Biological Control of *Fusarium Equiseti* Isolated from Wilt Disease Tomato Using *Trichoderma longibrachiatum* and *Penicillium Polonicum*

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Abstract— Two strains of pathogenic *Fusariun equiseti* and three antagonistic fungi including *Trichoderma longibrachiatum* and two strain of *Penicillium polonicum* were isolated from wilting tomato plant and the surrounding soil, respectiviely. Macroscopic, microscopic, and molecular analysis were used to identify all the isolated pathogenic and antagonistic fungi. Well assay method was applied to investigate the antifungal activity of the three antagonistic fungi against the two strains of *F. equiseti*. The results exhibit that, the best biocontrol for the two strains of *F. equiseti* occure in PDA media for 6 days at pH 3 – 5 and temperature range 25 -30 °C.

Index Terms— *Fusarium equiseti*; antifungal; pathogenic fungi; antagonistic fungi; wilt desease.

1 Introduction

Tomato is one of the most important crops throughout the world especially in Egypt. It covers about 32 % of the total growing land and with 16 % of the total vegetable production [1]. Tomato has ability to cultivate in wide range of climate in both plastic greenhouses and in heated glasshouses [2]. Also, tomato used as a row material in food industries and a fresh fruit market because it contains several valuable minerals and vitamins such as phosphorus, potassium, vitamin B and C. So, it used in diet against prostate and breast cancers. and food industries [3, 4]. Although tomato has an important economic issue, it is infected by several diseases. Tomato diseases is influenced by both environment factors and soil-borne pathogens. The statistical data mentioned approximately 20 types of tomato diseases effect on the production, quality of tomatoes which lead to huge economic losses [5]. This disease occurred by parasitic pathogen. This pathogen may be bacteria, virus, fungi and else. Bacteria can grow by degrading the organic matter in soil and then germinate in plant tissue. Viruses can cause streaking of the stem which affect the growth of plant. Fungi can ability to grow in presence of moisture to germinate spore then cause infection [6]. In favorable condition, this disease can spread from plant to another.

One of the most destructive fungal diseases in tomato is *Fusarium* wilt disease. This pathogen cause of serious disease such as wilting of vascular system, blights, rots, and cankers of many horticultural, field, ornamental, and forest crops in both agricultural and natural ecosystems and also produce poisonous secondary metabolites (mycotoxins) such as trichothecenes and fumonisins, that can pollute crops which makes the crop is not used for food or feed [7]. In addition, The *Fusarium* species is the main cause of some pollutions such as *Fusarium graminearum, F. sporotrichioides, F. poae, F. avenaceum, F. culmorum, F. accuminatum, F. langsethiae, F. verticillioides, F. proliferatum, F. oxysporum, F. anthophilum* and *F. paranaense* [8]. This pathogen can infect the tomato plant through germination of conidia in soil and then through root tips which facilitated by injury due to nematode feeding or separating leaves from stem or moving plant from place to another, then colonizing the cortex by hyphae of *Fusarium* sp [9] and therefore quickly germinate in the xylem which called water transporter system in the plant [10].

The xylem of pseudo stem infests by micro conidia which prevent the motion of upwards of water and nutrients, then conidia prevent from spreading through sieve cells of stem after that occur germination of spore through sieve cells to block the entire xylem and the wilt symptoms appear. When the plant dies, spore occur germinate in the surrounding tissue then form chlamydospores and come back into soil [11]. Chlamydospores can survive in the soil until adequate moisture for germination and then this cycle occurs again. This disease can be spread through polluted soil, infected plant tools, using irrigation water and by human motion around infected plant. The initial symptom of wilting disease is appeared on stunted seedlings and discoloration of lower leaves which become yellow color that occur frequently in one side of plant and then older leaves are falling. When disease development, wilting reach to stem and occur decaying [12]. The main symptom of this disease is browning of vascular system which appear in tissue section when cut stem near the root, despite this the stem is appear green on the outside. In some issue, plants which infect with diseases do not occur die, but the production is reduced [13]. *Fusarium* wilt disease is the most complex management so that in old decade, the researchers try to discover methods of management such as pesticide, resistant cultivars, and crop rotation but all methods are not effective because firstly pesticides can cause toxicity in the soil, pollute the environment, and kill beneficial microorganism [14]. Although resistant cultivars are effective and friendly environmental, new strain of pathogen are found which can

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inhibit the effect of this method. furthermore, crop rotation is not effective because pathogen inhabit in soil as a nature [15]. As a result, a new method was discovered to control this disease which is called as a biological control. This new method depends on the utilize of living organism such as bacteria and fungi to reduce the inoculum of pathogen and reduce the yield waste, so it has a safe effect on both the economic and environment [16].

