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Prevalence of citrus wither tip (*Colletotrichum gloeosporioides*) in Sargodha and its management

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Abstract
Citrus wither tip (*Colletotrichum gloeosporioides*) is most prevalent disease in Sargodha and is the most important fungal disease contributing towards citrus decline in this area. During field surveyed, it was recorded that citrus wither tip was 100% prevalent in tehsil Kotmomin, Bhalwal and Silanwali and lowest was recorded in Shahpur while highest disease incidence was recorded in Kotmomin 42.22%, Sargodha 36.11%, Sahiwal 34.44%, Silanwali 36.11%, Bhalwal 32.22% and lowest in Shahpur 26.11%. The fungicides were evaluated using poisoned food technique at three concentrations 100, 200 and 300 ppm. The design used was completely randomized design with three replicates. Among all the fungicides, Topsin M was the most effective fungicide against all four isolates followed by Mancozeb+Matalaxyl and Aliette. Topsin M performed exceptional and its effect almost remained same against all the isolates tested. The second and third fungicides showed inhibitory effect against Isolate 2 were Success and Copper Oxychloride that percent inhibition was 22.22% at 7th day. Topsin M gave highest inhibition as compared to other fungicides against Isolate3 and its inhibition was 74.22% at 7th day. Meanwhile, Mancozeb+Matalaxyl showed good results against Isolate3 followed by Topsin M and its percent inhibition was 41.11%. Same trend has been observed in case of isolate 4 on which Topsin M gave highest inhibition as compared to other fungicides and its inhibition was 77.78%. The overall result revealed that that all tested concentrations of Topsin M were found significantly effective for controlling *Colletotrichum gloeosporioides* causes of citrus wither tip.

Keywords: incidence, citrus, fungicides, poison technique, management

1. Introduction
Citrus is fruit of tropical and subtropical area. South East Asian area is considered as the originating point of citrus, and then its cultivars cultivated throughout the world [9]. In Pakistan citrus industry is much behind due to many problems which include biotic and abiotic factors [16]. Among biotic factors, various microbial pathogens of citrus that are significantly causing heavy losses throughout the world include fungal pathogens such as *C. gloeosporioides* is [12]. Spores of the fungi are brown to black. Size of acervuli varies a lot in shape those exudates pinkish conidia at maturity. It causes postharvest and field losses causing infection on almost 470 genera. Its host range is very wide causing anthracnose on apple, Arabica coffee, guava, passion fruits, citrus, etc. [22]. Humidity at 95% is required for the germination of conidia and aspersoria production. Growth of the pathogen is very high at 20 to 30 °C [22]. Sporulation is ceased when temperature is below 18 °C or above 30 °C. Dispersal of pathogen could be by air, wind splashes or even by insect [3].

There are various techniques that are being used since ancient times to control these microbes. Many fungicides have been used to control the pathogen and for effective control of the pathogen spray at the interval of 14-28 days is recommended. Various fungicides are used at post-harvest and pre-harvest stages in accordance with the requirement [24]. Fruits that are to be shipped remain infected with *C. gloeosporioides* are treated with fungicides. If weather conditions remain favorable for *C. gloeosporioides,* then copper-based fungicides are used. Azoxystrobin is performing best among all presently used fungicides [23]. Citrus wither tip is important disease-causing severe losses in orchards. It destroys orchards that leads to heavy economically losses [5]. So, it is need to combat that disease to overcome it drastic effects. During the last few decades, it has been observed that citrus wither tip found most openly due to favorable environmental conditions and currently its management is done remarkably with the use of different chemicals. Inappropriate use of chemicals lead to serious human health hazards so proper concentration of chemicals at proper intervals should be mandatory [14]. Therefore, keeping in mind of its devastating nature, the present study was performed to record incidence of the disease in citrus growing areas of district Sargodha, pathogen morphological characterization and use of different fungicides which was evaluated through food poisoned technique against pathogen.

2. Materials and Methods
The present research was carried out in the laboratory of Fungal Culture Bank, College of Agriculture University of Sargodha, during 2016-2018.

