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In Vitro Efficacy Testing of Fungicides on *Botrytis cinerea* causing Gray Mold of Tomato

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ABSTRACT: The tomato plant, during its vegetation period, is damaged by a number of diseases, such as bacterial wilt, early blight, Septoria leaf spot, late blight, gray mold, anthracnose, fusarium wilt. Among them, gray mold causes considerable damage to the crops. This article reveals information about the study of the effects of different fungicides of in vitro condition on the isolates of *Botrytis cinerea* fungus isolated from different infected parts (leaves, stem, fruit) of tomato plant. Herein, fungicides Difenoconazole and Cyprodinil with active ingredient have been used. The resistance of isolates of *B. cinerea* fungus was determined by considering their growth and development in culture media amended with different concentrations of fungicides. The results of the experiment showed that at natural population of *B. cinerea* fungus type, the isolates were supposed to be resistant in various levels to fungicides. It was identified that no any resistant isolates were noted towards Difen Super, 55% WP fungicide concentration of 0.08% with active ingredient – Difenoconazole, and fungicide Skor, 250 g/l EC with concentration of 0.05% and 0.07%, while in fungicide Chorus, 750 g/kg WDG concentration of 0.07 and 0.13% with active ingredient – Cyprodinil, *B. cinerea* fungus isolates were observed to have fungicide resistance.

Keywords: tomato, gray mold, *Botrytis cinerea*, isolates, in vitro testing, fungicide, resistance.

I. INTRODUCTION

Tomato plant, like other crops, is infected with several diseases, such as viral, bacterial and fungal diseases during its growth and storage period, and accordingly, these diseases cause a decrease not only in the yield of crops, but also the quality of their product. Tomato diseases like sprout diseases, fusarium, verticillium wilt, phytophthora, browning and stippled leaves (cladosporium), alternaria, graymold, viral diseases, non-infectious diseases, nematodes, damage all organs of the plant in growth period. Among these diseases, a damaging level of *Botrytis cinerea* Pers. ex. Fr. fungus which causes graymold disease is considered high enough in greenhouses [1, 40].

Botrytis cinerea Pers.; Fr. is a major pathogen of grapes and greenhouse crops, which causes graymold. In greenhouses, *B. cinerea* is a ubiquitous pathogen that causes severe losses in many fruit, vegetable and ornamental crops, and which can damage and even kill plants and affect the quality of the produce. The pathogen infects the leaves, stems, flowers and fruits in greenhouse plants [2, 3].

B. cinerea is a ubiquitous fungus. It causes graymold on almost all major greenhouse crops, initially entering greenhouses probably through open windows, cracks, on people and on young cuttings and seedlings. Later in production, infected plants or infected and decaying plant parts in the greenhouse form a source of inoculum. Young plant parts are generally not very susceptible but older plants or plant parts are more usually infected. The occurrence of *B. cinerea* is expressed in different crops in different phases. Tomato, cucumber and sweet pepper are the most widely grown vegetable crops. *B. cinerea* can cause severe problems in these and in many other vegetable, flower, potted plant and herb crops [3].

In tomato in non-heated greenhouses, the fungus infects flowers, fruits and leaves and can grow through the petiole into the stem [4, 5]. In heated greenhouses, *Botrytis* infection is almost completely limited to stem infection. Tomato is mostly grown according to the highwire method [6], with plants reaching lengths of 20-30 m with bundles of stems running along the floors and grown for up to 50 weeks. The leaves around the ripening fruits are removed thus causing pruning wounds. In heated greenhouses, *B. cinerea* mainly causes stem lesions by infecting such pruning wounds. Stem lesions may girdle the plant leading to plant death, though stem infections may remain quiescent for up to 12 weeks [7].

Chemical control remains the main way to reduce the incidence of graymold and other *Botrytis* diseases on major crops. The most common interventions consist of aerial spraying to the parts of plants with fungicides. The applied doses vary from 2000-3000 g/ha (e.g. maneb, thiram, dichlofluanid) to 400-500 g/ha (e.g. carbendazim, fludioxonil, pyrimethanil). The number of treatments during a season ranges from one or two, to more than twenty. Treatments of seeds or bulbs, as well as fungicide applications after the harvest of fruits, are also used [3, 8].

The chemical control of *Botrytis* diseases is impeded by the development of resistance to many fungicides and the negative public perception regarding the safety of pesticides. As a consequence, in many countries, the regulatory authorities have restricted the use of new and established pesticides [9, 39].

A number of research work has been conducted on the study of the fungicide resistance of *B. cinerea* isolates. Fungicide resistance of isolates of *B. cinerea* fungus isolated from sweet basil, grapes and tomato, has been investigated in Israel [10-12]. Occurrence of fungicide resistant strains is not the same in different countries. The occurrence of isolates of *B. cinerea* fungus which is slightly resistant to dicarboximide was observed after three years of application of these fungicides in England [13], while in Japan the strains which is slightly resistant to benomyl were determined in the fields where the treatment with this fungicide though was not performed [14].

The emergence of *B. cinerea* fungus isolates resistant to benzimidazole occurred a bit slowly in Hungary than in France, while in the fields where dicarboximide was applied, this case was not observed [15].

In Ontario province of Canada the occurrence of fungicide-resistant isolates was lower in vineyards where benzimidazole was applied against disease causing *B. cinerea* fungus compared to Europe [16].

