Antagonistic Activity and Characterization of Indigenous Soil Isolates of Bacteria and Fungi Against Onion Wilt Incited by *Fusarium* sp

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Abstract

Tuber rot disease due to phytopathogen *Fusarium oxysporum* f. sp. *cepae* (*Foc*) infection is one of the main factors causing the decreasing amount of global shallot production. This study aims to find bacteria and fungi candidates which have *Foc* antagonistic activity through *in vitro* tests using dual culture techniques. A total of five bacterial isolates and three fungal isolates isolated from the rhizosphere of healthy onion plants showed the ability to inhibit *Foc* growth. B1 and B4 bacterial isolates had an average inhibitory capability of 65.93% and 72.27% respectively. Whereas C1 and C2 fungal isolates have the ability to inhibit the growth of *Foc* by as much as 74.82% and 67.76% respectively. The four tested microbial isolates were able to significantly inhibit *Foc* activity *in vitro* based on the ANOVA test, with values $\alpha = 0.05$, and $n = 3$. Molecular analysis based on 16S-rRNA markers showed bacterial isolates B1 and B4 have an evolutionary relationship with *B. subtilis*. Whereas fungi C1 and C2 have evolutionary relationships with *Aspergillus tubingensis* and *Trichoderma asperellum* respectively, based on internal transcribed spacer (ITS) gene markers. The results of this study can be used to develop indigenous microbial consortiums as biological control agents for phytopathogenic fungi *Fusarium oxysporum* f. sp. *cepae* (*Foc*) on shallots.

1. Introduction

Shallots (*Allium cepa* var *ascalonicum* L) are one of the world's main commodities with production reaching 96.77 million tons per year. However, productivity fluctuates almost every year. In Indonesia, several regions show fluctuations in the amount of production each year (BPS 2018). Various factors, especially unfavorable environments such as drought, salinity, climate, nutritional imbalance and plant diseases, are the main obstacles in the production of shallots (Abdelrahman et al., 2016). Among a number of diseases caused by pathogens, *Fusarium* tuber rot or wilt disease caused by *Fusarium oxysporum* f.sp. *cepae* (*Foc*) is the most damaging and a serious threat to shallot production worldwide (Abdelrahman et al., 2016; Chand et al., 2017; Kalman et al., 2020). Symptoms caused by *Foc* include plants wilting rapidly, newly formed leaves curling and turning yellow, plants almost collapsing, white fungi colonies appearing at the base of the rotting layered bulb (Brayford 1996; Taylor et al., 2016). *Foc* is a pathogenic fungus that can infect a very wide range of plants as the hosts (Summerell et al., 2011; Armitage et al., 2018). This fungus can form chlamydospores so that it can last a long time in the soil (Brayford 1996; Cremer 2000; Kalman et al., 2020).

Management of this disease can be focused on integrating different prevention methods, including the use of mixed crops, crop rotation systems, use of pathogen-resistant cultivars, use of chemical fungicides and the use of biological agents (Mc Govern 2015; Gupta et al., 2020). In practice, the use of synthetic fungicides by shallot farmers has not been fully effective because of the residue left on crops, environmental pollution, and killing other organisms that are not targeted. Moreover, the continuous use of synthetic fungicides can lead to the emergence of resistant pathogenic populations (Mehnaz et al., 2013; Fournier et al., 2020; Tleuova et al., 2020). Biological control using microbes that are antagonistic

to pathogenic fungi is the right alternative because it does not have a negative impact on the environment (Lecomte et al., 2016; Jamil et al., 2020; Kalman et al., 2020).

Utilization of microbes as biological control agents ideally uses the potential of indigenous natural enemies with the hope that these microbes will work more effectively and are supported by appropriate environmental factors, do not cause changes in ecosystems, and are cheaper to formulate (Kalman et al., 2020). Therefore, the diversity of microbes from the root area and their propagation followed by their release back into the rhizosphere is a conservation measure that will provide promising prospects for biological disease control (Raaijmakers et al., 2009; Kandel et al., 2017.).

In the last decade, research on biocontrol and microbial metabolite products for pest and pathogen control has intensified (Jangir et al. 2018). Generally, this microbial group belongs to the genera *Bacillus*, *Pseudomonas*, *Streptomyces* and *Trichoderma* (Ramyabharathi et al., 2020; Jangir et al., 2018; Islam *et al.*, 2018; Kalman et al., 2020). This group of microbes is able to act as a biocontrol agent in reducing pathogenicity through a number of mechanisms such as antibiotic production, root colonization, induction of systemic resistance systems in the host, production of extracellular cell wall breakdown enzymes and formation of resistant spores (Ongena and Jacques 2008; Beneduzi et al., 2012). A number of studies have reported that the application of microbes, both bacteria and fungus, is effective in suppressing the growth of *Fusarium* pathogens, including using *Pseudomonas aeruginosa* (Islam et al., 2018), *Bacillus* sp. (Jangir et al., 2018), *Pseudomonas aeruginosa* DRB1 and *Trichoderma harzianum* CBF2 antagonist *Foc* Tropical Race 4 (Foc-TR4) (Wong et al., 2019), and *Trichoderma harzianum* (Jamil et al., 2020).