2.1 Disease incidence and prevalence
Orchards of district Sargodha were visited and disease incidence and prevalence were measured. Different five orchards were selected randomly in each tehsil (Bhalwa, Kotmomin, Sahiwal, Silanwali, Shahpur, Sargodha) of Sargodha and total 30 orchards were inspected during 2016-2018. In each orchard, 20 plants were selected at five sites,

four plants on each corner of the cubic orchard and four at right middle of the orchard. Then disease prevalence was measured by using following formula [21].

$$\text{Disease Incidence (\%)} = \frac{\text{Number of infected plants}}{\text{Total number of inspected plants}} \times 100$$

$$\text{Disease prevalence (\%)} = \frac{\text{Number of infected orchards}}{\text{Total number of orchards inspected}} \times 100$$

2.2 Sample Collection

A total number of 90 samples were collected from the healthy, partially infected and infected plants to confirm the presence of the pathogen. For association of the fungus total 3 samples were collected from each orchard. Samples contain infected shoots, bark, leaves and twigs were collected from trees of age 15-25. The samples were placed in polythene bag and labeled with required information such as farmer name, date, tree condition, area of orchard [21]. Then samples were brought to laboratory of Fungal Culture Bank, College of Agriculture, University of Sargodha for further processes.

2.3 Isolation of the Pathogen

The collected samples were used for the isolation of the pathogen by using standard method. The infected samples having prominent symptoms were cut in to small pieces of 4-5mm length. These cut samples were further divided in 8-10 small pieces. These pieces were further surface sterilized with 1% solution of sodium hypochlorite for 1-2 minutes. These sterilized pieces were then washed twice in distilled water in petri plates and then shifted on filter papers for drying of the tissues. All the material used during this study was autoclaved first. All the media ingredients (potato dextrose agar) were added into the flask of 1000ml and mixed. Autoclaved the media at 121°C for 20 minutes. Cooled the media up to back palm touch and poured 15ml media into autoclaved petri plates. The surface sterilized tissues of samples were placed on PDA plates with sterilized forceps. Five pieces were placed on each plate. Incubated all plates after wrapping at 25°C for 3-4 days. Fungi, that colonized these pieces was observed under microscope and relevant fungus was further purified by transferring aseptically on new PDA plates for seven days.

2.4 Identification of fungus *C. gloeosporioides*

Fungus *C. gloeosporioides* was identified morphologically based on their macroscopic and microscopic symptoms. For macroscopic symptoms, samples were observed visually under stereoscopic microscope. Different colonies colors were observed and growth pattern of the isolates. For microscopic symptomology prepared glass slides of all the isolates. Observed under compound microscope (10x, 40x and 100x). Different spore's shapes, hyphae growth and growth patterns were observed. After microscopic identification, 12 morphologically different isolates were selected. These had distinct colony color and morphology from each other [17].

2.5 Micrometry

To find more distinction evidences among the isolates micrometry was done. Spore size and hyphal length of all isolates were measured by using this technique.

For micrometry first of all calibration factor was calculated by using following formula;

$$\text{Calibration factor (C.B.F)} = \frac{B}{A} \times O.L$$

Where;

B= as stage division

A= ocular division

O. L= objective lens

After calculating CBF, measurement of spores and hyphae were done by using the formula;

Measurement = CBF × Size of sample (Du *et al*., 2017). All the 12 morphologically different isolates were micro metrically measured.

2.6 Growth Rate

Growth rate of all the isolates was measured. Plug of 5mm was placed on PDA plate of each isolate and incubated at 25°C. Growth rate was measured from day one to seventh. Isolates were marked as slow, medium and fast growing based on their growth rate comparison.

2.7 Pathogenicity Test

For pathogenicity of the isolates detach assay was performed. Fresh leaves were collected from the field. Spore suspension of all the isolates was prepared. Surface sterilized the leave samples and twigs, then washed with distilled water. Placed a 5mm plug on injured leaves and twigs with similar pattern. In other test, placed a drop of spore suspension on the leaves and twigs. Placed these leaves in plastic boxes and wrapped with wrapping tape to avoid the contamination [6].

After that, placed these boxes in growth room and observed the symptoms on daily bases up to one week. After successful detach assay two-year-old plants that were grafted a year ago were sprayed with spore suspension. Symptoms were observed after 7th day of inoculation and observed up to one month [7].

2.8 Evaluation of Fungicides

Four isolates were selected after pathogenicity test on the basis of macro and microscopic observations, which were used against different market available fungicides. There were six fungicides which were (Table 1).

