in TSB on an orbital shaking incubator at 200 rpm, at 30°C for 5 days. After centrifugation at 10,000 g for 10 min, cells free ECs was harvested for further studies.

Effect of temperature: The ECs was kept at different temperatures ranging from 40~100°C for 60 min. Then, 50 μl of ECs was loaded into a hole on *F. oxysporum* inoculated PDA plate, at 28°C for 2 days. The clear zones surrounding the holes were determined.

Effect of pH: The ECs was kept at different pH values ranging from 2~10 for 60 min. Then, 50 μl of ECs was loaded into a hole formed on *F. oxysporum* inoculated PDA plate, at 28°C for 2 days. The clear zones surrounding the holes were determined.

Effect of different solvents: Different solvents of chloroform, methanol, hexane, petroleum-ether and acetone were used to extract antifungal compounds from EC of strain L1 with a ratio of 1/3 (sample/solvent, v/v). Next, solvent was removed by evaporation rotator (BUCHI, Switzerland). The residual solid was dissolved in 70% (v/v) methanol, 50 μl of this solution was loaded onto a hole formed on *F. oxysporum* inoculated PDA plate, at 28°C for 2 days. The clear zones surrounding loaded holes were determined. The results from pre-experiment showed that

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methanol of 70% did not inhibit the growth of both *F. oxysporum* and *R. solani.*

Effect of protease K: The ECs was incubated with protease K at concentrations of 0.1, 0.25 and 0.5 mg/ml, at 37°C for 30 min. Then, 50 μl of ECs was loaded onto a hole on *F. oxysporum* inoculated PDA plate, at 28°C for 2 days. The clear zones surrounding loaded holes were determined.

Statistical analysis

The data was analyzed using one- and two-way analyses of variance (ANOVA) (α = 0.05), followed by a comparison of the means using Duncan's multiple range test (SAS Institute, Cary, NC, USA).

Results

The ECs of strains L1, L4, VN21 and VN391 inhibited markedly the growth of various plant pathogenic fungi. The antifungal activity of various ECs showed different levels among strains ($p < 0.001$) (Table 1). *In vitro*, almost ECs of antagonists except VN99 killed 49.9~78.82% of *F. culmorum,* 67.91~75.88% of *F. oxysporum,* 59.9~89.41% of *F. solani*, 61.76~72.94% of *F. graminearum,* 70.89~78.24 of *F. proliferatum,* 74.12~75.88% of *F. verticillioides,* 62.94~77.64% of *R. solani*. The ECs of strain L1 and L4 inhibited the growth of pathogenic fungi was stronger than other ECs ($p < 0.05$). The ECs of strain VN391 and VN99 killed a very low rate of mycelial death.

Activity of enzymes

The cell wall degrading enzymes play a crucial role in the growth inhibition of *R. solani* and *F. oxysporum.* The main composition of fungal cell wall was composed of protein, chitin polymers and β-glucan**.** The results shown that ECs of strains L1, L4, VN21, VN99 and VN391 contained chitinases, β-glucanases, cellulases and protease, among them protease showed higher activity than that of others (Table 2). However, cellulase activity in all most ECs was very low. The activity of protease and chitinase in ECs of strains L1, L4 and VN21 exhibited significantly higher than that of strains VN391 and VN99.

Growth chamber experiments

Effect of ECs on the growth of lettuces: The results showed that ECs of strains L1, L4, VN21, VN391 and VN99 markedly enhanced the biomass. As a result, biomass of fresh trunk-leaves was 36.42, 42.87, 36.49, 34.3 and 31.46 g by ECs of L1, L4, VN21, VN391 and VN99, respectively. However, the biomass of 30.39 g of un-treated ECs was lower than that of almost ECs treated plants. The dry weight of trunk-leaves of ECs treated plants was from

Table 2. Enzyme activity of ECs of different antagonists.

Enzyme[a]	Enzyme activity in ECs of strains				
	L1	L4	VN21	VN391	VN99
Cellulases	+[b]	+	+	+	+
Chitinase	++[c]	++	++	+	+
Proteinase	+++[d]	+++	+++	++	++
β-glucanase	++	++	++	+	+

[a]The ECs were obtained after 5 days of cultivation, on an orbital shaking incubator at 200 rpm and 25°C.
[b]Clear zone created on agar medium of corresponding substrate within 10 mm to 12 mm.
[c]Clear zone within 17 to 22 mm.
[d]Clear zone above of 22 mm.

Table 1. Mycelial death rate (%) of pathogenic fungi caused by ECs of antagonists *in vitro*.

