



# BIOLOGICAL CONTROL OF TOMATO DAMPING-OFF CAUSED BY *RHIZOCTONIA SOLANI* BY USING NATIVE ANTAGONISTIC BACTERIA

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## Abstract

The ability of antagonistic rhizobacteria that isolated from tomato rhizosphere soil from Mashhad, Iran to control tomato damping off disease was investigated. A total of twenty bacterial isolates were isolated and tested *in vitro* for their antagonistic activity against *Rhizoctonia solani* (*Thanatephorus cucumeris*) by using duel culture assay. The mycelial growth of pathogen was suppressed after placing *Rhizoctonia solani* and the isolates on the opposite side of the same petri dish. Cell wall degrading enzymes secreted by bacterial isolates were investigated to reveal their ability to degrade cell wall components of phytopathogens such as cellulase, protease and chitinase. Cellulase assay showed positive results in 16 bacterial isolates while 14 isolates of bacteria had protease positive result. Chitinase test gave positive result in 13 isolated bacteria. Among the isolates tested, six bacterial isolates choosing randomly were identified using universal primers and DNA sequencing as *Stenotrophomonas* sp. strain P7T6-4 (accession number MH665546.1), *Pseudomonas aeruginosa* strain P2R9-4 (accession number MH628051.1), *Pseudomonas aeruginosa* strain C2R4 (accession number MH628049.1) and *Pseudomonas aeruginosa* strain P1T30-9 (accession number MH628050.1), *Serratia* sp. strain E10-9 (accession number MH665545.1), and *Raoultella* sp. strain F9-1 (accession number MH665598.1). Our data revealed that antagonistic bacteria showed potential feasible alternative as ecofriendly biological control of *Rhizoctonia solani* damping off disease of tomato plants.

**Key words** : biological control, DNA sequencing, *Thanatephorus cucumeris*.

## Introduction

Tomato (*Lycopersicon esculentum* Mill.) is a member of *Solanaceae* with nutritional and economic values and potential health benefits as antioxidants (Assunta *et al.*, 2014). Tomato plants might be infected by several soil borne fungal pathogens that caused serious diseases leading to reduction in crop yield and quality (Saad 2006). Damping off is among the most important fungal diseases of tomato. This destructive disease can be caused by different pathogens which kill or make the seeds or seedling weak before or after germination (Grabowski, 2012). The contamination by *Rhizoctonia solani* Kühn (telemorph: *Thanatephorus cucumeris*) had been received much attention because of which is complex species of fungal pathogens infecting tomato worldwide. Application of chemical fungicides is the principle method used for controlling plant fungal diseases.

However, due to problems related to the environmental pollutant, fungicidal toxicity and development of resistant strains of pathogens lead to strict instructions and regulations of chemical fungicides usage needed (Jiang, 2014). The researchers try to focus their efforts for developing alternative biological agents to avoid the bad consequences of these chemicals (Baker, 1987). The aim of this research was to isolate and identify antagonistic bacteria and evaluate their effectiveness against *R. solani* destructive pathogenesis.

## Materials and method

### Isolation and purification of bacterial isolates

The bacterial isolates were obtained from soil rhizosphere from healthy tomato roots without any signs of infection, from different tomato farms in Mashhad, Iran. Tomato roots were cut well and washed three times by Distilled Water (DW). The suspensions were

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centrifuged in 100rpm to obtain bacterial isolates (Peng and Sutton 1991). Then, after serial dilution, 1 mL of each dilution was spread on King's B (KB) agar and incubated at 28°C for 4 days. Sterilized loop was used to transfer colonies to fresh Nutrient Agar based on color and morphological features to prepare pure cultures (Vidhyasekaran *et al.*, 1997). Bacterial suspensions were prepared by suspending 3 full transfer loops from bacterial culture in 5 ml of DW. Suspensions were adjusted to the desired concentration  $1 \times 10^6$  cfu/mL.

### Preparation of pathogen inoculum

An isolate of *Rhizoctonia solani* AG3 obtained from infected tomato plant and diagnosed previously based on morphological and molecular methods (Pourmandi and Taheri, 2014) was obtained from culture collection of Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran. Fungal culture was grown on Potato Dextrose Agar (PDA) at 25°C for 5 days.

### Inhibition of *R.solani* by isolated bacteria

A total of 150 isolates of bacteria were obtained from the soil rhizosphere of tomato roots and tested using a four point test to determine their inhibitory effect on growth of *R. solani*. Twenty bacterial isolates showed reduction in pathogen growth by producing inhibition zone *in vitro* assay.

### Dual culture

The 20 isolated bacteria were tested preliminary via *in vitro* methods to evaluate the antagonistic activity of bacterial isolates against *R. solani*. A loop of isolated bacterial was streaked on one side of Petri dish containing PDA medium agar by (1cm in from the edge), 5 mm mycelial disc from a 5 day old culture was placed at the distance of 5cm from bacteria. Then, the plates were incubated at 24°C and inhibition zone (mm) was recorded after 3 days of incubation by measuring the distance between edges of fungal mycelia and antagonistic bacterium according to the following equation:

$$L = [(C - T)/C] \times 100$$

Where, L is the inhibition percentage; C is the colony radius in control plate and T is radial growth of the pathogens in the presence of bacterial isolates. All *in vitro* antagonism assays were made in triplicate and repeated twice. The experimental design used was completely randomized (Vidhyasekaran *et al.*, 1997).

### Non-volatile compounds assay

Production of non-volatile antagonistic compounds was investigated by taking a full loop of bacteria that were inoculated in Nutrient broth (NB) then incubated in

a shaker at (120 rpm) for 72 h. at room temperature. After incubation, the cultures centrifuged at 5000 rpm for 15 min, then supernatant was taken for the assay. 1 mL of Culture filtrate was added to PDA medium and pH was adjusted at 6.8. Then, the medium was sterilized and poured in sterile Petri plates. After that, 4 cm diameter of *R. solani* from Potato Dextrose Agar plates (that incubated for 3 days) was placed in the center of this culture medium and incubated for 5 days at room temperature. Control Plates contained PDA medium and the pathogens only. Radial growth of fungal colony was measured at day three and five respectively and percentage of inhibition was calculated using the formula described by Jariwala *et al.*, (1991).

### Volatile compounds assay

The inhibitory effect of volatile compounds produced by isolated bacteria was assayed using the method described by Kazempour, (2004). Bacterial suspension (1 mL) contained  $2 \times 10^9$  cfu /mL was spread in the center of one half of King B agar. A disk of 5-mm<sup>2</sup> diameter of *R. solani* from '3 days culture was placed at center of another PDA Petri dish. Sandwich Petri dish was made by face up of isolated bacteria with a dish of the fungal pathogen face down then sealed by parafilm. Therefore, any physical contact between the fungus and bacteria was prevented. The petri dishes were incubated at 25±2°C for 5 days and the results were recorded by calculation of inhibition percentage of *R. solani* growth in presence of bacterial isolates.