The present study aims to isolate *Fusarium* sp from wilting tomato plant and antagonistic fungi from the surrounding soil. In addition, studying the antifungal activity of isolated antagonistic fungi against *Fusarium* sp.

2 EXPERIMENTAL

2.1 Collection of tomato samples

All samples were collected from various locations throughout the Damietta government. The infected and healthy tomato plants were carefully collected to preserve the adhering soil containing the antagonistic fungi, then placed separately in sterilized plastic bags and transported to the laboratory. This procedure was used to isolate both pathogenic fungi that cause tomato wilt disease and antagonist fungi that help with biocontrol of these diseases.

2.2. Isolation and purification of pathogenic fungi

The pathogenic fungi were isolated using the methods described below [17]. The infected vascular tissues of tomato roots and stems were washed separately under running tap water before being cut into small pieces ranging from 0.5 to 1 cm in length. Tissues were sterilized for 2 minutes in a 5% sodium hypochlorite solution before being washed several times in sterile distilled water. These tissues were dried between two layers of sterilized filter paper. They were then placed on the surface of potato dextrose agar (PDA) media containing traces of the antibiotic Chloramphenicol to inhibit bacterial growth and incubated in the laboratory at 25 27^{o}C for 5 to 7 days.

2.3. Isolation and purification of antagonist fungi

To control tomato wilt diseases, antagonistic fungi that act as a bio control were isolated [18]. This method consists of three steps. The first involved air drying a tomato soil adhering sample at room temperature and sieving to remove large particles. The second step was to dissolve 5 gram of dried soil sample in 50 ml of sterile distilled water and mix thoroughly for 20 minutes before performing a serial dilution from 10^{-1} to 10^{-10}. The third step was to inoculate 1ml of each dilution in a sterilized petri dish, followed by pouring PDA media with a temperature of approximately 40^{o}C to maintain the microorganisms' activities and incubating for 5 days in a laboratory setting at 25 ^{o}C. Finally, the frequency of occurrence of grown fungal colonies (percent) was recorded.

2.4. identification of purified isolate

The isolated fungi were purified and examined macroscopically and microscopically using light microscope to characterize the strain based on their morphological and cultural characteristics of the fungi [19]. The macroscopic examination includes mycelium growth density, color of surface and reverses mycelium, pigment. Microscopic examination was occurred by transporting a piece of the mycelium into microscopic glass slides. For better analysis of morphological characteristics, the slides samples were stained with Lacto phenol blue solution to reinforce contrast. The prepared slides were examined microscopically at 40× magnification.

2.5. Antagonistic activity by well assay method

Well assay method is also used for testing the antagonistic activities of isolated fungi against pathogenic fungi [20]. The isolated antagonistic fungi were grown in potato dextrose broth (PD) in 100 ml flasks on rotary shaker (180 rpm) for 7 days at 30^{o}c and repeat for three times for obtaining accurate results. The content of each flask was filtrated by syringe filter (0.22 um) to test this sterilized filtrate for antagonistic activity against pathogen. The molten PDA media were inoculated by disks of pathogen fungi in flasks and then were poured in sterilized Petri dish until solidification. Wells (5 mm diameters) were made in these plates and the sterilized filtrate of the isolated fungi was inserted into the wells and then incubated at 25 ^{o}C. The diameter of inhibition zone was measured after 5 days.

To obtain the optimum conditions, well assay method was applied in different media like PD, Czapex dox, dox modified, Sabroud dextrose and malt media. Also, the method was investigated at different incubation period from 3 to 7 days from cultivation. In addition, the effect of temperature and pH were studied at ranges 5-50^{o}C and 3-8, respectively.