ECs of strains[a]	Mycelial death rate (%)						
	F. cul[b]	*F. gra*	*F. oxy*	*F. pro*	*F. sol*	*F. ver*	*R. sol*
L1	73[b*]	69[b]	75.88[a]	77[a]	75[c]	75[a]	74[c]
L4	74[b]	72.94[b]	73[ab]	78.24[a]	89.41[a]	75.88[a]	77.64[a]
VN21	78.8[a]	69[b]	68[b]	70.89[b]	83[b]	74.12[a]	74[a]
VN391	49.9[c]	61.76[c]	67.91[b]	71[b]	59.9[d]	75[a]	62.94[b]
P	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

*Means within the same column followed by the same letter are not significantly different at $p < 0.05$ by using Duncan's Multiple Range Test.
[a]50 μl of EC of each antagonist was loaded into a hole created on dual PDA plate, at 25°C for 2 days. Each experiment was repeated for 3 times.
[b]*F. sol*: *F. solani*; *F. ver*: *F. verticillioides*; *F. oxy*: *F. oxysporum*; *F. cul*: *F. culmorum; F. gra*: *F. graminearum*; *F. pro*: *F. proliferatum*; *R. sol*: *R. solani*.

2.01 g to 2.31 g higher than 1.47 g in un-treated ECs (Table 3). The growth rate of roots was not significantly different between ECs treated plants and un-treated plants (P>0.05). The biomass of fresh roots was 6.97, 8.9, 8.46, 7.18 and 7.05 g of plants treated with ECs of strains L1, L4, VN21, VN391 and VN99 respectively, while that of un-treated plant was 6.66 g (Table 3).

Effect of ECs on the growth of tomatoes: The ECs of strains L1, L4, VN21, VN391 and VN99 enhanced significantly fresh weight of trunk-leaves in tomatoes (p< 0.05). As a result, fresh weight of trunk-leaves of tomatoes was from 20.99 g by ECs of L1 to 24.49 g by ECs of L4 higher than 18.26 g of un-treated plants (p>0.05). Similar tendency was found in growth rate of roots, fresh weight of roots was from 5.72 to 8.17 g in treated tomatoes significantly higher than 5.56 g of un-treated tomatoes (p<0.001), therefore, dry weight of roots enhanced significantly from 0.33 to 0.51 g while that of un-treated plants was 0.31 g (p<0.01) (Table 4).

Table 3. Effect of ECs of antagonists on the growth of lettuces in a climate chamber.

ECs of strains	Biomass (g) of			
	fresh weight of trunk leaves	dry weight of trunk leaves	fresh weight of roots	dry weight of roots
L1	36.42^{ab*}	2.01^{ab}	6.97^{a}	0.37^{a}
L4	42.87^{a}	2.22^{a}	8.90^{a}	0.56^{a}
VN21	36.49^{ab}	2.31^{a}	8.46^{a}	0.46^{a}
VN391	34.30^{ab}	2.27^{a}	7.18^{a}	0.51^{a}
VN99	31.46^{b}	2.10^{ab}	7.05^{a}	0.40^{a}
Control	30.39^{b}	1.47^{b}	6.66^{a}	0.38^{a}
P	0.137	0.194	0.5676	0.69

*Means within the same column followed by the same letter are not significantly different at p<0.05 by using Duncan's Multiple Range Test.
Experimental lettuces were carried in a climate chamber at 20°C, under 16 h/8 h (Light/Dark) photoperiod.

Table 4. Effect of ECs of antagonists on the growth of tomatoes in a climate chamber.

ECs of strains	Biomass (g) of			
	fresh weight of trunk-leaves	dry weight of trunk-leaves	fresh weight of roots	dry weight of roots
L1	20.99^{ab*}	2.68^{ab}	7.9^{a}	0.5^{a}
L4	24.49^{a}	3.0^{a}	8.17^{a}	0.5^{a}
VN21	22^{ab}	2.7^{ab}	7.6^{a}	0.46^{ab}
VN391	23^{ab}	2.83^{ab}	5.4^{b}	0.33^{dc}
VN99	23^{ab}	2.7^{ab}	5.72^{b}	0.41^{bc}
Control	18.26^{b}	2.27^{b}	5.56^{b}	0.31^{d}
P	0.376	0.269	0.0003	0.0002

*Means within the same column followed by the same letter are not significantly different at p<0.05 by using Duncan's Multiple Range Test.
The experimental tomatoes were carried in a climate chamber at 25°C, under 16 h/8 h (Light/Dark) photoperiod.