### Hydrogen cyanide and siderophore production

The production of Hydrogen cyanide (HCN) was examined by bacterial strains streaked on KB agar plates that supplemented with glycine (4.4 g/L). Then Petri dishes were inverted and a piece of filter paper was saturated in picric acid solution (2.5g picric acid, 12.5 g Na<sub>2</sub>CO<sub>3</sub>, 1000 ml distilled water) and placed in the upper lid then sealed with parafilm and incubated at 28°C for 72 h. The change in color of filter paper to orange-brown or reddish brown was occurred according to the amount of HCN produced (Bakker & Schipper, 1987).

Siderophore production was assayed by using FeCl<sub>3</sub> with chrome azurol S agar (CAS) as described by Schwyn and Neiland (1987). The inoculum (10 µL) was taken from bacterial isolates and dropped on the center of CAS plates, then incubated at 25°C for 3 days. The production of siderophore was detected by the change of medium color from blue to orange.

### Enzymatic evaluation

Cellulase production qualitative assay for isolated bacteria was done on Carboxy Methyl Cellulose (CMC)

agar medium. Bacteria were cultured in LB medium for 24 h then 4 µL bacterial isolates were spotted on Carboxy Methyl Cellulose agar plate and incubated for 2 days at 30°C. After incubation period, the plates were treated first with 0.1% Congo red, and then it allowed to stand for 15 min. followed by 1M NaCl for 15 min. When cellulose degraded, a clear zone was formed around the colonies (Gomashe *et al.*, 2013).

Antagonistic bacteria were assayed on the basis of proteolytic activity by protease enzyme to produce nitrogenous compounds, which are soluble in nature. Antagonistic bacteria were cultured in Luria – Bertani (LB) medium for 24 h then 5 µL of bacterial suspensions were spotted on skim milk agar (Pancreatic digest of casein 5 g, skim milk solution 7% 100 ml, Glucose 1 g, Yeast extract 2.5 g, Agar 15 g, Distilled water 1000 ml). Clear zones formed around colonies were detected. (Anson 1938).

Chitinase assay was used to detect chitin degradation by bacterial chitinase enzyme. Bacterial isolates were cultured in LB media for 24 h and 5 µL of the bacteria were spotted on Colloidal Chitin Agar (CCA) at 25°C and the clear zone that resulted due to chitin hydrolysis was recorded after 7 days of incubation (Shanmugaiah *et al.*, 2008).

### Light microscopy

Interaction of the bacterial isolates and the fungal pathogen was studied using light microscope. In the dual plate assay, the samples were taken from the pathogen and antagonist interaction zone and observed under the light microscope (OLYMPUS cover-018, MODEL BX41TF, Japan).

### DNA extraction and PCR amplification

DNA extraction of bacteria was done by using DNA extraction kit (*Prime Prep* genomic extraction kit from tissue, GenNet Bio. Korea). The universal SSU primer set included forward primer uni-for (5'TGCCAGCAGCCGCGGTA 3'), reverse primer uni-rev (5' GACGGGCGGTGTGTACAA 3') were used for identification of bacteria (Giovannoni *et al* 1990). A reaction mixture containing approximately 2.5 µL of extracted DNA in this study and 12.5 µL master mix (Taq DNA polymerase, reaction buffer, MgCl<sub>2</sub>, dNTP, with nuclease free water), which were mix it with 8 µL distilled water and 1 µL of each forward and reverse primers. PCR products were separated in a 1% agarose gel in parallel with DNA ladder as a molecular size standard. After electrophoresis, the gel was visualized under UV light. The PCR products were sequenced by Macrogen Inc. (Korea), and then these sequences were aligned

with the sequences in BLAST database of NCBI site and record their accession number.

### Experimental design and data analysis

The experiments were performed using a complete randomized design and all data were obtained from biocontrol assays were analyzed by using t test spss statistical analysis. The phylogenetic analysis was done by phylogeny. fr site.

## Results

Twenty unknown bacterial isolates obtained from rhizosphere of tomato plants were evaluated for their ability to control *R. solani* the causal agent of tomato damping off disease.

### Direct antagonism assay

There is significant difference between bacterial isolates in the inhibition zones percent of dual culture, non-volatile and volatile assays respectively as shown in table 1.

The dual culture was assayed by using Potato Dextrose Agar medium. It was found that H12-2, E10-9, P1T27-14, C2R4, P2R1-2, P4T7-10 had the highest inhibition zone percentage in Dual culture assay while the lowest inhibition zone percent was 47% by E8-1. For non -volatile antifungal assay, E10-9 showed 60% as a highest inhibition zone percentage and the lowest inhibition zone was 15% by P.F bacterial isolate. Many bacterial isolates gave zero percent of inhibition by volatile compounds as in H12-2, P1T27-14, E4-9, P1R14-14, E8-1, D4-9 and E9-9 while highest inhibition zone percentage (28%) was by C2R4 as shown in fig. 1.

### Siderophore and Hydrogen Cyanide

It was found that only 6 bacterial isolates had positive results for both siderophore and hydrogen cyanide as a defense mechanism. Table 2 indicate the presence and absence of HCN and siderophore, also shown in Fig. 5, 6 respectively.

### Enzymatic evaluation

A group of hydrolytic enzymes were investigated with their ability of degrading the cell wall components of phytopathogens such as cellulase, protease and chitinase using different diagnostic media containing specific substrate for each enzyme. Cellulase assay showed positive results in 16 bacterial isolates while 14 isolates of bacteria had protease positive result. Chitinase test gave positive result in 13 isolated bacteria as shown in table 3.

### Microscopic analysis

In the area of inhibition zone which observed under

**Table 1:** Comparison between different bacterial isolates inhibition zones in dual culture, non-volatile and volatile assays respectively using t test.

Inhibition zone percent	mean±SE	P value
Dual culture	56.5±2.4*	0.001
Non volatile	48.7±2.1*	0.001
Volatile	10.75±2.5*	0.003

\*Significant difference when  $P < 0.05$ .

There is significant difference of inhibition zone percent between bacterial isolates in dual culture, no- volatile and volatile assays respectively.

**Table 2:** The results of sidrophore and HCN production.

Bacterial isolates	Sidrophore	Hydrogen cyanide
H12-2	–	–
P1T27-14	–	–
C2R4	+	++
P2R18-8	–	–
E4-9	–	–
P1T30-9	+	+++
PIR14-14	–	–
F3-8	–	–
P3R10-5	+	+++
P2R1-2	–	–
F9-1	–	–
A7-1	–	–
E10-9	–	–
E8-1	–	–
D4-9	–	–
P7T6-4	–	–
E9-9	–	–
P4T7-10	+	+
P2R9-4	+	+++
P.F	+	+

The color of positive result was ranging from faint yellow (+) to brown (++) until reddish dark brown (+++) according to HCN concentration while sidrophore positive result occur by iron chelating ability of these biocontrol bacteria.

microscope, considerable changes occurred in *R. solani* hyphal morphology when compared to fungal growth in the control plate. The hyphal structure became thinner, the heads appeared deformed and vacuolated with stunted growth and the branches were proliferated near the tips (Fig. 8).