2.6. Genomic DNA and phylogenetic analysis

fungal genomic DNA was isolated from the cell using Solgent purification head. The 18S rRNA gene was amplified by using universal pairs of primers – Forward (ITS1: 5'-TCC GTA GGT GAA CCT GCG G-3') and Reverse: (ITS4: 5'-TCC TCC GCT TAT TGA TAT GC-3'). The amplified PCR product was sequenced using Solgent EF-Taq. PCR Machine name :9700(ABI). BLAST was used to match the best similarities with other related sequences in the database for the resulting 18S rDNA sequence. The most similar DNA sequences to our 18S rDNA region were obtained from NCBI GenBank and aligned using CLUSTAL Omega. Unalienable regions were manually removed, and sequences from the same species as well as unidentified organisms were discarded. Finally, using MEGA version 4, phylogenetic tree analyses were viewed and analyzed [21]. Maximum composite likelihood methods were used for neighbor-joining). Only values of 20 and higher were considered and displayed next to the phylogenetic tree branches, with confidence levels estimated using 1000 bootstrap replicates.

3.RESULT AND DISCUSSION

3.1. Collection and isolation of fungal samples

Twenty Samples were collected from different four places in Damietta governorate in order to obtain several antagonist and pathogen fungi. All the collected samples were cultivated

in PDA media and 22 different fungi were isolated from rhizosphere. Table SI.1 presents the relative frequency percentage of all the isolated fungi from rhizosphere. The relative frequency percentage (RF) was calculated according to equation 2.

RF = (no. of isolated fungi /total no. of fungi) ×100 (2).

3.2. The characteristic properties of the purified isolated fungi

The macroscopic characteristic properties of the purified isolated fungi which are grown on PDA media including color of surface mycelium, color of reverse mycelium, pigment and density are presented in table SI.2. Starting with color of surface mycelium, species 2,3,17,18,19,20 and 22 have green surface mycelium color while species 5, 6, 9, 14, 23, 25, 26, and 27 have white surface mycelium color. Also, Species (1, 8, 12, 15), (7, 11), (4), (10, 24), (21), (16) appear black, gray, buff, white to gray, white to green and carpet (green, yellow, and orange) surface mycelium colors, respectively. Moving into color of reverse mycelium, species 2, 3, 17, 22 have green reverse mycelium while species 4, 5, 6, 14 have brown reverse mycelium color. Also, species (7, 8, 12), (11, 15), (9, 20), (13), (10), (21), (16) appear black, gray, buff, orange, white to gray, white to green and carpet reverse mycelium color, respectively. Species 1, 18, 19 have no color of reverse mycelium. In addition, all the species do not have the soluble pigment except species 16 and 17 appear red pigment. The densities for all species are varied from low, medium, and heavy growth.

The molecular identification of pathogen and antagonistic fungi by DNA sequences obtained from pathogenic and antagonistic fungi were trimmed and assembled in Geneious software and then phylogenetic tree analyses were viewed in figure 1.

3.3. Antagonistic activity by well assay method

The antagonistic activities are measured by inhibition the growth of pathogenic fungi which isolated from wilting tomato plants by producing antimicrobial compounds [20]. The isolated purified fungi are cultivated on potato dextrose broth (PD) media. The supernatants which filtered by syringe filter are taken and put in wells in the inoculated PDA media with all the pathogen species to measure the potentiality of the isolated filtered fungi to antagonist pathogenic fungi. The obtained results are presented in figures 2-3. The results indicate that, the potentiality of the filtrate of the isolated fungi to inhibit the growth of the pathogen are the most effective by the isolated fungi *Tr. longibrachiatum, P. polonicum st.1* and P. *polonicum st.2* appear the highest inhibition zone against *F. equiseti st.1*and *F. equiseti st.2*.

3.5. Factor affecting the antagonistic activities of the selected antagonistic strains against pathogen.

The diameter of inhibition zone of pathogenic fungi is affected by several factors such as incubation period, media, temperature, and pH to obtain the optimum condition for inhibition the pathogenic fungi.

3.5.1. Effect of different incubation period

The incubation period was found to have great effect on the antagonistic activity against pathogen. Therefore, well assay method is studied at different incubation time from 3 to 7 days by using the three selected antagonistic fungi. The effect of incubation period of the antagonistic fungi *(Tr. longibrachiatum, P. polonicum st.2* and *P. polonicum st.1*) against the patho-