Table 1: Fungicides used for evaluation

Sr. No.	Fungicide	Active ingredient	Company name
1	Aliete	Fostyl-A	Bayer
2	Topsin M	Thiophenate Methyl	Arysta
3	Matalaxyl+Mncozeb	Metalaxyl+Mancozeb	Green zone
4	Kumulus	Sulfar	FMC
5	Copper oxychloride	Copper oxychloride	Capricorn
6	Success	Chlorothalonil &Metalaxyl	Arysta

2.9 Preparation of stock solution
Stock solutions were prepared to make further concentrations. Stock solutions were prepared by dissolving 1g of respective fungicide in 999ml of water [20].

2.10 Preparation of concentrations to be tested:
Concentrations of 100ppm, 200ppm and 300ppm were prepared by dissolving 100, 200 and 300ml of stock solutions of respective fungicides in distilled sterilized water having volume of 900, 800, 700ml, respectively [15].

2.11 Poisoned Food Technique
To evaluate the fungicides food poisoning technique was used. Five-ml of prepared concentrations were added in 15 ml of PDA in autoclaved beaker to poison the media. After thoroughly mixing was poured in petri plate and was kept in laminar flow chamber till solidification. A plug of 5mm was placed on the poisoned media. Three replicates were made of each concentration of each fungicide against all the pathogen isolates. Two plates of PDA without fungicide for each isolate were poured and plug of 5mm was placed on it to maintain as control. Plates were incubated at 25°C and percent inhibition was calculated [15].

$$I = \frac{C - T}{C} \times 100$$

I= Percent inhibition
C= Fungus growth in control
T= Fungus growth in treated plate

3. Results
3.1 Disease prevalence and incidence
Survey was conducted in six Tehsils of district Sargodha. Nine orchards were selected randomly in each tehsil anddisease incidence and prevalence was measured. It was found that citrus wither tip was 100% present in tehsil Kotmomin, Bhalwal and Silanwali while it was 97.77% in Sargodha and Sahiwal, lowest was recorded in Shahpur (Figure 1) while highest disease incidence was recorded in Kotmomin 42.22%, Sargodha 36.11%, Sahiwal 34.44%, Silanwali 36.11%, Bhalwal 32.22% and lowest in Shahpur 26.11% (Figure 2).