This study aims to evaluate the antagonistic activity of indigenous microbial strains isolated from shallot growing areas in Enrekang Regency, South Sulawesi, Indonesia. *In vitro* analysis was conducted using *Fusarium* isolates which were isolated from shallot plants showing symptoms of tuber rot. All isolates that showed potential in inhibiting the growth of the *Foc* pathogen were identified molecularly using specific primers for the 16S rRNA gene and the nuclear ribosomal internal transcribed spacer (ITS) region using specific primers ITS1 and ITS4. The isolates obtained are expected to be able to contribute to the inventory of genetic diversity in the region, with possible future applications for the control of *Fusarium* pathogens in plants, especially in shallots.

2. Materials And Methods

2.1 Isolation of *Foc* Fungi

Foc fungi were isolated from shallot rhizosphere soil samples which showed tuber rot symptoms in the shallot cultivation area in Enrekang regency. The isolation was carried out based on techniques described in Miao et al. (2016) by using potato dextrose agar medium (PDA, Merck) and incubated for 5 to 7 days at 25 ± 2°C. Isolates were determined based on their microscopic morphological characteristics.

Microscopic observation using the fungal slide culture method was used to observe the hyphae growth under a microscope (Harris 1986).

2.2 *Fusarium*-Antagonist Bacterial and Fungal Isolations

Foc-antagonist bacteria and fungi were both isolated from healthy rhizosphere areas of shallot plants by the serial dilution method. The rhizosphere bacteria isolation technique is based on Jangir et al. (2018) with modifications. The dilution results were grown in Nutrient Agar (Merck) medium at 30°C for 48 hours. Whereas the fungal isolation technique is based on Miao et al. (2016) by growing the results of 10^{-3} dilution in PDA medium at 25 ± 2°C for 5–7 days. Next, the bacterial and fungal isolates were purified in the same medium and maintained at 4°C. Further preservation used glycerol stock (25%) and was stored at a temperature of -80^{o}C. All the isolates which were successfully identified were characterized based on morphological and biochemical parameters.

2.3 In *vitro* Tests of *Fusarium* Antagonist Isolates

Foc-antagonist microbes screening was conducted using the dual culture method (Skidmore and Dickinson 1976). A culture block with a diameter of 8 mm from antagonist isolate and another from *Foc* isolate were placed opposite each other in a PDA medium, 3 cm away from the edge of the petri dish. As a control a single *Foc* culture disc was placed alone in another petri dish without the antagonist isolate. The petri dish was then incubated at a temperature of 25 ± 2°C for 5–7 days. Observation of the inhibition zone (growth inhibition, GI) was done every two days. Observation was terminated when the colony in the control reached maximum growth. The percentage of antagonist inhibition (G)I was calculated using the formula:

GI = [(R1-R2)/R1] x 100%

In which, R1 is the radius of radial growth to the opposite direction in the control petri dish and R2 is the radius of radial growth in the treated petri dish. The tests were done three times to acquire the mean of the inhibition zone for each isolate. The GI data were analyzed using one-way ANOVA with values α = 0.05 and n = 3.

2.4 DNA Extraction and PCR Amplification

Isolation of fungal genomic DNA was carried out using the Plant Genomic DNA Mini Kit (Geneaid) in accordance to the manufacturer's standard protocol. The nuclear ribosomal internal transcribed spacer (ITS) region was amplified using a universal primer set (ITS 1: 5’-TCC GTA GGT GAA CCT GCG G- 3’ and ITS 4: 5’-TCC TCC GCT TAT TGA TAT GC-3’) (White et al. 1990). The PCR reaction consisted of 1 µl DNA template (100 ng/µl), 5 µl NZYTaq II 2X Green Master Mix, 0.25 µl ITS 1 primer (10 pmol/ µl), 0.25 µlITS 4 primer (10 pmol/ µl), 3.5 µl dH_2O so that the total reagent volume was 10 µl. PCR was run with a thermal cycler for pre-denaturation at 95°C for 5 minutes, for denaturation at 95°C for 30 seconds, for annealing at 52°C for 30 seconds, for extension at 72°C for 30 seconds, the reaction being repeated for 35 cycles, and post-PCR at 72°C for 5 minutes.

The total bacterial genome was isolated using Presto™ Mini gDNA Kit (Geneaid). according to the manufacturer's protocol. The 16S-rRNA gene amplification was performed using specific primer pairs (63 F: 5’ -CAG GCC TAA CAC ATG CAA GTC- 3’ and 1387 R: 5’ -GGG CGG WTG GTA CAA GGC- 3’). The mix composition and PCR program were made the same as the ITS gene amplification procedure in fungi. PCR products were analyzed using 1% agarose gel in 1x TAE buffer. The gel was then electrophoresed at a voltage of 100 V for 28 minutes and stained using ethidium bromide staining. The visualization of the electrophoresis results was carried out using a UV-Transilluminator. PT Bioneer Indonesia conducted the PCR product sequencing.