#### Molecular identification of the bacterial isolates

To identify the antagonistic bacteria, DNA extracts were amplified using the universal primers. After sequencing the amplified fragments, they compared with available DNA sequences by BLAST and the bacterial isolates were diagnosed which all of them have a biocontrol effect. Phylogenic identification revealed the following

strains: *Stenotrophomonas* sp. strain P7T6-4 (accession number MH665546.1) and showed 100% similarity with the nucleotide sequences from Gene Bank corresponding to *Stenotrophomonas maltophilia* Mt5, source of isolation is rhizosphere. *P. aeruginosa* strain P2R9-4 (accession number MH628051.1) that showed 99% similarity to *Pseudomonas aeruginosa* CX17, source of isolation was soil. *P. aeruginosa* strain C2R4 (accession number MH628049.1) which showed 99% similarity to *P. aeruginosa* CR1, source of isolation was soil. *P. aeruginosa* strain P1T30-9 (accession number MH628050.1) showed 99% similarity to *P. aeruginosa* PBS29, source of isolation was soil. *Serratia* sp. strain E10-9 (accession number MH665545.1) showed 100% similarity to *Serratia merscens* S2I7, source of isolation is soil. *Raoultella* sp. strain F9-1 (accession number MH665598.1) showed 98% similarity to *Raoultella terrigena* strain 10<sub>1</sub>5 and source of isolation was soil and water as shown in fig. 9).

### Discussion

Biological control considered as a promising alternative way to reduce the use of chemical fungicides in controlling plant diseases worldwide and improve crop yield and quality (Heydari and Pessaraki 2010). The most important objective of this study was to isolate and identify bacteria and investigate their ability to control tomato damping off disease caused by *R. solani*. Most bacterial isolates were obtained from rhizospheric soil showed ability to suppress destructive pathogenic development.

*R. solani* AG3 was used in this study as a pathogenic agent on tomato. The AG3 of this pathogenic fungus has been divided into three genetically distinct subgroups: AG3-PT (potato type), AG3-TM (tomato type), and AG3-TB (tobacco type), based on sequence variation in ribosomal DNA (rDNA) and  $\beta$ -tubulin genes, culture appearance, fatty acid profile, and pathogenicity (Misawa and Kuninaga, 2010).

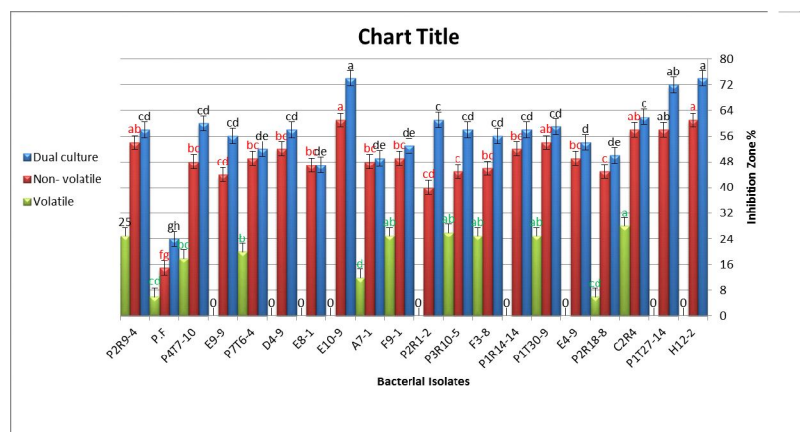
The experimental data presented in this research revealed that 20 bacterial isolates had the best antagonistic effect against *R. solani*. Inhibition zones produced in dual culture assay can be due to the ability of bacterial isolates to produce toxic or antifungal metabolites, antibiotics and sidrophores. The diameter of these inhibition zones might be due to the amounts and diffusion capacity of the inhibitory factors secreted by each antagonistic bacterium (Mounry *et al.*, 2016).

Non-volatile secondary metabolites have a high polarity which makes them water soluble and due to their effect in short distance, it gave a stronger bioactive control by toxins or antibiotics (Olaf *et al.*, 2017). Antagonistic

**Table 3:** The enzymatic assay results and EI ratio for bacterial isolates.

Bacterial Isolates	Cell wall degrading					
	Cellulase	EI	protease	EI	chitinase	EI
H12-2	+	0.8	+	1.2	+	1.2
P1T27-14	+	0.75	+	0.95	+	1.2
C2R4	+	0.85	+	0.4	+	0.66
P2R18-8	—	—	+	0.1	+	0.2
E4-9	+	1	—	—	+	0.5
P1T30-9	+	1.8	+	0.27	+	1.65
P1R14-14	+	0.5	—	—	—	—
F3-8	+	0.6	—	—	—	—
P3R10-5	+	1.5	+	0.24	+	1.8
P2R1-2	+	1	+	0.22	+	1.2
F9-1	—	—	+	0.26	—	—
A7-1	+	0.7	+	0.18	—	—
E10-9	+	1	+	1.1	+	1.7
E8-1	—	—	+	0.3	—	—
D4-9	+	0.5	—	—	—	—
P7T6-4	—	—	+	1	+	1.75
E9-9	+	0.8	—	—	+	0.45
P4T7-10	+	1.9	+	0.28	+	0.6
P2R9-4	+	1.8	+	0.35	+	1.5
P.F	+	0.25	—	—	—	—

It showed positive cellulase result in 16 bacterial isolates, 14 bacterial isolates gave positive protease results, while chitinolytic effects showed in 13 bacterial isolates, EI: Enzymatic Index=diameter of halo zone/ diameter of colony.