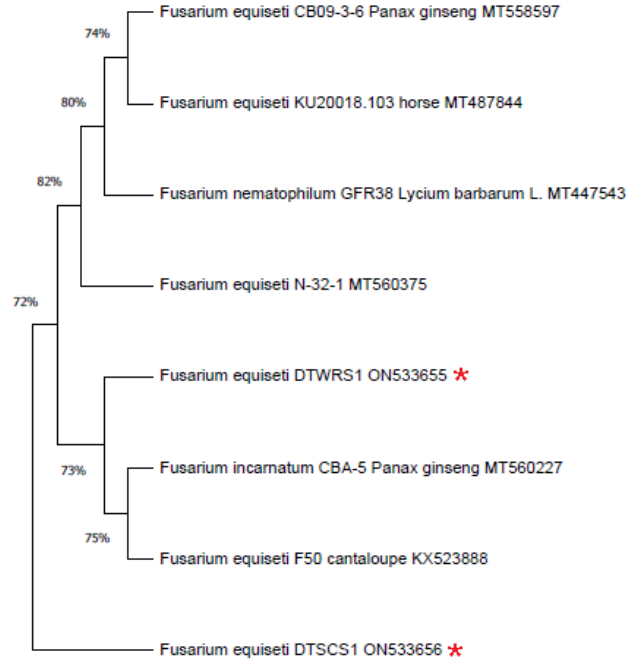

Figure 1. Phylogenetic tree analysis based on 18S rDNA sequence alignment for *Fusarium equiseti* with some other related species, which possessed the highest similarity. The neighbour-joining was performed using the maximum composite likelihood methods.

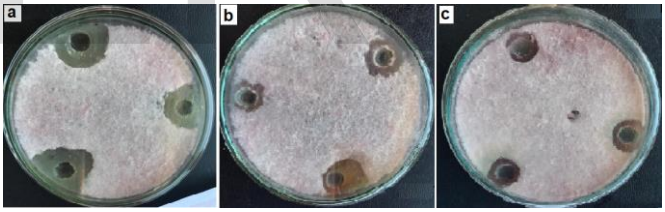

Figure 3. Antagonistic activity of isolated *Tr. longibrachiatum* (a)*; P. polonicum st.2* (b); *P. polonicum st.1*(c) against *Fusarium equiseti* st .2. using well assay methods.

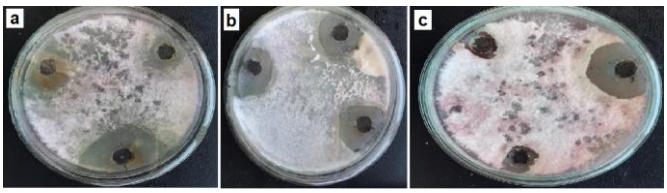

Figure 2. Antagonistic activity of isolated *Tr. longibrachiatum* (a)*; P. polonicum st.2* (b); *P. polonicum st.1*(c) against *Fusarium equiseti* st .1. using well assay methods.

genic fungi (*F. equiseti st.1* and *F. equiseti st.2*.) are presented in figures 4-5. These results indicate that, the inhibition zone against the three pathogens is increased with increasing the incubation period and are relatively constant after the six days of cultivation of antagonistic fungi. So, six days old culture is the most suitable growth to inhibit the growth of pathogen.

3.5.2. Effect of different culture media

The different culture media are affected on the growth of biomass dry weight of the three species of antagonistic fungi and the diameter of inhibition zone affected by three antagonistic fungi against two pathogenic fungi. The effect of different media on the biomass dry weight of antagonistic fungi which cultivated in shacking culture media (180 rpm) at 25°c for 6 days are presented in table SI.3 which indicate the most suitable media for all the three antagonists to give high biomass is potato dextrose media (PD). And also investigate the effect of filtrate of different media on the diameter of inhibition zone produced by the three antagonistic fungi (*Tr. longibrachiatum, P. polonicum st.2* and *P. polonicum st.1*) against two pathogenic fungi which are presented in figures 4-5. These results indicate that the most optimum media for all antagonistic fungi is PD media which obtain the highest inhibition zone of the pathogen.

3.5.3. Effect of hydrogen ion concentration

The different hydrogen ion concentration pH affected on the growth of biomass dry weight of the three species of antagonistic fungi and also the diameter of inhibition zone affected by three antagonistic fungi against two pathogenic fungi a. The effect of different pH on the bio mass dry weight of antagonistic fungi which adjusted pH by adding drop of HCl or NaOH to obtain varying value of pH by using pH meter and cultivated in shacking culture media (180 rpm) at 25°c for 6 days are presented in table SI.4 which indicate the most appropriate pH for all the three antagonist to give high bio mass is different value of acidic medium which *Tr. longibrachiatum* at Ph 6, *P. polonicum st.1* at Ph 3 and *P. polonicum st.2* at Ph 5. And the filtrate of the antagonist fungi are tested against the two fungi pathogen by well assay method to investigate the effect of different pH on the diameter of inhibition zone which produced by the three antagonistic fungi (*Tr. longibrachiatum, P. polonicum st.2* and *P. polonicum st.1*) against two pathogenic fungi (*Fusarium equiseti st.1* and *Fusarium equiseti st.2)* which are presented in figures 4-5. These results indicate that the most optimum pH for all antagonistic fungi is Ph 5 against *Fusarium equiseti st.1* while the best value of pH is pH 3-4 against *Fusarium equiseti st.2* to obtain the highest inhibition zone against both of *Fusarium equisetist.1* and *Fusarium equisetist.2.*