2.5 Construction of Phylogenetic Trees

The 16S and ITS sequences for all bacteria and fungi were constructed to determine their evolutionary relationships based on phylogenetic analysis. The sequences are aligned using data from the GenBank database (https://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were performed using Bio Edit's CLUSTAL W program. Phylogenetic tree construction was carried out using the neighbor-joining method from the MEGA version 10.0 program. Each clade obtained was then determined using bootstrap analysis with 1000 replications and then Kimura's two-parameter model was used.

3. Results

3.1 Isolation and Identification of Fungal Pathogens

The isolates suspected as *Fusarium* were isolated from the rhizosphere of the shallot plants which showed tuber rot symptoms. Observation of the morphology of fungal isolates was based on the characteristics described which include parameters of color, colony, texture and air hyphae. All parameters showed characteristics matching *Fusarium oxysporum* f.sp. *cepae* (*Foc*). Furthermore, the observations showed that on the upper surface, the mycelium was purple, while the lower surface was white. In addition, microscopic characteristics such as macroconidia, microconidia and chlamydospores were successfully observed under a microscope at magnification of 100x (Fig. 1) with the appearance of a colorless round microconidium, and a crescent-shaped macroconidium that was colorless and had 3–5 septums while chlamydospores are single-celled.

3.2 *In vitro* tests of Antagonist Microbes vs. *Foc* Fungi

A total of 8 fungi isolates and 20 bacterial isolates were isolated from rhizosphere soil samples. From the results of initial *in vitro* testing against *Foc*, three isolates of fungi and five isolates of bacteria showed inhibitory activity reaching 50% against *Foc* mycelium growth. In the second test, the percentage of inhibitory potential was measured against the growth of *Foc* grown in dual culture with the test isolate. Tests were carried out three times to determine the average inhibition. From the results of analysis of variance (ANOVA) on all isolates, it was found that almost all tested microbes had inhibitory activity above 50%. Isolates B4 and B1 showed the highest inhibitory activity of the five bacterial isolates with an average of 72.27% and 65.3%, respectively. Whereas the inhibitory activity of fungi against *Foc* was

discovered to have a higher growth rate than the bacterial activity. The lowest inhibitory activity was shown by the C3 fungal isolate with an inhibition value of 41.26%. All data are presented in Table 1.

Table 1
Percentage of inhibition of *Foc* growth by bacteria and fungi from the rhizosphere of shallot plants on PDA medium

Isolate Code	Inhibition (%)				
	Day 1	Day 3	Day 5	Day 7	Mean
B1	54.84	65.39	70.61	72.87	65.93[b]
B2	49.34	59.67	61.04	65.38	58.86[c]
B3	48.32	50.3	60.6	65.39	56.15[c]
B4	68.94	67.36	73.28	79.51	72,27[a]
B5	49.3	51.7	58.58	65.53	56.28[c]
C1	68.35	73.77	77.65	79.51	74.82[a]
C2	62.12	65.32	70.79	72.79	67.76[b]
C3	25.6	41	46.81	51.08	41.12[d]

Numbers followed by the same notation do not show a significant difference based on analysis of variance with values α = 0.05 and n = 3.

The capability of bacteria and fungi to inhibit *Foc* growth seems to be correlated with different growth rates. Visually, the growth of fungi in colonizing the growth medium was seen to be faster than bacteria (Fig. 2). From all isolates, B1 and B4 bacteria can be considered to have the best potential as antagonists in suppressing *Foc* growth *in vitro*.

Further analysis was carried out to determine the capability of the isolates to suppress the growth of *Foc* mycelium. Microscopic observations were carried out on the outer part of the *Foc* mycelium growth zone. From the observations, it was found that hyphae damage occurred which is assumed to have been due to the activity of the antifungal compounds produced. In contrast to the control, hyphae in *Foc* were seen to undergo fragmentation (Fig. 3).

3.3 Molecular Identification of Bacterial and Fungal Isolates

The five bacterial isolates were analyzed molecularly to identify species based on their evolutionary relationships. The phylogenetic tree construction from the alignment results of 16S gene amplification products with the GenBank database showed that all *Foc* antagonist bacteria were related to the genus *Bacillus* and all of them belong to different species evolutionarily (Fig. 4). There are four isolates

belonging to the species *B. subtilis*. Isolates B1 and B3 are closely related to species *B. subtilis* strain X2 with a gene similarity rate of 94% for the two isolates. The B2 and B4 isolates were closely related to *B. subtilis* strain S11. Meanwhile, isolate B5 showed a closer relationship with species *B. cereus* strain EM6 and had a homology level of 97%.

The results of the BLAST analysis were different for each fungal sample. From the results of phylogenetic constructs, it was found that the C1, C2, and C3 samples were of different species. Sample C1 belongs to the species *Apergillus* sp. which has a similarity rate of 99.6% with the species *Aspergillus tubingensis*. As for the C2 sample, based on BLAST analysis and phylogenetic trees have a level of kinship with the species *Trichoderma* sp. with a homology level of 99% for the *T. asperellum* species. The BLAST results of the C3 sample belong to the species *Issatchenkia orientalis* with a similarity level of 99.2% (Fig. 5).