**Fig. 1:** Diagram of dual culture, non-volatile and volatile effect of bacterial isolates.

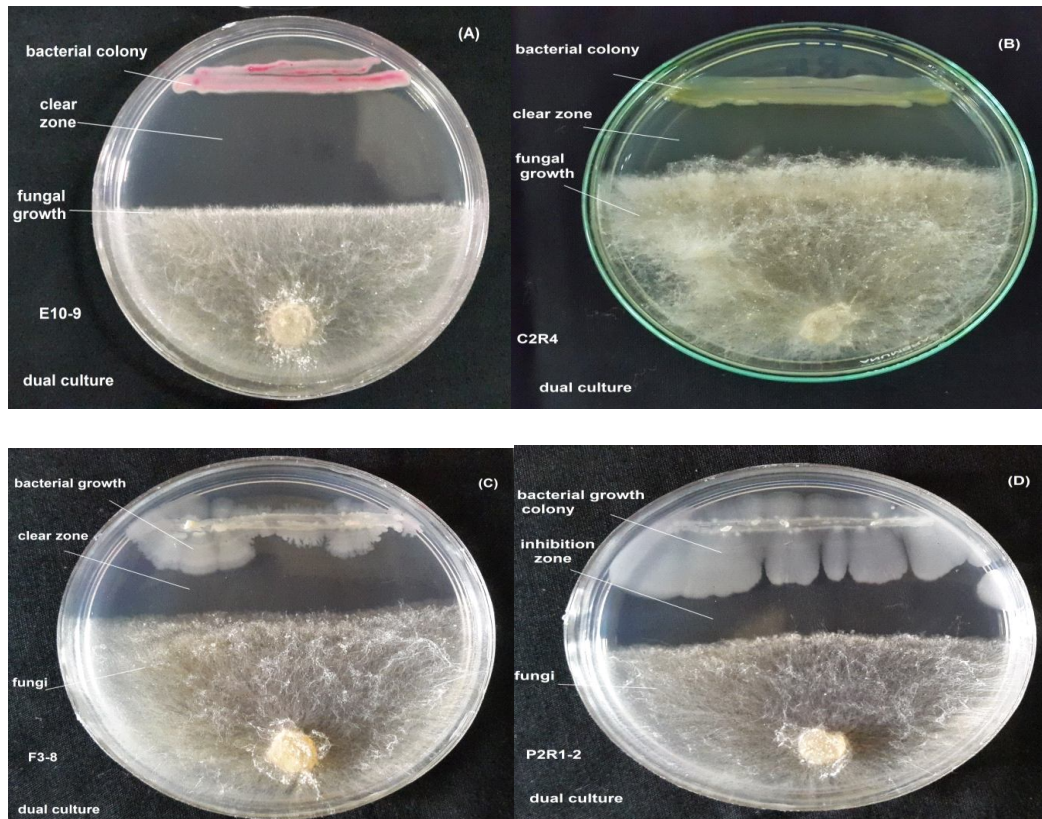
activity of nonvolatile metabolites produced by different bacterial isolates significantly inhibited the fungal growth. Volatile organic compounds were lipophilic components with vapor pressure derived from microbial metabolic pathways with low molecular weight, which can reduce fungal growth, impair hyphae, and/or promote the plant growth. Volatile metabolites could improve the interactions between physically separated microorganisms when compared with non-volatile metabolites which need physical contact to interact with other organisms. (Effmert *et al.*, 2012; Kai *et al.*, 2009; Weisskopf, 2013).

Ossowicki *et al.*, (2017) revealed that volatile compounds obtained from tomato rhizosphere isolate *Pseudomonas donghuensis* had potential antifungal activity related to the effect of volatile metabolites against *R. solani* fungal pathogen.

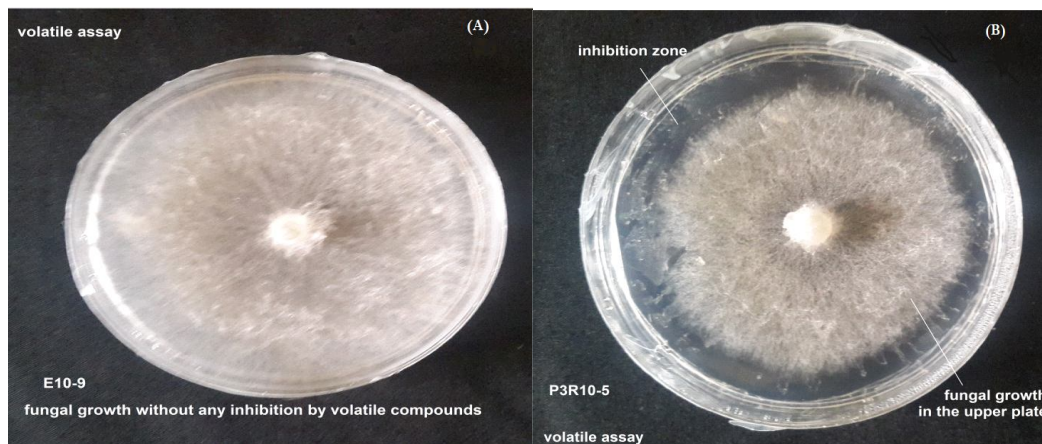
The secondary metabolite that produced commonly by bacteria is Hydrogen Cyanide, which is a gas in nature with potential mechanism as biological control. Difference between biocontrol activity and harmful strains was related to the consequence pattern of different HCN production on the surface of plant, inside the plant tissue or both (Tropentag and Hohenheim 2008; Tomaz and Ales 2016). HCN acts as a metal-complexing factor in the soil. It can form a complex either with the excess of microelements like iron, cobalt and others or sequester them from compounds like phosphates to form insoluble precipitate (Dunbar and Heintz, 1997). Physiological response for HCN production is induced by iron under the influence of quorum sensing. The excess amounts of iron in the rhizosphere, which form complex with phosphate cannot be handled by siderophore chelating mechanism and needs for irreversible complex formation by HCN production to observe the difference in plant growth (Pessi and Haas, 2000). Our study revealed that 6 bacterial isolates had positive results for HCN production with variation in color produced according to HCN amounts. Siderophore production is one of biocontrol mechanisms by producing complex with heavy metals, including iron. Rhizobacteria can produce siderophores with fluorescent color, which have the ability to chelate the available iron preventing its nutrition by pathogen. Bacteria producing siderophore can induce systemic resistance with suppressive effect to the soil that contributes in controlling many plant diseases (Sayed & Patel 2011). Siderophore assay in this study revealed that the bacteria which produced HCN showed the capability of producing siderophore. Therefore, these bacteria might be *Pseudomonas* species.

Cell wall degrading enzymes are able to degrade the structural components of fungal cell wall. Therefore, these enzymes secreted





**Fig. 2:** Dual culture assay (A, B, C, and D) showed examples of inhibition zone by dual culture assay using PDA medium. The bacteria growth in different phenotype appearance with different inhibition zone percent against *R. solani*.