3.5.4. Effect of different temperature

Temperature has a significant impact on both the dry weight of the biomass of the three different antagonist fungi and the diameter of the inhibition zone which produced by antagonist fungi against two pathogenic fungi. The effect of different temperature on the biomass of antagonistic fungi which cultivated in the optimum media (PD) and incubated at different temperature between 5 to 50°C for 6 days are presented in SI.5

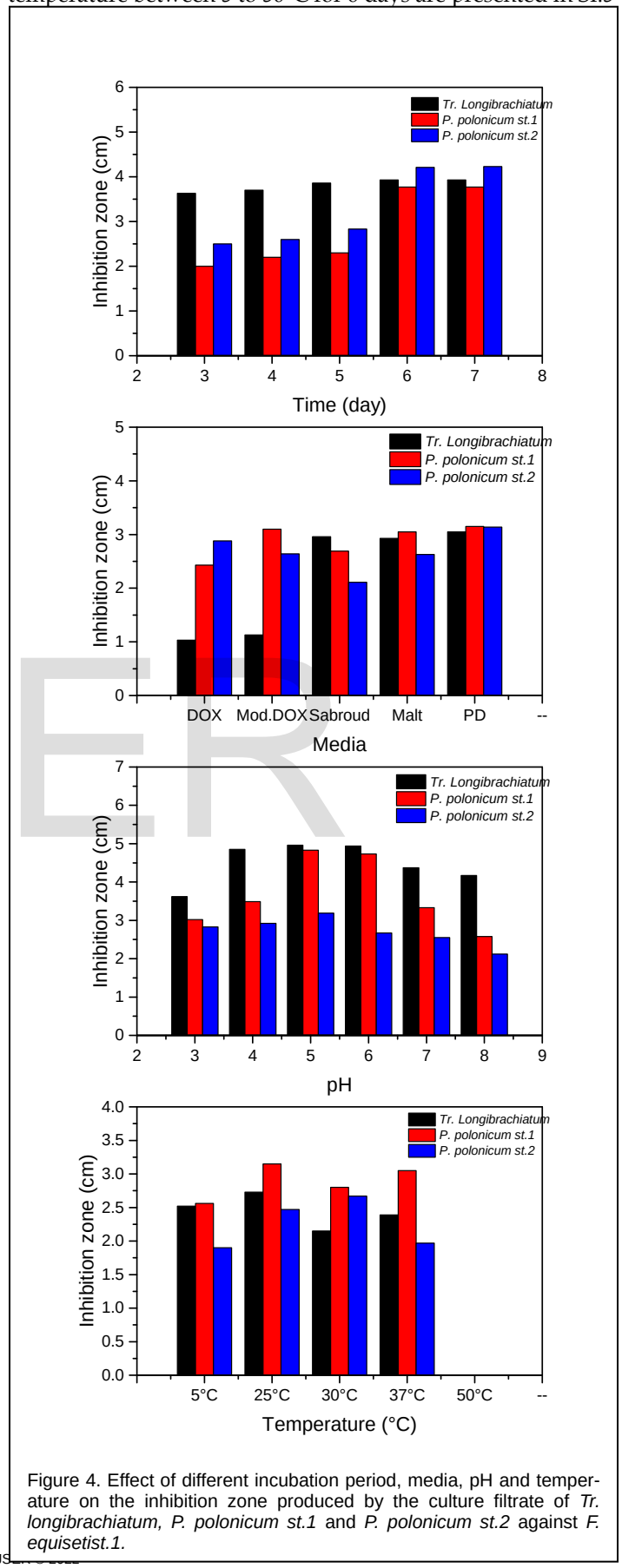

Figure 4. Effect of different incubation period, media, pH and temperature on the inhibition zone produced by the culture filtrate of *Tr. longibrachiatum*, *P. polonicum st.1* and *P. polonicum st.2* against *F. equisetist.1.*