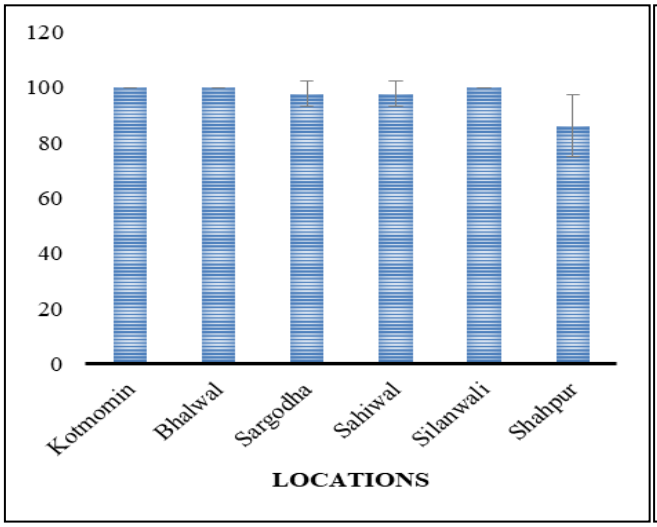

Fig 1: Prevalence of citrus wither tip in district Sargodha

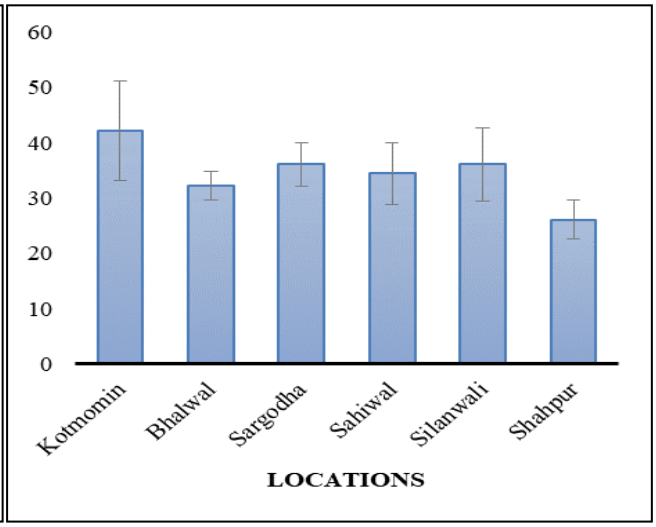

Fig 2: Disease incidence of citrus wither tip in district Sargodha

3.2 Morphological Study
Colony morphology of all selected isolates was studied (Fig 3). There were four isolates that were identified on visual basis. Isolate1 was initially white and later on turned greyish black; there was buffy mass on it with 35µm × 0.15µmsetae and 0.6µm × 0.23µm conidia. Isolate-2 was initially white and turned to creamy white on latter stages, buffy mass was present on it with 95µm × 0.3µm setae and 1.1µm × 0.25µm. Isolate-3 was initially white butwith passage of time turned to creamy white with netting and ringed slightly buffy mass appearance, setae and conidia were 65µm × 0.19µm, 0.9µm × 0.2µm, respectively. Isolate-4 was initially white but turned to reddish creamy with netting appearance in old cultures, setae were 65µ × 0.2µm and conidia were 0.5µm × 0.25µm

Isolate 1 and 2 were fast growing as their growth was 21mm and 19mm, respectively up to 3rd day and increased up to 71mm and 69mm till 7th day. On other hand, isolate 3 and 4 were slow growing as their growth remained 15mm and 16mm, respectively and 58mm and 62mm, respectively from 3rd to 7th day (Table 2; Fig 4).

Table 2: Morphological characteristics of selected isolates

Isolate	Colony color	Conidial mass	Setae (um)	Conidia	Growth rate (mm)			
					D2	D3	D4	D7
1	Initially white, then turned Greyish black	Buffy mass	35µm × 0.15µm	0.6µm × 0.23µm	21	30	44	69
2	Initially white, then turned to creamy white	Buffy mass	95µm × 0.3µm	1.1µm × 0.25µm	19	31	45	71
3	Initially white, then turned creamy	Slightly buffy and netting mass with rings	65µm × 0.19µm	0.9µm × 0.2µm	15	21	33	58
4	Initially white, then turned to creamy reddish	Flat colony with netting mass	65µ × 0.2µm	0.5µm × 0.25µm	16	27	38	62

Fig 3: Colony morphology of selected isolates

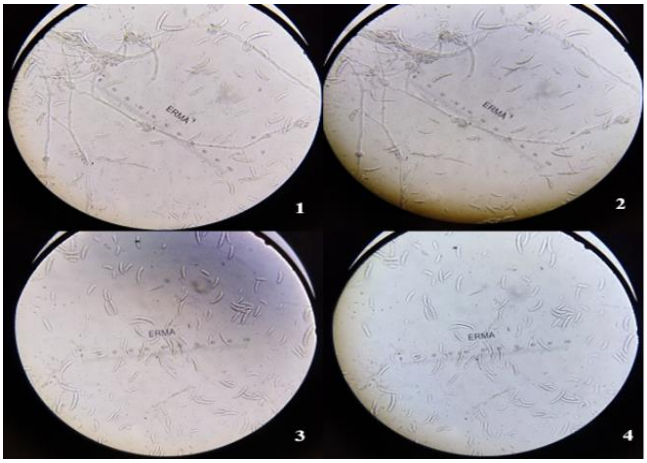

Fig 4: Micrometry of the selected isolates

3.3 Pathogenicity Assay

Virulence of isolates of *C. gloeosporioides* was checked *in vitro* by using plate assay. All four selected isolates produced lesions on inoculated leaves and shoots representing their pathogenic behavior. There were no lesions on control. Isolate1 exhibited high pathogenic behavior and produced symptoms after 3 days of inoculation followed by isolate-2which exhibited symptoms after 4 days of inoculation. Isolate-3 and isolate-4 showed similar behavior and produced symptoms after 6 days of inoculation (Fig 5).

Fig 5: Formation of lesions on leaves and twigs of citrus by selected isolates of *C. gloeosporioides*

3.4 Fungicides Evaluation

Six market available fungicides were evaluated against all the selected isolates. These fungicides were selected on the basis of mode of action. The fungicide Topsin M against I isolate showed 29.66% and 58.66%, 63% percent inhibition followed by Mancozeb+Matalaxyl (28.66%, 40% and 54.33%) and Aliette (37.33%, 39.33% and 39.66%) at 100ppm, 200ppm and 300ppm concentrations respectively. In case of isolate 2, After day7, at 100ppm, 200ppm and 300ppm concentrations, Topsin M showed 32%, 75.33% and 73.67% percent inhibition followed by Success (21.33%, 39.67% and 42.675) and Copper Oxychloride (19.67%, 7.67% and 9.67%) While, Kumulus and Aliette remained least effective to control the pathogen. The same trend has been observed in case of isolate 3 in which Topsin M showed 65.33%, 78.67% and 78.67% percent inhibition followed by Mancozeb+Matalaxyl (23.67%, 40.67% and 59%) and Aliette (24.67%, 46.33% and 49%) while, Kumulus and Copper Oxychloride was least effective at same concentrations used earlier (Fig 6). The overall results clearly depicted that, Topsin M fungicide was showed best inhibitory effect against all isolates tested to control pathogen and can be recommended for practical use in the field in calculated manner.