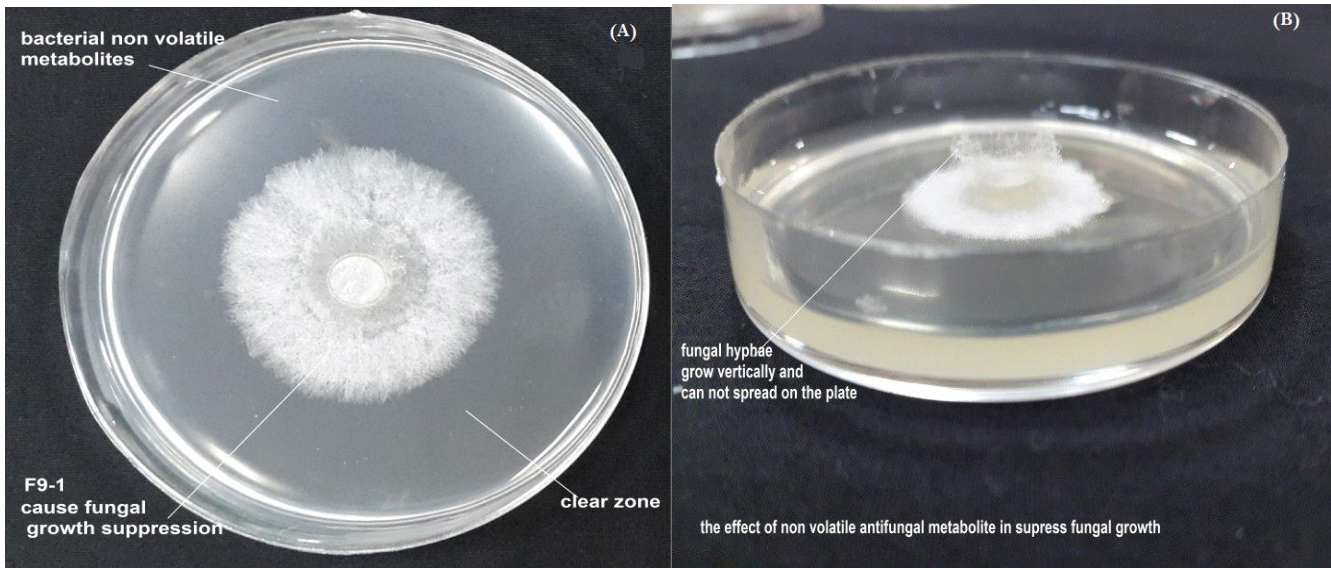
**Fig. 3:** Volatile compounds assay

(A) Showed fungal growth without any inhibition, (B) showed inhibition zone caused by volatile compounds

by bacteria can be considered as antifungal factors. Bacterial cellulase has a role in pathogen suppression through effects on the cellulosic cell walls of fungal hyphae causing fungal cell wall lysis (Jadhav & Sayyed 2016). Bacterial proteolytic effect was showed by hydrolyzing fungal cell wall because chitin and other constituents of the cell walls are embedded into the protein matrix. Proteases are able to inactivate extracellular enzymes of fungal pathogen (Christian *et al.*, 2014).

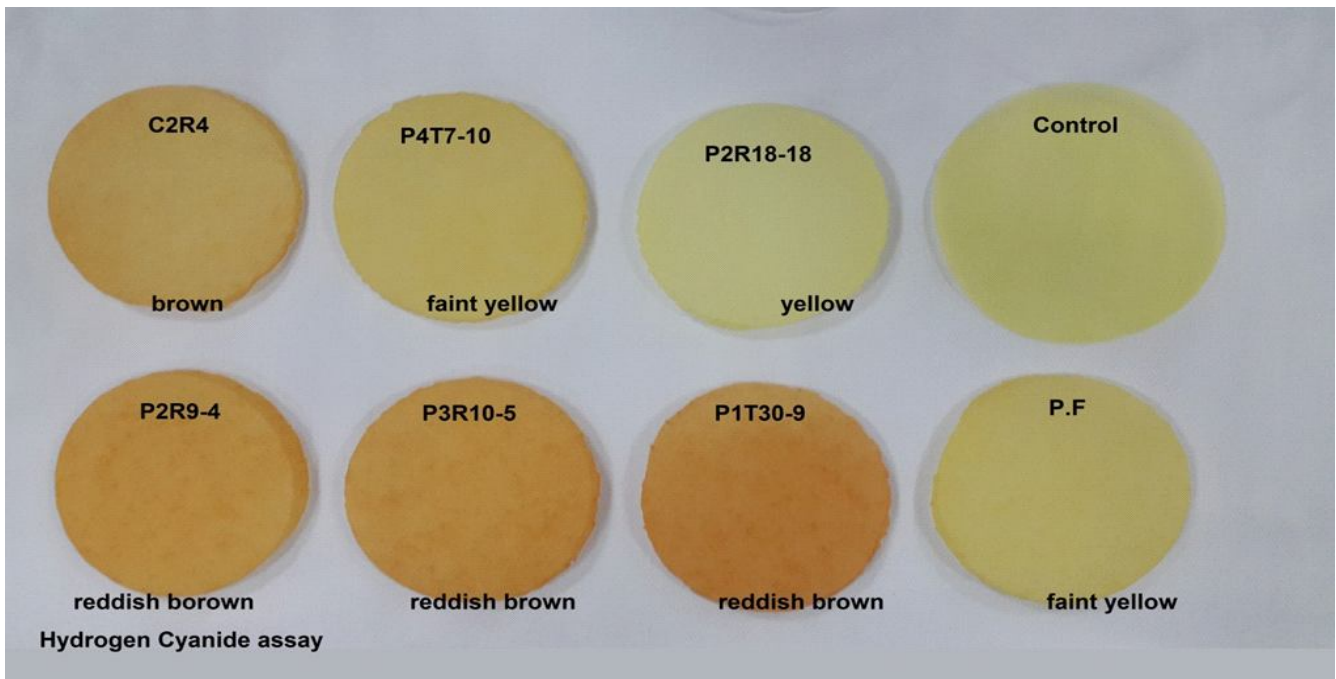
Chitinase enzyme secreted by some isolated bacteria was able to degrade chitin polymer in the cell wall of fungal pathogens. (Kim *et al.*, 2003). Our study showed that chitinolytic effects were found in 13 bacterial isolates. The higher enzymatic index for cellulase was by the isolate P4T7-10, protease higher enzymatic index was by the isolate H12-2, and higher chitinase effect by the isolate P3R10-5.

Results of molecular identification of the bacterial



**Fig. 4:** Non-volatile assay

It showed the inhibiting effect of non-volatile metabolites that extracted from bacteria and spread in the plate which suppress the growth and spread of fungi. These pictures are captured after the incubation periods that required for each assay.