Which indicates the most suitable temperature for all three antagonist fungi to give high biomass is 30°C as well as 25°C for *Tr. longibrachiatum*. And investigate the effect of different temperature on the diameter of inhibition zone (*Tr. longibrachiatum, P. polonicum st.2* and *P. polonicum st.1*) against two pathogenic fungi (*Fusarium equiseti st.1* and *Fusarium equiseti st.2)* by well assay method which are presented in figures 4-5. These results indicate that the most optimum temperature for *P. polonicum st.2* is 30°C while the best temperature is 25°C for *Tr. Longibrachiatum* and *P. polonicum st.1* against *Fusarium equisetist.1*. while the optimum temperature for all antagonistic fungi as *Tr. longibrachiatum, P. polonicum st.2* and *P. polonicum st.1* against *Fusarium equisetist.2* is 25°C to obtain the highest inhibition zone of the pathogen.

4 Conclusion

In conclusion, the growth of *F. equiseti* which cause wilting tomato desease can be effectivielly biocontrolled using *Tr. longibrachiatum, P. polonicum st.1* and *P. polonicum st.2* in PDA media for 6 days at pH 3 – 5 and temperature range 25 -30 °C.

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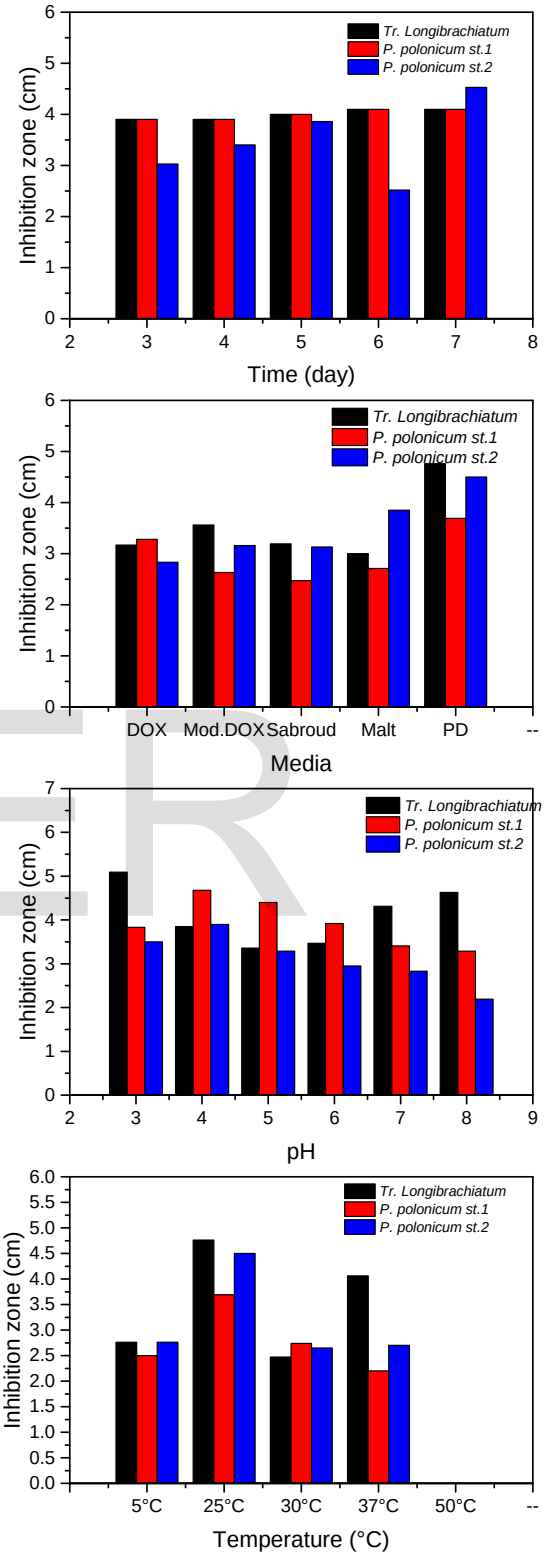

Figure 4. Effect of different incubation period, media, pH and temperature on the inhibition zone produced by the culture filtrate of *Tr. longibrachiatum*, *P. polonicum st.1* and *P. polonicum st.2* against *F. equisetist.2.*

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