4. Discussion

Fusarium wilt or tuber rot in shallot plants due to *Foc* infection causes enormous losses annually to global agriculture. Treatment using synthetic fungicide is not entirely effective because *Foc* can last a long time in the soil thanks to its chlamydosporic structure (Fig. 1.D) which is resistant to extreme environmental stress (Gupta et al., 2020).This is also considered uneconomical and a source of environmental pollution, so that alternative pathogen control with antagonistic microbes (biocontrol) is more promising and sustainable (Abbey *et al*., 2018; Fournier et al., 2020; Tleuova et al., 2020). In this study, a number of indigenous microbes showed antagonistic activity against *Foc* growth *in vitro* (Fig. 2).

Five bacterial isolates and three fungal isolates showed inhibitory activity of *Foc* mycelium growth. B1 and B4 bacterial isolates were significantly ($p < 0.05$) able to inhibit the growth of radial mycelium *Foc* when compared to controls with inhibition percentages of 65.93% and 72.27%. Meanwhile, the other three bacterial isolates did not statistically have a significant percentage of inhibition when compared to isolates B1 and B4 (Table 1). When compared with bacterial isolates, the *Foc* inhibition capability of fungal isolates was much higher. Although the rate of bacterial cell proliferation is faster, the expansion capability of fungal hyphae in the test medium is much faster, so this is thought to be correlated with its antagonistic activity in suppressing the growth of *Foc* mycelium. Kalman et al. (2020) reported that the *Foc* growth rate reached 0.83–0.87 cm / day. The activity of rhizosphere bacteria in suppressing pathogen growth can be through a number of mechanisms of action, including; synthesis of hydrolytic enzymes such as chitinase, β-1,3-glucanase, and proteases that can lyse pathogenic fungal cells (Lopez *et al*.,2020), (2) competition for nutrition and colonization of the rhizosphere niche (Rana et al., 2019), and (3) production of siderophores and antibiotics (Kumar et al., 2018; Panchami et al., 2020). But generally, the mechanism of inhibitory action by bacteria occurs due to the synthesis of a number of bioactive compounds, especially antibiotics (Jangir et al., 2018; Panchami et al., 2020; Ramyabharathi et al., 2020).

From the results of molecular analysis using 16S-rRNA markers, it was found that the five bacterial isolates were included in the genus *Bacillus* (Fig. 4). The isolates with the highest inhibitory capability, B1 and B4, have evolutionary similarity to *B. subtilis* strains X2 and S11.The interesting thing is that isolates B1 and B3 have a percent identity of 94% when compared to *B. subtilis* strain X2, where both share the same branch. A number of studies have reported the capability of *Bacillus* to suppress the growth of various phytopathogenic fungi so that it is commonly used as a biocontrol agent in both monoculture and consortium forms (Khan et al., 2017). Cucu *et al*. (2019) reported that *B. subtilis* QST713 was able to suppress the growth of *F. oxysporum* f.sp. *lycopersici* (*Fol*). *Bacillus* sp. B44 Anatagonist *Fol* (Jangir et al., 2018). In contrast to bacteria, of the three antagonistic fungi isolates tested with the dual culture method, isolates C1 and C2 showed significant inhibitory activity while isolate C3 was the lowest among the three (Table 1) with an inhibitory percentage of 41%. The results of molecular analysis showed that the C1 isolate had high homology (99.4% -100% similarity) (Gupta et al., 2020) with *Aspergillus tubingensis* strain ND8. Whereas C2 and C3 are identical to *Trichoderma asperellum* strains CHI3 and *Issatchenkia orientalis*.

The application of fungi in controlling the growth of the *Fusarium* pathogen is not only related to its high proliferation capability so that it is able to colonize the environment quickly, especially habitats exposed to pathogens (rhizosphere, phyllosphere, and plant organs) but is also related to its capability to produce bioactive compounds (Ghorbanpour *et al*., 2018). A number of previous studies have reported that *A. tubingensis* has antifungal activity. Zhao *et al.* (2018) reported that *A. tubingensis* QF05 was able to inhibit the activity of the pathogenic fungus *Botrytis cinerea* in tomato plants. Whereas Kriaa *et al*.(2015) reported that the activity of glucose oxidase (β-d-glucose: oxygen-oxidoreductase EC 1.1.3.4) which was partially purified from *A. tubingensis* CTM 507 effectively inhibited *F. solani*. This enzyme activity causes the mycelium to undergo lysis, cytoplasmic vacuolization, premature formation of chlamydospores, and mycelium induction through anastomosis between hyphal filaments.