Greenhouse experiments

Lettuces: After 7 days of *R. solani* inoculation, disease symptoms began to be found. ECs of almost antagonists except VN99 reduced different levels of *R. solani* root rot of lettuces (p<0.001). The ECs of strain L1, L4, VN21 and VN391 suppressed markedly *R. solani* root rot of lettuces. The reduction rate of severity disease was 75 and 66.6% by ECs of L1 and L4, respectively, while ECs of VN21 and VN391 reduced lower rate of 50% and 16.7% respectively. In plants un-treated with ECs, the rate of *R. solani* root rot- and bottom rot symptoms of lettuces was 100%. In consequence of this, *R. solani* caused significantly a reduction of biomass of fresh - and dry trunk-leaves within 3 weeks (Table 5).

The lettuce biomass was significantly increased by

Table 5. Effect of ECs of antagonists on the disease severity of *R. solani* bottom root- and biomass of lettuce in a greenhouse.

ECs of strain[a]	(%) reduction of disease symptom	Biomass of lettuce/plant		
		Fresh weight (g)	dry weight (g)	Number of leaf
L1	75.0^{a*}	47.21^{a}	2.0^{abc}	13.8^{a}
L4	66.6^{ab}	35.1^{ab}	1.7^{c}	13.4^{ab}
VN21	50.0^{ab}	37.9^{ab}	2.0^{abc}	13.5^{ab}
VN391	16.7^{dc}	34.1^{ab}	1.9^{abc}	13.2^{ab}
VN99	0.0^{d}	32.4^{ab}	1.8^{bc}	13.2^{ab}
Control 1[b]	0.0^{d}	32.4^{ab}	2.0^{abc}	13.9^{a}
Control 2[c]	0.0^{d}	16.2^{c}	1.1^{d}	10.4^{c}
P	<.0001	0.0065	0.0011	0.0036

*Means within the same column followed by the same letter are not significantly different at p<0.05 by using Duncan's Multiple Range Test.
Experimental lettuces were carried in a greenhouse at 20°C, 16 h/8 h (L/D) photoperiod.
[a]*R. solani* inoculated lettuces and then treated with ECs of antagonist.
[b]Lettuce without both *R. solani* and ECs of antagonists.
[c]Only *R. solani* inoculated lettuce.

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almost ECs as compared to that of pathogen control (only *R. solani* treated plant). In contrast, lettuces inoculated with *R. solani*, biomass decreased markedly compared with that in control ($p < 0.001$). Thus, the weight of fresh trunk-leaves ranging from 32.4 g to 47.2 g increased significantly compared with 16.2 g in un-treated plants ($p < 0.01$). Similar tendency, the leaf number (13.2~13.8) of each treated plant was found while 10.4 leaves in un-treated plant ($p < 0.01$) (Table 5). Interesting point was the highest weight of fresh trunk-leaves (47.2 g) and dry trunk-leaves (2 g) found in plants treated with ECs of strain L1 and VN21 (Table 5).

Tomatoes: The ECs of strain L1 and L4 reduced significantly *F. oxysporum* root rot- and disease symptoms of tomatoes ($p < 0.001$), and the reduction rate of severity *F. oxysporum* root rot differed significantly among ECs treated plants ($p < 0.001$). Almost ECs of antagonists except ECs of VN99 reduced markedly bottom rot symptoms. The ECs of strain L1 and L4 reduced 76.3% and 75% of *F. oxysporum* root rot of tomatoes, respectively, while reduction level of *F. oxysporum* root rot was 41.7% and 25% by ECs of strain VN21 and VN391, respectively (Table 6).

The biomass of tomatoes enhanced markedly in almost ECs treated plants compared with that of pathogen control (only *F. oxysporum* inoculated plants). The weight of fresh trunk-leaves was 52.7 g to 56.7 g higher than 39.3 g of pathogen control ($p < 0.05$). Moreover, the height (69.51 cm to 72.83 cm/each plant) and the number (10.33 to 10.7 leaves/each plant) increased markedly ($p < 0.05$) compared with that of pathogen control (9.73 leaves/each) (Table 6). Consequence of this, EC of strain L1 exhibited to be potential strains to reduce level of *R. solani* - and and *F. oxysporum* root rot in plants. Because the ECs of strain L1 provide to be the most effective bio-control agent against plant pathogenic fungi *in vivo* and *in vitro.* Therefore, EC of this strain could become a good candidate for developing commercial products against plant pathogenic fungi. For clarifying this point, this study investigated some critical characteristics of EC from strain L1.

