DISEASE CAUSING PHYTOPATHOGENIC MICROMYCETES IN CITRUS IN UZBEKISTAN

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ABSTRACT

This research work provides information on phytopathogenic micromycetes that cause major and emerging diseases posing a significant threat to the cultivation of citrus in Uzbekistan. The purpose of the study is to develop strategies for the management of diseases by biological methods and to improve the methods of eliminating the causative agents of diseases, as well as to determine the effectiveness of micromycetes with high antifungal properties in the cultivation of citrus. The issues affecting the citrus industry and scientific recommendations to overcome those issues are discussed. Micromycetes of phytopathogens that cause diseases in the roots, rhizosphere and leaves of lemon and tangerine in closed ground conditions, including Fusarium vasinfectum, Verticillium dahliae, Alternaria alternata, F. semefectum, Aspergillus oryzae, A. flavus, Penicillium funiculosum, F. sporotrichiella, Aspergillus sp., A. terreus, P. chrysogenum, P. digitatum, F. solani, P. sp.1, A. sp.2, A. sp.3, F. sp.1, P. sp.2, F. sp.2, A. sp.4, F. sp.3 were identified.

Keywords: Trichoderma harzianum 55, lemon, tangerine, micromycete, phytopathogen, antagonism.

INTRODUCTION

Citrus fruits are among the top ten and the most productive fruits in the world, grown in more than 100 countries and ranked first in international fruit trade by value (Khanouch et al., 2017). In Uzbekistan, lemons and tangerines are grown mainly in closed ground conditions (greenhouses), and are also imported from Turkey and Pakistan to meet the needs of the population. The constant use of chemical fungicides against disease-causing micromycetes in the cultivation of citrus has led to the emergence of resistant strains of phytopathogenic fungi (Sánchez-Torres, 2011). Every year, in total of 25% of fruit crops are infected with phytopathogenic microorganisms, resulting in up to 50% loss of its yieldin the world (Barkai, 2001; Díaz, 2020). In optimal humidity and temperature, the phytopathogenic microflora present in the soil develop very rapidly and damages the plants. Recently, the problem of decrease in the yield of perennial citrus plantations has become an urgent issue. Mostly the risk of the mass loss of the fruit yield is being increased due to pathogens in citrus products (Kulyan, 2017; Cronje, 2002). Alternaria alternata phytopathogenic fungus infects lemons and tangerines causing 7 different diseases considered as dangerous for citrus (Tsuge, 2013). Phytopathogen micromycetes such as, A. alternata, A. brassicicola, A. solani, Stemphylium loti, S. calilstephi, S. trifolii, S. sarciniforme, S. lancipes, Stemphylium solani were isolated from lemon plantations, and their mechanisms of propagation and pathogenicity features have been determined (Peever, 1999). Phytopathogenic microorganisms belonging to Dothiorella viticia, Lasiodiplodia theobromae, Neoscytalidium hyalinum, Phaeoacremonium (P.) parasiticum, P. italicum, P. iranianum, P. rubrigenum, P. minimum, P. croatiense, P. fraxinopensylvaniram, Phaeoacremonium sp., Cadophora luteo-olivacea, Biscogniauxia (B.) mediterranea, Colletotrichum...
gloeosporioides, C. boninense, Peyronellaea (Pa.) pinodella, Stilbocrea (S.) walteri, Pestalotiopsis and Fusarium genera were identified in infected parts and tissue of citrus trees in Iran (Espargham, 2020) Diatrypaceae sp. micromycetes have been isolated from citrus leaves in Australia and Greece (Elena et al., 2006; Trouillas, 2010). In Australia, Argentina, Brazil, the United States, and the Philippines, phytopathogenic microorganisms C. paradisi, C. maxima and C. aurantium belonging to the genus of Eutypella were found to induce diseases in lemons. Phytopathogenic micromycetes because dangerous diseases such as root rot, gray rot, dry root rotin plants that affect the yield.