The inhibitory activity of *Fusarium* by the fungus C2 with a percentage of 41.12% was strong. The results of molecular analysis showed that C2 had an evolutionary relationship with *T. asperellum* with a similarity percentage reaching 99.2% with *T. asperellum* strain CHI13. *Trichoderma* is one of the fungi that has the capability to produce a number of metabolites that can inhibit or kill pathogenic fungi, so it is the most common biocontrol agent (Ghorbanpour *et al*., 2018). A number of bioactive compounds with the antifungal activity of *Trichoderma* have been reported, such as 3-octanone and 1-octen-3-ol which are both fungistatic and strong fungicides (Okkull *et al*., 2003), 6-pentyl-2H-pyran-2-one produced. by *T. koningii, T. harzianum, T. virens*, and *T. viride* (Worsatit et al., 1994) and sesquiterpenes from *T. harzianum* (Lee *et al*., 2016). The mechanism of inhibitory action by *Trichoderma* can be either direct contact or the result of diffusion of the compound being excreted into the environment. De la cruz-Quiroz *et al*., (2018) reported that there are two mechanisms to inhibit the activity of *Phytophthora capsica* and *Colletotrichum gloeosporioides* by *Trichoderma*, namely the production of antibiotic compounds, which work during the growth of *Trichoderma* hyphae to touch the phytopathogenic biomass, and the second is the mycoparasitic mechanism, which works when these organisms come into contact. Furthermore, Das et al., (2019) reported that *T. asperellum* was able to effectively inhibit the growth of *Ralstonia solani* and

Phytophthora capsica through mycelium colonization of pathogens. *T. asperellum* was also reported to be able to suppress the growth *of F. oxysporum*f.sp. cucumerinum (May *et al.*, 2019).

From this research, all tested isolates have great potential to be applied as a field biocontrol to suppress *Foc*. However, the capability for antifungal activity by both bacteria and fungi can be further optimized through bioformulation in the form of a consortium. A large number of studies have stated that the application of fungi and a number of bacteria, especially *Bacillus*, is able to inhibit or even kill the growth of phytopathogens through a number of mechanisms (Cucu *et al.*, 2019; Karuppiah et al., 2019; Jangir *et al.*, 2019). Furthermore, Wong et al., (2019) stated that a BCA consortium (biological control agents) is more effective in controlling plant pathogens than single strains due to the involvement of various modes of action of antagonists in suppressing phytopathogens. Apart from acting as a biocontrol agent against phytopathogens, the application of fungi and bacteria as biocontrol agents is also correlated with supporting plant growth through the mechanism of action of providing metabolites synthesized by bacteria, for example phytohormones, or facilitating the absorption of certain nutrients from the environment (Beneduzi et al., 2012; Jangir et al., 2018). However, further testing is still needed to obtain a more comprehensive understanding of all isolates obtained.

5. Conclusion

In this study, bacterial isolates B1 and B4 as well as fungal isolates C1 and C2 isolated from the shallot planting area showed the strongest inhibitory activity against the pathogen *Fusarium oxysorum* f.sp. *cepae*

Declarations

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Compliance with Ethical Standards

Hereby, I Dr. Hilda Karim consciously assure that for the manuscript Antagonistic Activity and Characterization of Indigenous Soil Isolates of Bacteria and Fungi Against Onion Wilt Incited by Fusarium sp. the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by

using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper and will take public

responsibility for its content.

I agree with the above statements and declare that this submission follows the policies of Solid-State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

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Dr. Hilda karim

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Conflict of Interest:

The authors declare that there is no conflict of interest.

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Figures

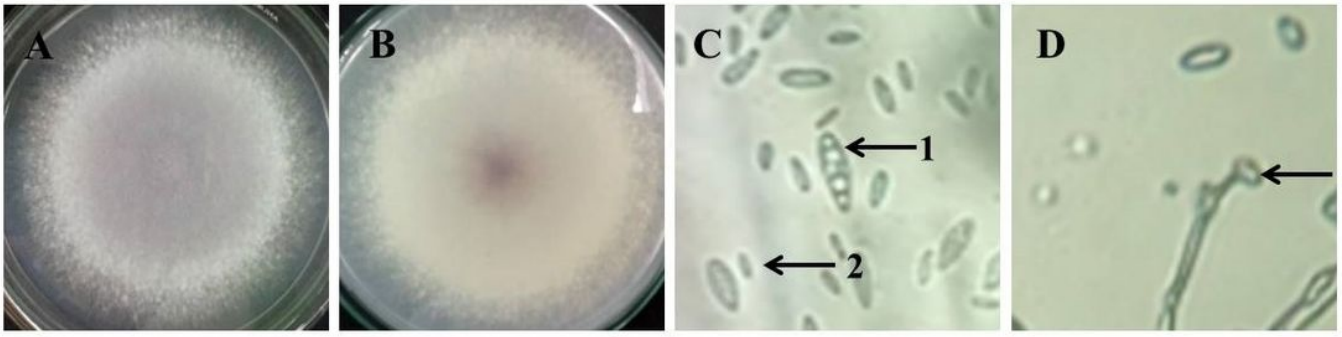

Figure 1

Morphological and microscopic characteristics of 7-days-old Fusarium isolated from the rhizosphere of shallot plants. A. The upper surface of the colony; B. The basal surface of the colony; C. Microconidia (1) and Macroconidia (2); D. Chlamydospores. Microscope observation at 100x magnification.