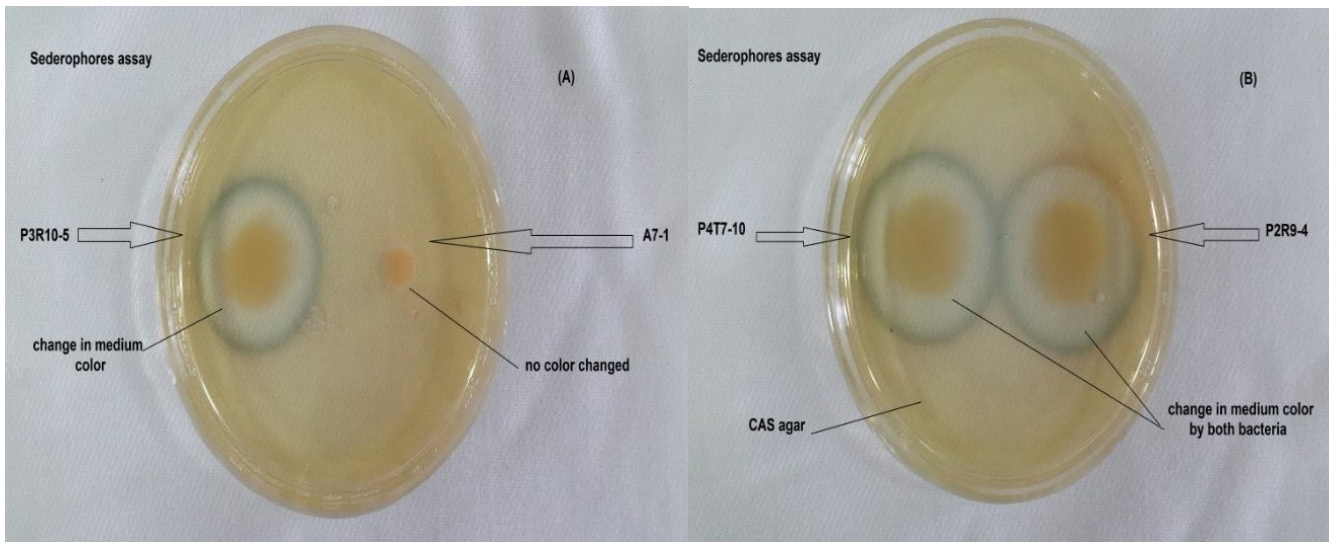


**Fig. 5:** Hydrogen cyanide production assay.

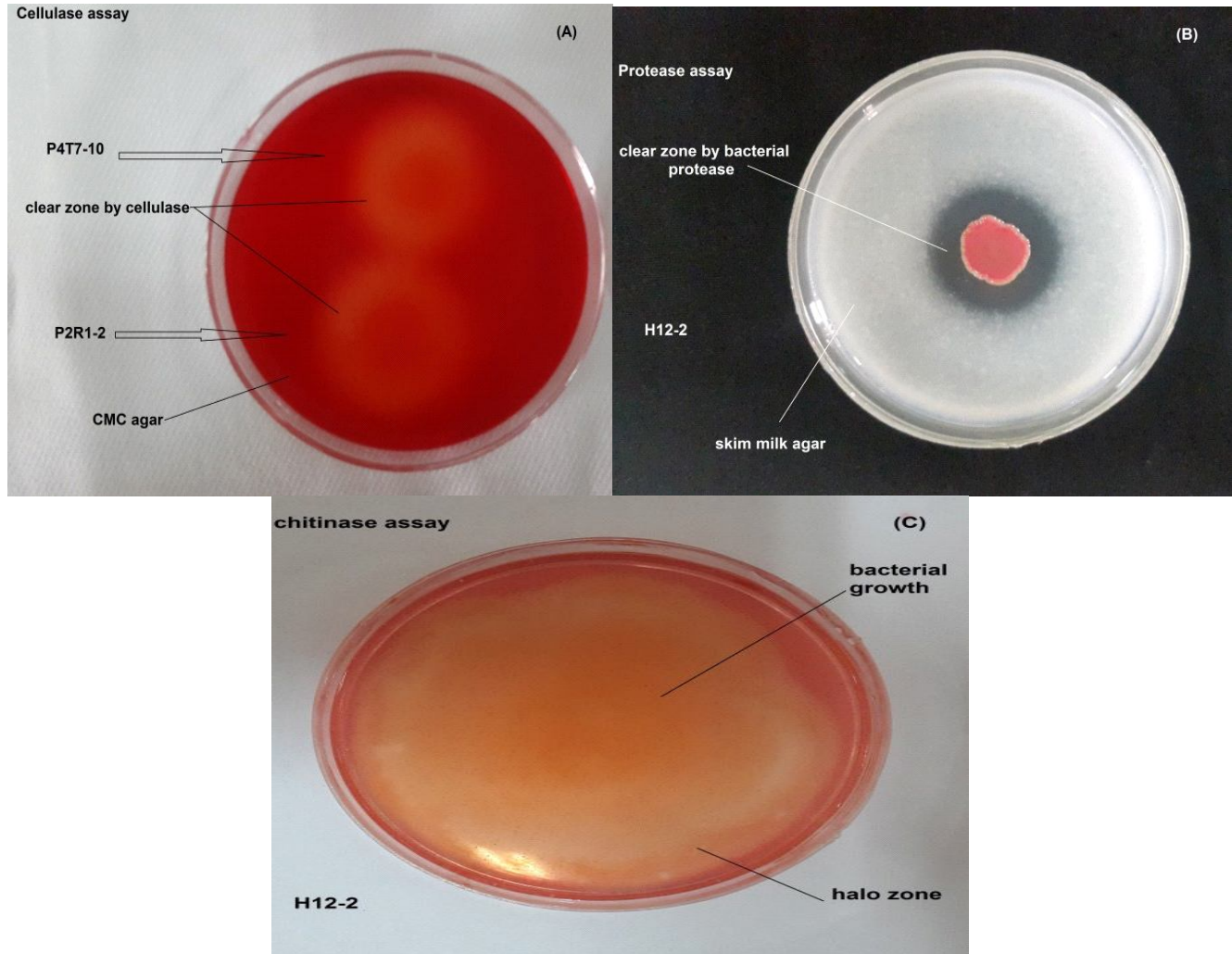
isolates showed that the isolate E10-9 was *Serratia merscens* which many studies approved its biocontrol effect against plants pathogens. Induction of systemic resistance is a mechanism involved in the biological control of root pathogens by *Serratia* species (Owen *et al.*, 2018). Another study showed the effective biocontrol capacity of *Serratia merscens* to control fungal pathogens especially *R. solani* (Haydari & Pissaraklli 2010) which is in accordance with our findings.