D. limoncola and D. melitensis micromycetes spread gray rot disease, which is the most common disease in citrus, leading to complete loss of yield. These phytopathogenic fungal strains develop very rapidly and maintain viability in plant stems, leaves, roots and soil even under extremal stress conditions (Guarnaccia, 2017). The major threat group of phytopathogens common in agriculture around the world is Fusarium species. A healthy and strong development of the root system of the seedling is required in growing seedlings in the establishment of citrus plantations. Fusarium phytopathogens infect lemon and tangerine seedlings with dry root rot, which has become a serious problem in some countries. F. solani micromycetes strain causes dry root rot disease and leads to yield loss due to its destructive effects on lemon cultivation. The varieties of orange Valencia Temprana, Valencia Tardía, Navel, Comondú and Sur are grown in Mexico and considered as main export product. Up to 40% of tangerine yields are los teaches year due to damage caused by micromycetes of the genus Aspergillus, Fusarium and Penicillium. Particularly, A. flavus, F. oxysporum, P. digitatum, P. italicum and P. variabiles strains of phytopathogenic micromycetes were identified (Ochoa, 2007). In the lemon plantation, 30% of the fruits of 400 trees were found to be covered with white mycelium and Green conidia. Their infected parts became rotten and soft, and in the samples taken from the affected parts of trees; P. italicum micromycetes were detected and isolated for morphologic analysis. Investigations have shown that phytopathogens cause gray rot in fruits (Hernández-Montiel, 2007). Mold fungi can also be included in the group of phytopathogenic fungi that damage citrus. Mold fungi cause fruit spoilage. A. niger micromycetes isolated from lime were found to develop actively at high temperatures (Sandoval-Contreras, 2010). High temperature and humidity in the closed groundsoils create favorable conditions for the development of the phytopathogenic fungus A. niger.

From various citrus fruits, 294 species of phytopathogenic micromycetes have been isolated and their feature of synthesizing secondary metabolites (toxins) have been studied. The fungi belonging to Penicillium, Aspergillus, Alternaria and Fusarium species actively synthesized the mycotoxins with strong toxic features. Deathwas observed in animals when the toxins synthesized by phytopathogenic micromycetes were tested in them (Albinas, 2002; Leila et al., 2014). P. expansum and P. crustosum phytopathogenic micromycetes are widespread fungi that threaten the storage of citrus (lemon, tangerine), pear and apple fruits. They develop in plant tissues and infect fruits (Gonzalez-Candelas, 2010). A common post-harvest disease in citrus is a green mold disease spread by the phytopathogenic fungus Penicillium digitatum that can cause 90% loss of total yield in dry and subtropical climates (Eckert, 1989; Moraes Bazioli, 2019). P. italicum (blue mold) is one of the most dangerous pathogens of citrus and can cause damage to citrus fruits even at low temperatures (<10 °C) that are strictly controlled for transportation and storage (Palou, 2002; Tingfu, 2020). A. fumigatus, Apergillus niger, A. flavus pathogenic fungi causing various putrefactive diseases were detected in sweet orange (Citrus sinensis L.) in Sokoto Metropolis city that may cause fruit spoilage by 22, 17, 25 and 36%, respectively. When healthy oranges were artificially infected phytopathogenic fungi, Rhizopus stolonifer, A. flavus were found to form a decay zone of 45 mm and 35 mm, A. niger 25 mm (Tafinta, 2013).