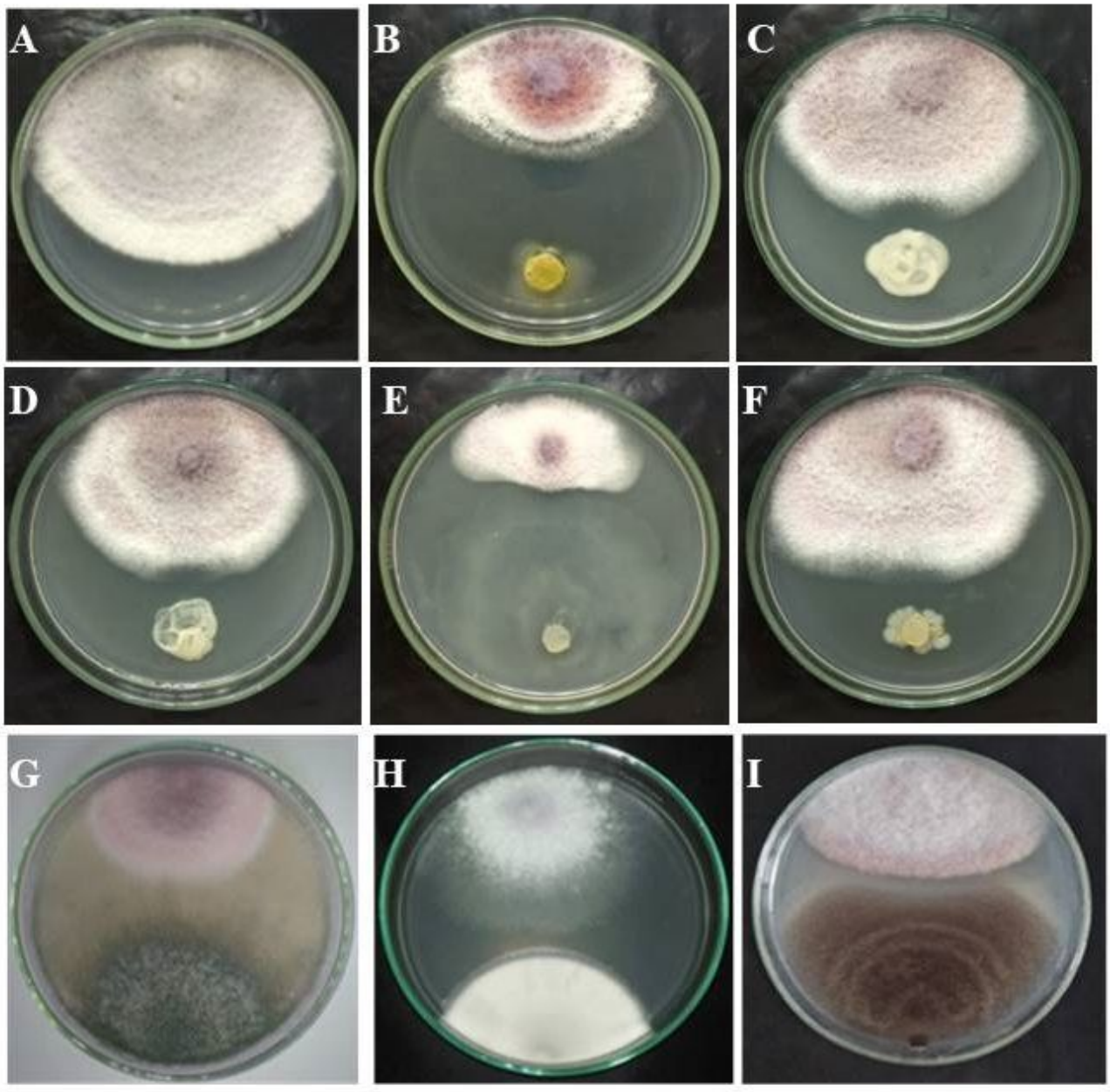

Figure 2

Inhibitory activity of bacteria and fungi against the pathogen Fusarium sp. origin of shallot rhizosphere in PDA medium on day 7. A. Control; B. B1 bacterial isolate; C. Bacteria B2; D.Bacteria B3; E.Bacteria B4; F. Bacteria B5; G. Fungus C1; H. Fungus C2; I. Fungus C3.