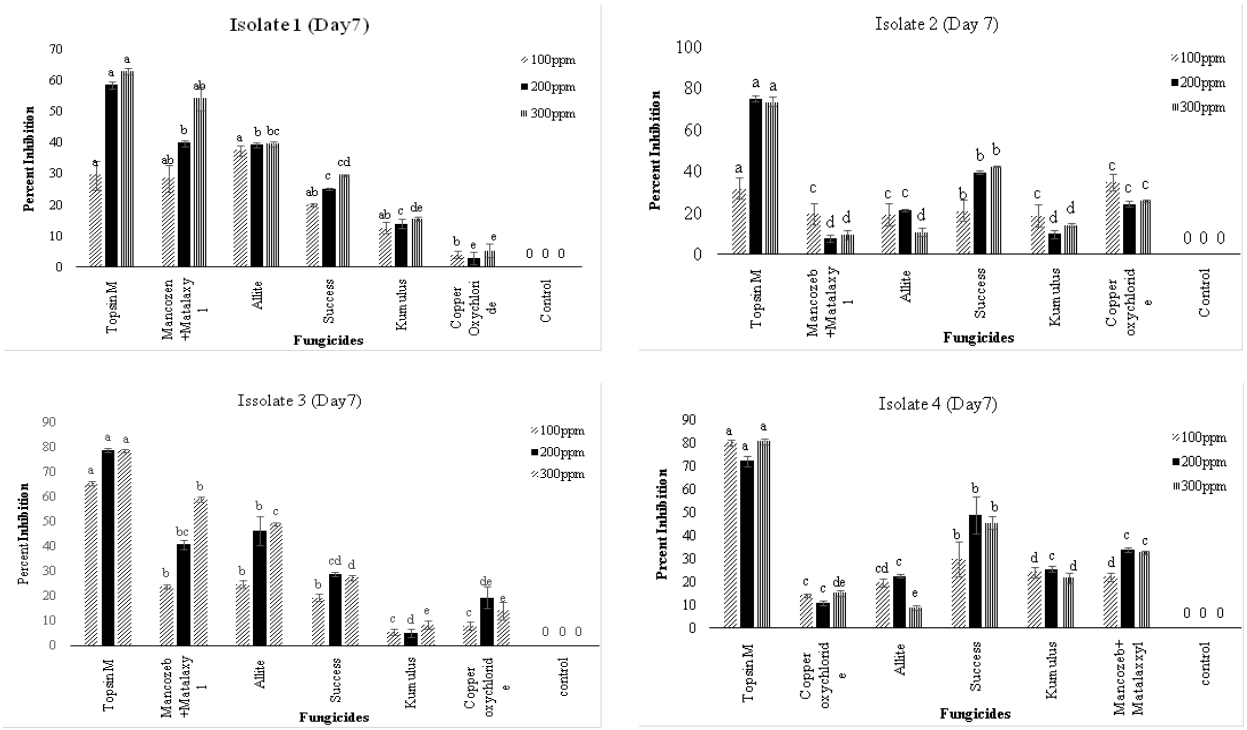

Fig 6: Efficacy of fungicides against tested isolates

4. Discussion

Citrus is an important fruit of tropical and subtropical areas. In Pakistan, citrus industry is much far behind due to many problems which include biotic and abiotic stress. Among them, the pathogen *C. gloeosporioides* is destructive fungal pathogen that infects quality and quantity of citrus fruit. *C. gloeosporioides* is a common pathogen throughout the world [19]. In this study, we assessed the incidence of citrus wither tip across Sargodha area which shares half of the production of citrus industry in Pakistan. Citrus wither incidence was found at varying degrees in different localities of Sargodha region. Citrus wither tip prevailed maximum in tehsil Kotmomin (100%), Sargodha (97%) and Bhalwal (97 %). In past, many studies have been conducted to assess the incidence of citrus wither tip and losses caused by this disease. In Brazil, disease incidence of this disease was recorded in the range of 25-60%. McGovern *et al*. (2012) and Du *et al*. (2017) reported that *C. gloeosporioides* can cause 25-35% yield losses in citrus.