The isolates P2R9-4, C2R4 and P1T30-9 were identified as *Pseudomonas aeruginosa*. Biocontrol mechanisms of these bacteria were approved by many studies. Protective effect of *P. aeruginosa* showed on Chilli plant in china (Bakthavatchalu and Shivakumar, 2016). A study of Minaxa and Saxena (2010) revealed abnormal morphology of pathogenic fungi caused by *P. aeruginosa* such as perforation, swelling, fragmentation and lysis of hyphae. These researchers also reported production of siderophore and HCN by this biocontrol





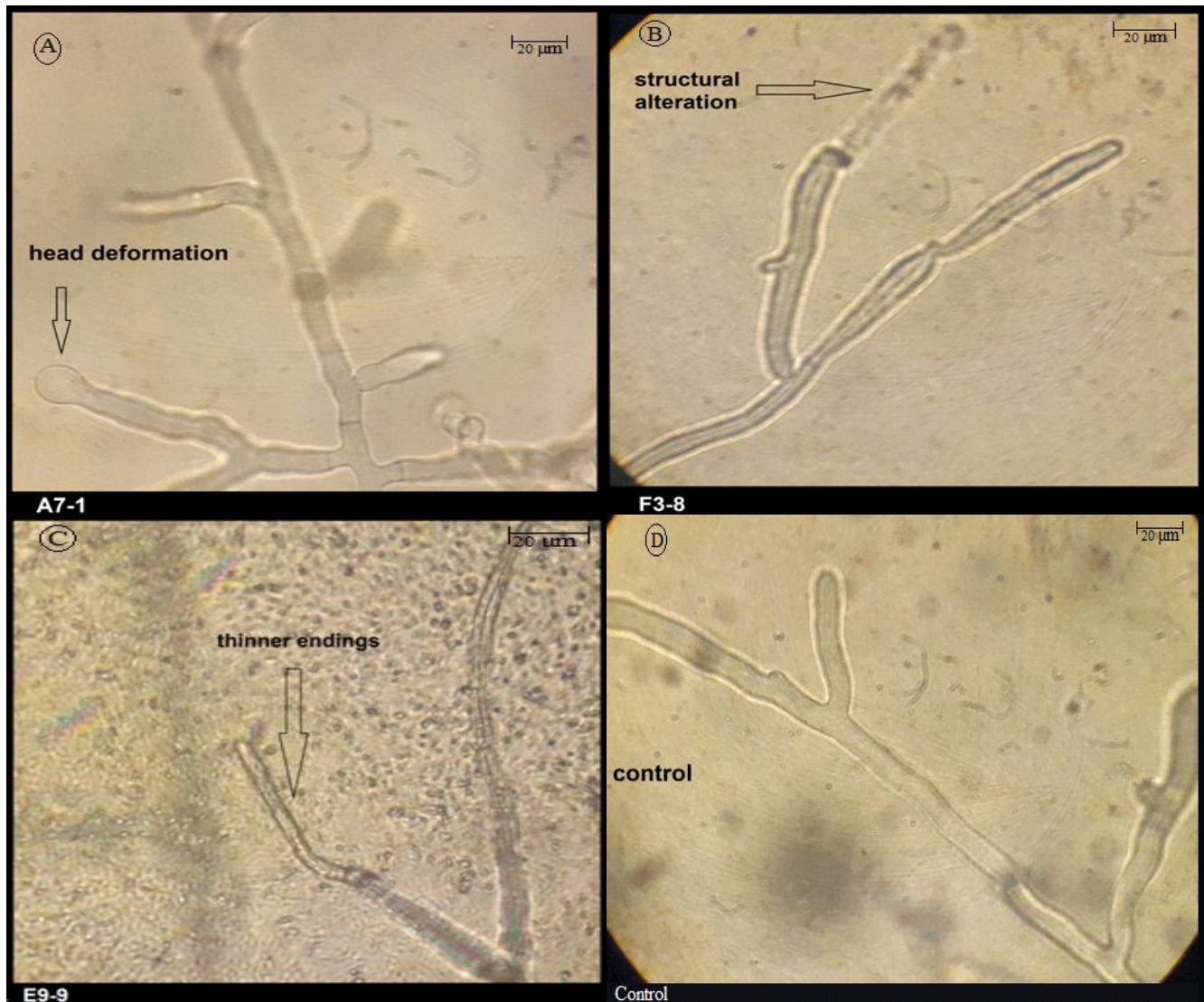
**Fig. 6:** Siderophore production assay.



**Fig. 7:** Cellulase, protease and chitinase assays

(A) The positive result of cellulolytic enzyme assay as clear zone surrounding the colony in CMC media. (B) Showed the halo zone of proteolytic enzymes by bacterial isolates in skim milk agar while (C) showed the chitinolytic effect of bacterial isolate in CCA medium.





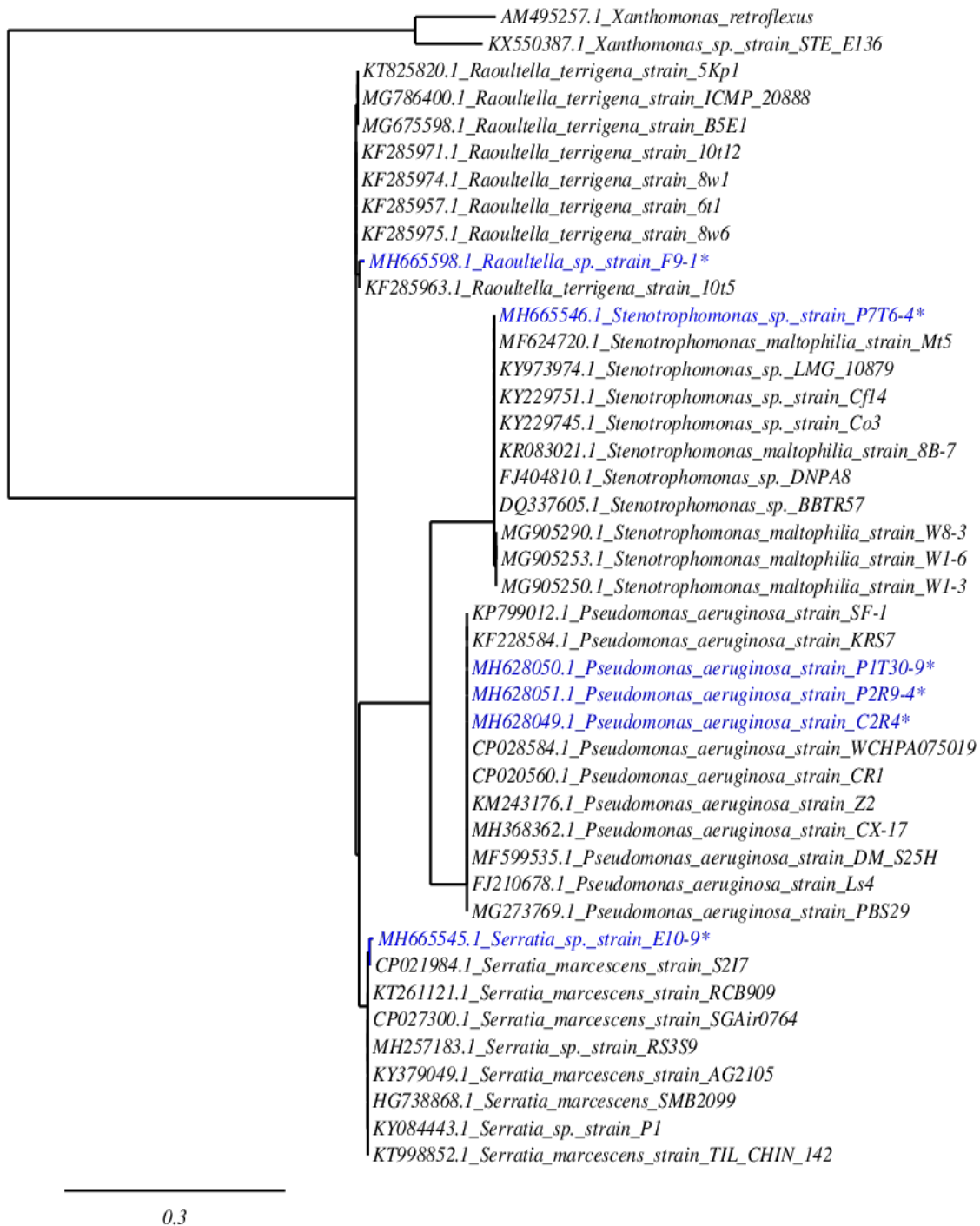
**Fig. 8:** Microscopic examination of *Rhizoctonia solani* hyphal changes due to bacterial biocontrol.