Effect of various factors on the anti fungal activity of EC of strain L1

Temperature and pH: EC of strain L1 kept at different temperatures of 80, 90 and 100°C for 60 min caused the antifungal activity of ECs completely lost. However, EC kept at 50, 60 and 70°C, the residual antifungal activity retained 92.84 and 76%, respectively. Interesting point was that EC kept at 40 to 50°C, the antifungal activity retained 100% (Table 7).

The antifungal activity of strain L1 was not stable at low pH value. Thus, EC kept at pH value of 2 to 4 influenced markedly, as a result, the antifungal activity decreased significantly and retained 0.16 and 36% of its origin, respectively. But EC kept at pH 5 resulting antifungal activity retained 80% of its origin. Conversely, the antifungal activity of ECs was very stable at higher pH value of 6 to 10, after keeping ECs at these pH values, the antifungal activity

Table 6. Effect of ECs of antagonists on the disease severity of *F. oxysporum* bottom root- and biomass of tomatoes in a greenhouse.

ECs of strain[a]	(%) reduction of disease symptom	Biomass of tomato/plant			
		fresh weight (g)	dry weight (g)	height (cm)	number of leaves/plant
L1	76.3^{ab*}	56.7^{a}	4.9^{a}	69.51^{ab}	10.67^{a}
L4	75.0^{a}	50.2^{abc}	4.4^{ab}	72.33^{ab}	10.17^{ab}
VN21	41.7^{bc}	51.1^{abc}	4.4^{ab}	72.75^{ab}	9.67^{ab}
VN391	25.0^{dc}	53.6^{ab}	4.4^{ab}	71.13^{ab}	10.0^{ab}
VN99	0.0^{e}	52.7^{abc}	4.5^{ab}	72.83^{ab}	10.33
Control 1[b]	0.0^{e}	59.1^{a}	4.2^{ab}	68.67^{bc}	10.67^{a}
Control 2[c]	0.0^{e}	39.3^{c}	2.99^{c}	63.36^{c}	9.73^{ab}
P	<.0001	0.0263	.0117	0.0821	0.194

*Means within the same column followed by the same letter are not significantly different at $p < 0.05$ by using Duncan's Multiple Range Test.

Experimental tomatoes were carried out in a greenhouse at 25°C, 16 h/8 h (L/D) photoperiod.

[a]Tomatoes inoculated with *F. oxysporum* and then treated with ECs of antagonist.

[b]Tomatoes without both *F. oxysporum* and ECs of antagonists.

[c]Only *F. oxysporum* inoculated tomatoes.

Table 7. Antifungal activity of ECs of antagonists at different temperatures.

ECs at temperature (ºC)*	Clear zone (mm)	(%) reduction rate of mycelial growth
None treatment	18.0	100
40	18.0	100
50	16.5	92
60	15.2	84
70	13.7	76
80	0.0	0
90	0.0	0
100	0.0	0

*ECs of various antagonists at different temperatures of 40 to 100ºC for 60 min, then antifungal activity of ECs was determined on dual agar plate.

Table 8. Antifungal activity of ECs at different pH values.

ECs at pH value of*	Clear zone (mm)	(%) reduction rate of mycelial growth
None treatment	20	100
2	0	0
3	8	16
4	12	36
5	18	81
6	20	100
7	20	100
8	20	100
9	20	100
10	19	90

*ECs of various antagonists at different pH values for 60 min, then antifungal activity of ECs was determined on dual agar plate.

retained 100% (Table 8). Besides, EC of strain L1 showed to be thermostable at high temperature of 100ºC.

Protease-K and solvent: The antifungal activity in EC of strain L1 retained 100% after treatment with protease-K (Table 9). The results provided that antifungal activity in ECs of strain L1 was not negatively influenced by protease K. Besides, methanol at 70% did not affected fungal activity of ECs. Similar trend, antifungal compounds extracted by either chloroform or hexane inhibited 84.21% the growth of *F. oxysporum.* Methanol, petroleum ether affected slightly antifungal activity of EC, as a result, antifungal activity of EC retained 78.94 and 68.42%, respectively. Conversely, acetone affected negatively ECs, therefore, the antifungal

Table 9. Antifungal activity of ECs of antagonists at different concentrations of protease K.

Protease K (mg/ml) treated ECs	Clear zone (mm)	(%) reduction rate of mycelial growth
0	16	100
0.1	16	100
0.25	16	100
0.5	16	100

*ECs of various antagonists treated with different concentrations of protease K for 60 min, then antifungal activity of ECs was determined on dual agar plate.