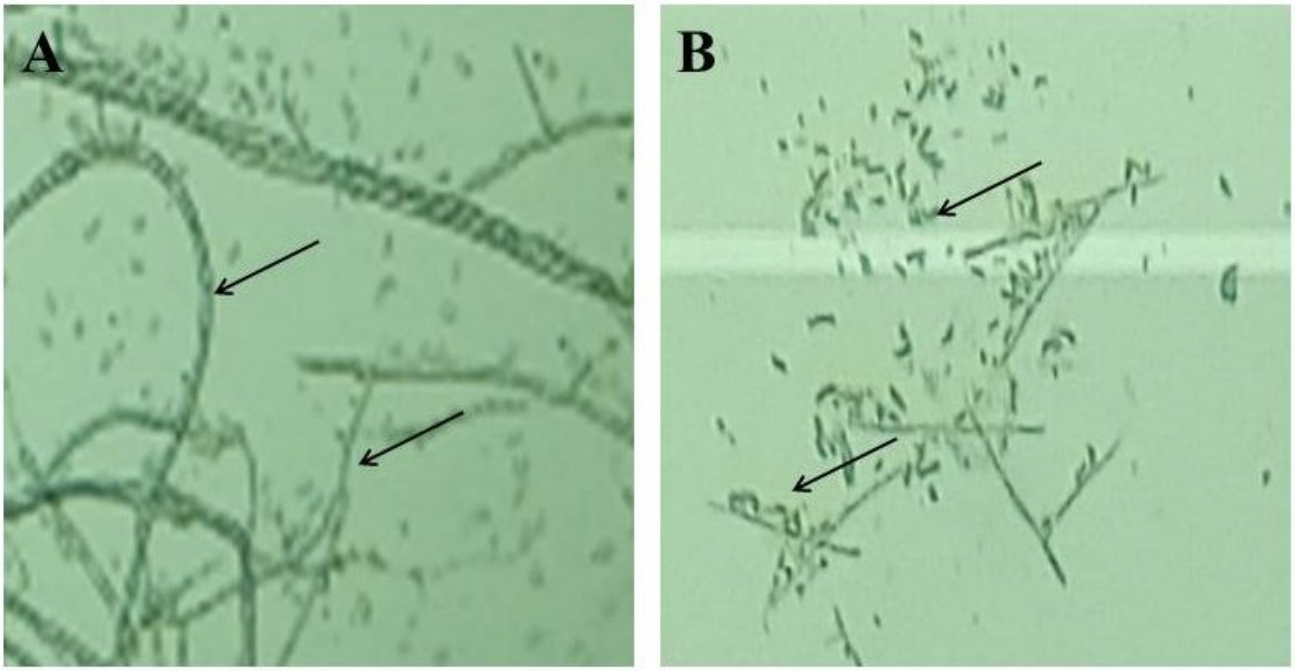

Figure 3

Comparison of growth conditions for Fusarium hyphae at day 7 in PDA medium. A. Hyphae condition in control; B. The condition of the Foc hyphae tested. Arrows show hyphae in both treatments.