When using fungicides against phytopathogenic micromycetes, it is important to determine the fungicide resistance of the isolates because a very large fine is paid for any fungicide applied against a phytopathogen with high fungicidal resistance (Dukare et al., 2018). Global climate change and the need for sustainable agriculture require biological control of phytopathogenic diseases in citrus using antagonistic microorganisms, and acceptance of biocontrol as an alternative to the use of fungicides, and the production of ecologically pure products (Vega, 2014). Several mechanisms work in the tripartite plant-
phytopathogen-antagonist interaction system to stop disease and phytopathogen development (Spadaro, 2016). The main affecting mechanisms of the antagonist include competition in the nutrient medium and proliferation, antibiotic production according to the nature of the formation of substances of antibiotic nature, the formation of resistance induction through the synthesis of enzymes affecting mycoparasitism and the pathogenic cell wall (Dukare et al., 2018). As a result of the use of chemicals, there is an accumulation of pathogenic pests in the soil, a decrease in soil microflora, in particular, antagonists, and an increase in resistance to phytopathogens. Diseases transmitted by phytopathogenic microorganisms develop very rapidly, damaging the plant, the yield and the soil. During the years of strong development of diseases, in some districts of Uzbekistan, crop damage was observed at 50-70% (Green, 1999; Howell, 2003). This situation demonstrates the need for the use of biological control agents in protecting crops from disease-causing microorganisms. The biological control method is based on the use of natural antagonists in the struggle against phytopathogens (Wells, 1988; Harman, 2000). Biological control is an important strategy to combat phytopathogenic fungi and the diseases they spread. This principle is based on the use of living cells of antagonists in the elimination of economic damage and the struggle against phytopathogenic microorganisms (Cuthbert, 2018; Hernandez, 2019; Safdarpour, 2019). Micromycetes belonging to the family Trichoderma synthesize a number of biologically active substances as primary metabolites (enzymes), secondary metabolites (phytohormones) and more than 100 antibiotics and are used worldwide as a biocontrol agent (Elad, 1983; Hammond-Kosack, 1995; Sivasithamparam, 1998). The fungal strain T. harzianum 55 was found to synthesize phytohormone activity, secondary metabolites gibberellin acid (GA) and indole acetic acid (IAA) and antagonistic features against phytopathogen fungi (Fusarium, Alternaria, Verticillium, Aspergillus, Scopulariopsis, Rhizoctonia species) that is, A. flavus, F. vasenflectum, F. solani, S. brevicaulis, A. tenuis, R. solani, F. verticilloides, S. carbonaria, F. oxysporum, F.avenaceum, F. semitectum, F. gibbosum, F. sambucinum, F. javanicum, F. culmorum (Harman, 2004; Turaeva et al., 2020). IAA activates cell division in the growing part of the plant root and enhances root development, eliminates toxic metabolites produced by phytopathogenic microorganisms and directly controls root pathogens (Turaeva et al., 2019). Micromycetes protect plants from phytopathogens, increase seed germination capacity, enhance plant growth, increase metabolism of substances, expand leaf plate surface, improve soil structure, and increase porosity; they is considered as highly effective biological control agents with a mechanism of polefunctional effect on soil and plant. T. asperellum MG/6 and T. T-30 strains have high antagonistic activity against F. sambucinum and F. sporotrichioides strains (Inbar, 1994; Chet et al., 1998). Antagonistic effect of T. virens 3X and T. lignorum M-10 strains was studied against phytopathogen fungi F. oxysporum, F. solani, F. sporotrichiella Blai, Fusarium sp. that cause root rot and Fusarium diseases, white rot pathogens - Sclerotinia sclerotiorum, rhizoctonia pathogen - Rhizoctonia solani, it was also found that fungal strains belonging to the family Trichoderma had a complete dominance over pathogens (Lorito, 1998; Alizadeh et al., 2020; Vafaie, 2018; Rosado, 2007). Rational use of biocontrol agents to protect crops from phytopathogens in closed ground conditions, to obtain high and qualitative yields gives effective results (Srinivasa et al., 2014).

MATERIALS AND METHODS
Sample collections: Samples were brought from the local zonal varieties of lemon (F-1 Tashkent, F-2 Yubileyniy), tangerine (Tashkent), pamella (Zaymidin) grown in greenhouses (Fakhriddinov et al., 2020). From the leaves, roots, fruits and root rhizosphere of the diseased lemon and tangerine plant the samples were taken for analysis. Samples from roots and leaves were purified with 3% hydrogen peroxide, alcohol, and distilled water. The samples were ruptured in the cell shell using crushed glass under laboratory conditions and inoculated in nutrient media in a sterile state. 1 g was taken by weighing from samples obtained from the root rhizosphere and diluted to 0.5 ml in 1–10 test tubes filled with 5 ml of sterile water. 3-4 and 5-6 diluted samples were inoculated in nutrient media.

Samples prepared for microbiological analysis were grown in meat-peptone agar (MPA), potato-dextrose agar (PDA), Mandels, Oats agar (OA), agar Chapek nutrient media and placed in thermostats at temperatures from 20°C to 38°C. XSP-136 B and OLYMPUS BX 41 light magnifiers (capable of magnification up to 400 times) were used to identify
the types of microorganisms. In identifying species of microscopic fungi determiners of Pidoplichko N.M. (Pidoplichko, 1971), Litvinov M.A. (Litvinov, 1967), Bilay V.I., Aristovskaya T.V., (Bilay, 1982; Bilay, 1977; Aristovskaya, 1962; Tepper, 2004; Heng Mei Hsuan, 2011; Garibova, 2005) were used. Also, phytopathogenic fungi were detected by a mass spectrometry (MALDI TOF) in the sanitary-hygienic laboratory under the Ministry of Healthcare of the Republic of Uzbekistan (Kazakov, 2017). The antagonistic properties of Trichoderma harzianum 55 micromycete strain against phytopathogenic micromycetes isolated for the development of biological control measures against phytopathogenic fungi were determined by agar block method (Turaeva et al., 2019; Karimov et al., 2020; Turaeva et al., 2016).