Identification of fungal pathogen is major hurdle in the management of different plant diseases. Proper identification of microbe could lead towards better management of plant diseases. Less costly techniques are being used for the identification of fungal pathogens. In this study, micrometry, spore production, oil immersion technique, detach assay and colony morphology was used for the identification of different isolates of *C. gloeosporioides*. Whitish to yellow, dark brown to blacken center and ring forming colonies were found for different isolates. Setae were tapering towards posterior and their size rangedbetween 35µm × 0.15µm and conidia were little bended just like crescent size and ranged between 0.6µm × 0.23µm. Fungus was finally declared as *Colletotrichum* spp. after confirming from different literature. Different studies were also cited to confirm the presence and identification. Chai *et al*. (2014) identified the presence of *Colletotrichum* spp. on cucurbitaceae. He isolated the *C. capsica* from infected samples of pumpkin. He confirmed the presence based onthree tests; pathogenicity, microscopic and molecular characterization. He inoculated pumpkin with the isolate and then re-isolated the same fungusand confirmed that it was *Colletotrichum* sp. on the basis of macroscopic identification. The colony colour of fungus was pale yellow, acervuli were dark brown to black and brown, and setae were tapering towards ends.

Chemical control is not eco-friendly but still there is no way that could replace chemical management of plant diseases with better and efficient option. So, we have to rely on synthetic chemicals due to unavailability of eco-friendly/bio-based products. However, if we could identify most appropriate fungicides for the control of target fungus with minimum dose that is highly effective to avoid extensive use of chemicals. This also helps saving non-judicious use of fungicides on farmers' fields that leads towards avoiding environmental pollution and reduces chances of resistance in fungi against fungicides. In this study, we evaluated different fungicide products against different isolates of *C. gloeosporioides* causing citrus wither tip to discuss the resistance of different isolates against these fungicides and to evaluate their efficacy. Our results are in accordance with previously conducted researches [1, 4, 10].

In present study we evaluated different chemicals against *C. gloeosporioides* but Topsin M showed highest results as compared to all others. This is in line with the findings of Khan *et al*. (2009). Topsin M has different mode of actions to kill fungi. It activates plant defense mechanism to some extent. Thiophenta methyl inhibits DNA synthesis and inhibits nuclei production in fungi [18]. In present study, six fungicides Topsin M (Thiophenate methyl), Mancozeb+Matalaxyl, Aliette (Fostyl A), Success (Chlorothionil), Kumulus (Sulfur) and copper oxychloride (copper oxychloride) were selected. Their efficacy was checked at three different concentrations 100, 200 and 300ppm with time interval of 3-7days. Efficacy of treatments was checked by using ANOVA and Tukey multiple range tests. Topsin M was most effective followed

by matalyxal+ mencozeb and Aliette at 300 ppm on 3rd day of incubation in case of first two isolates (Isolate1 & Isolate2), while other fungicides did not inhibit significantly but their inhibition at different concentrations was near to each other. Isolate 3 and 4 showed resistance against some fungicide like Mancozeb, so it means Isolates 3 and 4 have resistance against this fungicide. This confirms the results of Gang *et al*. (2015). In their studies, different fungicides were evaluated against *Colletotrichum* spp. causing anthracnose in persimmon fields. Resistance by this fungus was found against the applied fungicides despite of regular sprays. They found further that there were various pathotypes of this fungus which were resistant against applied fungicides [8].

Hussnain *et al*. (2006) reported that efficacy of fungicides decreases with time intervals against *C. falcatum* and sprays at regular intervals are necessary. In present research, percent inhibition decreased after the exposure of longevity of time. However, Success (Chlorothalonil) showed effective control against all isolates on different longevities.

5. Recommendations

The major challenge of the widespread use of alternatives to control *Colletotrichum gloeosporioides* causes of citrus wither tip is to meet the requirement of a low production cost. Therefore, it is necessary to develop eco-friendly approaches with no or little toxicity that control post harvest pathogens even when applied at low concentrations.

6. Acknowledgement

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7. Conflict of Interests

"The author(s) declare(s) that there is no conflict of interests regarding the publication of this article"

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