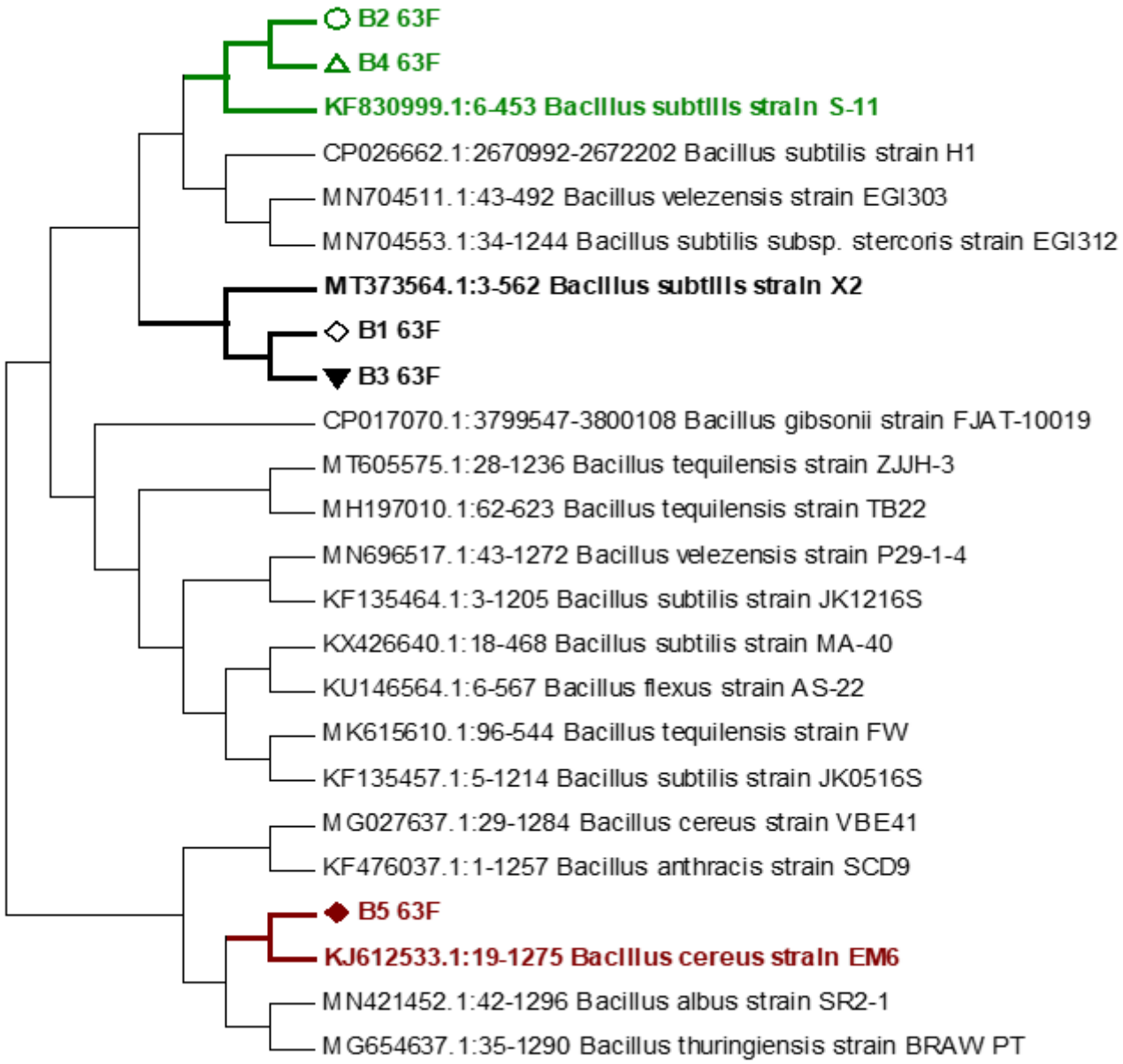

Figure 4

Phylogenetic trees constructed using neighbor-joining method at 1000 times bootstrap using Kimura's two-parameter model. The results showed the position of the isolates and related Bacillus species based on the 16S rRNA gene sequence.

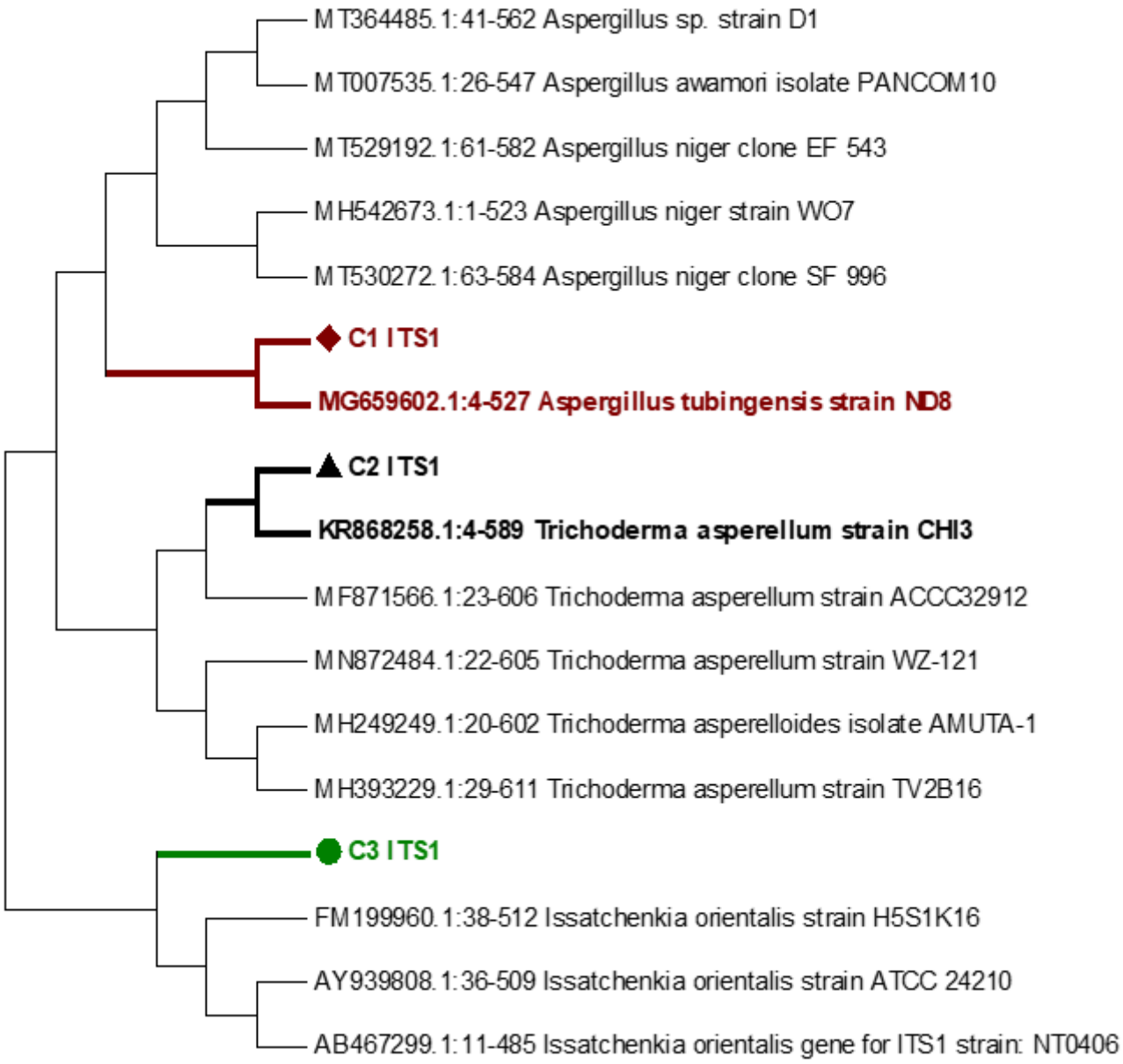

Figure 5

Phylogenetic trees constructed by neighbor-joining method at 1000 times bootstrap using Kimura's two-parameter model. The results show the position of the related isolates based on the ITS gene sequence.