STATISTICAL ANALYSIS

The experiments were carried out in three independent replicates and the results were expressed as mean standard deviations (SD). The SD values (represented as deviation bars) were determined using Microsoft Excel 2016 (Microsoft Corp., Redmond, Washington, DC, USA). The data were subjected to one-way analysis of variance (ANOVA) followed by a Tukey’s post hoc test where applicable, with the significance evaluated at p < 0.05.

Antagonistic property: The antagonistic property of microorganisms (micromycetes, bacteria, actinomycetes) is explained by the fact that they inhibit the growth of phytopathogenic micromycetes due to the synthesis of substances of antibiotic nature or form a growth ring against them (Bekmukhamedova, 2020).

Figure-1. Citrus plants grown in greenhouse and infected pamella (Zayniddin) fruit.
Antagonistic properties of the fungal strain of *T. harzianum*-55 in relation to phytopathogens isolated from citrus: The antagonistic property of the *T. harzianum*-55 fungal strain was determined by the agar block method. In agar block method the following were used: agar Mandels media with 2% saccharose (g/l): KH$_2$PO$_4$ - 2.0; (NH$_4$)$_2$HPO$_4$ - 1.4; MgSO$_4$ - 0.5; CaCl$_2$ - 0.3; saccharose - 2; agar - 16; microelements - 1 ml; (mixture of microelements: 500 mg FeSO$_4$; 156 mg MnSO$_4$ 4H$_2$O; 167 mg ZnCl$_2$; 200 mg CoCl$_2$; 1 ml 19% HCl in 100 mL distilled water) (Mandels, 1962). It was grown on a Petri dish for 6 days and 8 mm agar blocks were prepared. Phytopathogenic fungi isolated from citrus were inoculated in Chapek nutrient medium (g/l: KH$_2$PO$_4$ - 1.0; MgSO$_4$ - 0.5; NaNO$_3$ - 3.0; KCl - 0.5; glucose - 2; agar - 16; microelements - 1 ml; (mixture of microelements: 500 mg FeSO$_4$; 156 mg MnSO$_4$ 4H$_2$O; 167 mg ZnCl$_2$; 200 mg CoCl$_2$; 1 ml 19% HCl in 100 mL distilled water)) in 24 * 150 mm test tubes and grown in a thermostat at 28 °C for 6 days, and then poured 5 ml of sterilized water, 0.1 ml of the prepared suspension was taken and then planted in Petri dishes. 20 minutes after planting phytopathogenic fungi, 8 mm agar blocks of *T. harzianum*-55 fungal strain were placed. Grown and monitored for 5 days in a thermostat at 30 °C. The experiments were performed in three replications, the antifungal activity of the antagonist fungus was determined by the formation of a growth ring relative to the pathogen and the diameter of the ring.

Table 1. Antagonistic effect of *T. harzianum*-55 fungus on phytopathogens (4 days)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Strains of phytopathogen micromycetes</th>
<th><em>T. harzianum</em>-55 antagonist strain formed ring relative to phytopathogen micromycetes (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Fusarium vasinfectum</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>2</td>
<td><em>Verticillium dahliae</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>3</td>
<td><em>Alternaria alternata</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>4</td>
<td><em>Fusariumssemefectum</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>5</td>
<td><em>Aspergillusoryzae</em></td>
<td>60 ±1,1</td>
</tr>
<tr>
<td>6</td>
<td><em>Aspergillus flavus</em></td>
<td>55 ±1,2</td>
</tr>
<tr>
<td>7</td>
<td><em>Penicilliumfuniculosum</em></td>
<td>63 ±0,9</td>
</tr>
<tr>
<td>8</td>
<td><em>Fusariumsporotrichiella</em></td>
<td>70 ±0,3</td>
</tr>
<tr>
<td>9</td>
<td><em>Aspergillus</em></td>
<td>80 ±0,9</td>
</tr>
<tr>
<td>10</td>
<td><em>Aspergillusterreus</em></td>
<td>70 ±0,7</td>
</tr>
<tr>
<td>11</td>
<td><em>Penicillum chrysogenum</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>12</td>
<td><em>Penicillumdigitatum</em></td>
<td>60 ±0,6</td>
</tr>
<tr>
<td>13</td>
<td><em>Fusariumsolanii</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>14</td>
<td><em>Penicillium sp. 1.</em></td>
<td>45 ±0,7</td>
</tr>
<tr>
<td>15</td>
<td><em>Aspergillus sp.2.</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>16</td>
<td><em>Aspergillus sp.3</em></td>
<td>80 ±0,9</td>
</tr>
<tr>
<td>17</td>
<td><em>Fusarium sp.1.</em></td>
<td>65 ±1,1</td>
</tr>
<tr>
<td>18</td>
<td><em>Penicillium sp.2.</em></td>
<td>45 ±0,9</td>
</tr>
<tr>
<td>19</td>
<td><em>Fusarium sp.2.</em></td>
<td>70 ±0,6</td>
</tr>
<tr>
<td>20</td>
<td><em>Aspergillus sp.4.</em></td>
<td>Complete stop of pathogen growth</td>
</tr>
<tr>
<td>21</td>
<td><em>Fusarium sp.3.</em></td>
<td>45 ±0,9</td>
</tr>
</tbody>
</table>