It showed morphologic changes of fungal hyphae due to bacterial biocontrol under microscope when (A) show head vacuolation and deformation, (B) show thinning hyphal endings. (C) Indicate cellular hyphae degradation and structural alteration. (D) Control *Rhizoctonia solani* under 100XP light microscope.

agent. *P. aeruginosa* strains were able to colonize the rhizosphere of plants and reduce fungal disease (Bushra *et al.*, 2018). These bacterial species released HCN as antifungal volatile metabolites.

‘The isolate P7T6-4 was identified as *Stenotrophomonas* sp. strain P7T6-4 with biocontrol effect. It was previously reported in an Egyptian study, isolated from rhizosphere of eggplant, with antagonistic potential effect against *Ralstonia solanacearum* the causal agent of potato brown rot (Messiha *et al.*, 2007). *S. maltophilia* produced cell-wall degrading enzymes such as protease, pectinase and chitinase in addition to other antifungal metabolites (Ayyadurai *et al.*, 2007) Another Study showed that *Klebsiella*, *Stenotrophomonas*, *Rhizobium*, and *Citrobacter* strains

had low siderophore production. Costa *et al.*, 2014 agreed with our study that showed no siderophore production by *Stenotrophomonas* sp. Raoultella terrigena (G-584), *Bacillus amyloliquefaciens* (G-V1) and *Pseudomonas fluorescens* (2R1-7) were found with the highest inhibitory effect on the mycelial growth of both *Phytophthora* spp. For the management of both fungal diseases the antagonistic bacteria were further evaluated under greenhouse and field conditions Raoultella terrigena (G-584), *Bacillus amyloliquefaciens* (G-V1) and *Pseudomonas fluorescens* (2R1-7) were found with the highest inhibitory effect on the mycelial growth of both *Phytophthora* spp. For the management of both fungal diseases the antagonistic bacteria were further evaluated under greenhouse and field conditions Raoultella terrigena



**Fig. 9:** The phylogenetic tree of the selected five bacterial isolates

It recorded in NCBI site with their accession number. The phylogenetic analysis was done by phylogeny. fr site.

(G-584), *Bacillus amyloliquefaciens* (G-V1) and *Pseudomonas fluorescens* (2R1-7) were found with the highest inhibitory effect on the mycelial growth of both *Phytophthora* spp. For the management of both fungal diseases the antagonistic bacteria were further evaluated under greenhouse and field conditions

Bacterial isolate F9-1 was identified as *Raoultella* sp. strain F9-1 which was closely related to *Klביםiella*

spp. *R. terrigena* is considered an environmental species as it has been isolated from soil and water (Farmer *et al.*, 1985). Our study revealed an antagonistic effect of this bacterial isolate against fungal pathogen. German study showed that *Raoultella terrigena* found with higher antagonistic effect and inhibit the mycelial growth of *Phytophthora* fungal pathogen causing reduction in red core and crown rot disease of strawberry in both

greenhouse and field condition (Anandhakumar and W. Zeller 2008). Many studies provide evidence for the occurrence of opportunistic human pathogens such as *P. auroginosa* in the rhizosphere with very little information on their virulence relative to the clinical trials is available. Therefore, the risk assessment for each strain is necessary for strains that could be analysed for the occurrence of specific virulence determinants (Gabriele *et al.*, 2005).

This research is an attempt to achieve possible application of isolated bacteria to protect tomato against damping off disease. It is necessary to evaluate culture conditions for commercial production of these bacterial isolates to control *R. solani* damping off. Future studies seem to be necessary for evaluating antifungal effect of these bacteria against *R. solani* and determining the possible biocontrol mechanisms.

### Conclusion

1. We can conclude that all unknown isolated rhizobacteria had *in vitro* biocontrol effects against *Rhizoctonia solani* causing tomato damping off disease.
2. The antagonistic bacteria inhibit the fungal pathogen by antibiosis (volatile and non volatile) or enzyme production, siderophore formation or combination of these mechanisms.
3. DNA sequencing revealed six bacterial strains which had safe biological control effect. P7T6-4 bacterial isolate recorded in NCBI site as *Stenotrophomonas* sp strain P7T6-4 sequence ID: MH665546.1, while P2R9-4 recorded as *P. aeruginosa* strain P2R9-4 sequence ID: MH628051.1. The isolate C2R4 recorded as *P. aeruginosa* strain C2R4 sequence ID: MH628049.1. The isolate P1T30-9 recorded as *P. aeruginosa* strain P1T30-9 with sequence ID: MH628050.1 and for E10-9 isolated bacteria it was identified as *Serratia* sp. strain E10-9 sequence ID: MH665545.1. The isolate F9-1 was identified as *Raoultella* sp. strain F9-1 with sequence ID: MH665598.1.
4. *Serratia* sp. strain E10-9 was showed the highest inhibition zone among bacterial isolates in dual culture assay with high non-volatile antagonistic compounds production. It secreted fungal cell wall degrading enzymes.
5. *Stenotrophomonas* sp. strain P7T6-4 showed inhibitory effects by volatile and non volatile antagonistic compounds and hydrolytic enzymes effect.
6. The three identified *P. aeruginosa* isolated strains showed volatile and non volatile metabolites. They characterized by producing siderophore and HCN biocontrol agents. *Raoultella* sp. strain had volatile and non volatile antagonistic effects.

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### Compliance and ethical standards

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

### Conflict of interest

All authors declare that they have no conflict of interest.

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