Table 10. Antifungal activity of ECs of antagonists extracted by various solvents.

Solvents*	Clear zone (mm)	(%) reduction rate of mycelial growth
None	19	100
Chloroform	16	84.21
70% methanol	19	100
Methanol	15	78.94
Hexane	16	84.21
Petrolium ether	14	68.42
Acetone	11	57.89

*ECs of various antagonists were extracted by various solvents for 60 min, the solvent phase was taken and evaporated to dryness, then residual solids was dissolved in 70% methanol for determination of antifungal activity on dual agar plate.

activity retained 57.8% after treatment (Table 10).

Discussion

The ECs of almost antagonists inhibited the growth of pathogenic *Fusarium, R. solani*. *In vitro*, ECs of strain L1, L4, VN21 and VN391 inhibited markedly the growth of *R. solani* and *F. oxysporum*. The report of [19] showed that ECs of *B. subtilis* and *P. fluorescens* reduced 51 to 89% of growth of both *Fusarium* and *R. solani.* Similar finding was observed in this study, ECs of strain VN21, L1, and L4 inhibited 74.0, 74 and 77.64% of the growth of *R. solani,* respectively. Herein, we did not report process of extraction and purification of antifungal compounds from antagonists. However, some papers proved that genus of *Pseudomonas* sp. produced a wide range of antibiotic including phenazine [17], 2,4-diacetylphloroglucinol [23] while species of *B. subtilis* produced bacillomycin [15], and fengymcine [16], these compound exhibited to be less toxic to plants and protect plants from infecting pathogenic *Rhizoctonia* [16].

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Pseudomonas represent a large proportion of the total bacterial communities in the fields, so far, the antagonists belonging to genus *Pseudomonas* have been studied and used as bio-control agents [9].

The reduction level of *R. solani* root rot of lettuces was markedly different and depended on antagonists. Thus, the ECs of strain L1, L4, and VN21 reduced 75.0, 66.6 and 50.0% of *R. solani* root rot on lettuces, respectively. The reduction levels of *F. oxysporum* root rot of tomatoes was 76.3, 75.0 and 41.7% by ECs of strain L1, L4 and VN21, respectively. This finding was similar with that of report [6], *Bacillus* isolates 212, 83 and 179 reduced 12, 94 and 100% *Rhizoctonia* infection, respectively, while *Pseudomonas* isolates 144, 174 and 69 reduced 96, -20 and 69%, respectively [6].

In a green house, the biomass of *R. solani* inoculated lettuces and inoculated ECs of either strain L1 or L4 enhanced markedly of 29.13 and 21.67%, respectively, while biomass of in pathogen control (only *R. solani* inoculated lettuce) reduced markedly ($p < 0.001$). The reduction level of *R. solani* root rot of lettuces in ECs treatment was higher than that of pathogen control. The similar trend was found that biomass of tomatoes infected *Fusarium* and then treated with ECs of either strains L1 or L4 enhanced markedly of 14.42 and 12.7%, respectively. Besides, the reduction level of *Fusarium* root rot of tomatoes treated with ECs of L4 and L1 was 75.0 and 76.3%, respectively. In the report [7] showed that the ECs of *P. fluorescens* B1 reduced *R. solani* disease severity by 52% on potatoes while the disease suppression effect on potatoes was 37.33% and 31% by *P. fluorescens* B2 and *S. plymuthica* B4, respectively, whereas, tuber yield increased up to 12.6 to 17%. *Pseudomonas fluorescens* WCS417 showed to be effective against *Fusarium* wilt of banana [5], *P. fluorescens* strain from India showed a degree of protection against *F. oxysporum f.sp. cubense* on banana plantlets [24], *Pseudomonas* sp. reduced the severity of wilt of banana [18]. While *Bacillus* spp. reduced the incidence of *Fusarium* wilt of chickpea [11], cucumber [10], banana [18]. Our further objective is to combine ECs of several antagonists in a formulation to obtain high level of disease suppression effect and enhanced yield in some kinds of crops.

In conclusion, EC that of strain L1 was found to be the best agent which inhibited 74 and 75.88% of the growth of *R. solani* and *F. oxysporum,* respectively, *in vitro*. In greenhouse, EC of strain L1 reduced 75 and 76.3% of *R. solani* -, *F. oxysporum* root rot, respectively. Besides, EC of strain L1 enhanced markedly 29.13 and 14.42% biomass of lettuce and tomatoes, respectively. Therefore, ECs of strain L1 demonstrated to be a potential bio-control agent for next applications to suppress root rot of plants.

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