**RESULTS AND DISCUSSION**

*Trichoderma harzianum*-55 fungus mani fested the highest level of antagonistic activity against phytopathogens *Fusarium vasinfectum*, *F. solani*, *Verticillium dahliae*, *Alternaria alternata*, *F. semefectum*, *Penicillium chrysogenum*, *Aspergillus sp.4* and it was found to stop growth of phytopathogen micromycetes (Figure 2). Pereira, (2014); Silva et al. (2016) noted in their research that *Trichoderma* fungi had high antifungal feature against micromycetes of phytopathogen *F. solani*. Hoffmann et al., (2015) reported that isolates of *Trichoderma* sp. micromycetes manifested antifungal activity up to 80 % against Fusarium sp. phytopathogen fungi. Tapwal et al. (2011) studied in the research that fungi of *Trichoderma* genus had high antagonistic activity against phytopathogen fungi of *Alternaria and Fusarium* genera. Al-Askar et al., (2016) reported that *Trichoderma* sp. fungus is considered as biological control agent against *Rhizoctonia solani* pathogenic fungi.
Fusarium vasinfectum

Verticillium dahliae

Alternaria alternata

Fusarium semefectum

Aspergillus oryzae

Aspergillus flavus

Penicillium funiculosum

Fusarium sporotrichiella

Aspergillus sp.1.

Aspergillus terreus

Penicillium chrysogenum

Penicillium digitatum
Figure 2. Antifungal activity of *T. harzianum* UzCF 55 fungus relative to phytopathogen fungi.

On the 4th day of the research experiment, *T. harzianum*-55 fungus was found to stop growth ring in relation to phytopathogen micromycetes *Aspergillus oryzae* (60-80 mm), *A. flavus* (55-70 mm), *P. funiculosum* (63-70 mm), *F. sporotrichiella* (70-80 mm), *A. terreus* (80-70 mm), *P. digitatum* (60-85 mm), *P. sp. 1.* (45-50 mm), *A. sp. 3* (80-70 mm), *F. sp.1.* (65-70 mm), *F. sp.2* (70-80 mm), *F. sp.3* (45-50 mm) and *P. sp.2* (45-73 mm) (Table 1).

**CONCLUSION**

The use of biological preparations based on the fungal strain *Trichoderma harzianum*-55 as a biological control agent in the control of disease-causing micromycetes in the cultivation of citrus can be used as the main means of protecting citrus from phytopathogenic micromycetes and diseases they spread. Thus, the results of the study testify to the development of a drug with a complex effect based on a local micromycet strain that protects against various phytopathogenic microorganisms and diseases they spread in the cultivation of citrus. The creation of biopreparations on the basis of local strains ensures the
efficiency of ecologically clean and high yields from crops due to the suitability of the soil and climatic conditions of the region.

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**Contribution of Authors:**

- **Bakhora I. Turaeva**: Conduct trials and wrote manuscript
- **Azamjon B. Soliev**: Helped in manuscript write up and research trials
- **Husniddin K. Karimov**: Prepared tables, figures and graphs
- **Nodira S. Azimova**: Helped in conducting research trials
- **Guzal J. Kutlieva**: Helped in manuscript write up
- **Khurshida M. Khamidova**: Supervised the study
- **Nigora Y. Zuxritdinova**: Conceive the idea of research and contribute in write up