In Germany since 1978 Botryticide fungicide with dicarboximide basis had been used against this disease in vineyards, where in 1979 fungicide-resistant isolates were observed. The occurrence of fungicide-resistant isolates varied depending on regions [17, 18]. Use efficiency of ronylan, rovral, procimidin fungicides in the fields began decreasing constantly from 1981 [19].

In Italy, incubation of resistant isolates of *B.cinerea* fungus occurred slowly. It was recorded that pathogen isolates which were resistant to preparations with active ingredients decarboximide and benzimidazole, were also observed in greenhouses and vineyards.

From different provinces of England *B. cinerea* fungus isolates were collected and they performed various levels of resistance to different concentrations of iprodione and benomyl. The strains of *B. cinerea* isolated from different greenhouses of Netherlands were found to have resistant forms of 0 to 100%to benomyl.

It was noted that isolates of *B. cinerea* that were resistant to benomyl, were contrarily non-resistant to the fungicides with active ingredient benzimidazole.

Most systematic fungicides are inactivated by fungi enzymes [20]. Under mutation impact, fungicides lose their inhibitory effect. Therefore, resistance of pathogens to systematic fungicides increases due to the changes of metabolites in fungus cell.

Taking into account aforementioned scientific references, it is known that Botrytis types perform different resistance to different fungicides. However, in Uzbekistan special research work haven't been conducted yet on the study of efficacy of fungicides against graymold and the occurrence of resistance to them. Considering this case, several fungicides were tested against graymold disease of tomato.

II. MATERIALS AND METHODS

On the study of graymold disease of tomato plant, the research work in the form of experiments were conducted during the years 2018-2019 in "Innovative work and counseling center in agriculture" State unitary enterprise at Tashkent state agrarian university and in greenhouses of "Fresh rose" LLC in Urtachirchik district of Tashkent region. On the study of efficacy of fungicides at in vitro condition and the disease, the experiments were carried out in the laboratory Agro-biotechnology at Tashkent state agrarian university.

Preparation of nutrient media. In the experiment, pure culture of isolates of Gray mold pathogen was isolated from the parts (stem, leaf, flower, fruit) of infected tomato. PGA-Potato glucose agar and Czapek dox agar nutrient media were used to isolate pure culture of graymold pathogen and for further investigations. For the preparation of potato glucose agar, 200 g potato, 20 g glucose and 20 g agar were used. Nutrient medium was prepared in the following procedure: (i) After washing and peeling the potato, it was cut into small cubes. (ii) Then, these cubes were left in 1000 ml water for 20-30 min. to boil on low heat. (iii) Having boiled the potato, it filtered through gauze fabric, then added boiled water unless it reached to 1000 ml. (iv) Added 20 g of glucose and 20 g of agar, boiled to dissolution. (v) Ready nutrient medium was poured into test-tubes by 10-15 ml, closed with corks, then, sterilized in autoclave for 15-20 min at 121ºC under 1 atmospheric pressure. Sterile test-tubes were put in a dish or bed and covered with paper or gauze. In autoclave the sterilized test-tubes were placed inclined, and nutrient media was alloyed [21-23]. Czapec dox nutrient medium was prepared from magnesium sulfate (0,5 g), dehydrated phosphate potassium (1,0 g), potassium chloride (0,5 g), iron sulfate (0,01 g), sodium nitrate (2,0 g), dextrose (30 g), agar (20 g), distilled water (1000 ml).

Isolation of pure culture. The tomato plant parts under the testing were cleaned from microorganisms, that is, its external parts were sterilized. Testing part of a plant was kept in 0, 5-1% solution of sodium hypochlorite (NaOCl) for 30 seconds or in 96% solution of ethanol for several seconds. Then it was washed three times with sterile water in the glasses. Besides this, 1% solution of potassium permanganate was used too for the sterilization of tomato plant parts. For this, the part of a plant was kept in the solution for 1 min. and then accurately washed with sterile water. Moisture chamber was used to isolate pure fungus from the parts of sterilized tomato by the abovementioned ways [24-26]. For this, filter paper was spread on the bottom of Petri dish and then sterilized at 120℃ under 1 atmospheric pressure for 20 min. in autoclave. Petri dishes with filter paper were moistened with sterile water in front of spirit lamp flame, and then the tomato plant was cut into 1-3 cm parts with scalpel heated at the flame. These parts were placed on each dish by 4-6 pieces. Petri dishes, in which plant parts were placed, were put in thermostats with 24-26ºC and observed from the third day. Fungi mycelia and spores appearing on the surface of plant parts were inoculated with microbiological hook in inclined agar nutrient media in the test-tube. When the fungi in the test-tube grew well, their species were identified [25-27].

In vitro tests of fungicides efficacy in nutrient media. Different concentrations of fungicides Difen Super, 55% WP (200 g/kg Difenoconazole + 350 g/kg Thiamethoxam), Skor, 250 g/l EC, Chorus, 750 g/kg WDG were tested during the experiments in the laboratory at in vitro condition against isolates of *Botrytis cinerea* isolated from different parts (leaves, stem, flower, fruit) of tomato plant infected with graymold disease.

Table 1: Description of fungicides used in the experiments.

S. No.	Active ingredient	Trade name	Formulations*	Chemical class	Application concentration
1.	Difenoconazole	Difen Super	WP, 200 g/kg	Triazole	0.07
					0.08
2.	Difenoconazole	Skor	EC, 250 g/l	Triazole	0.05
					0.07
3.	Cyprodinil	Chorus	WDG, 750 g/kg	Anilinopyrimidine fungicide	0.07
					0.13

**WP- Wettable Powder; EC- Emulsifiable Concentrate; WDG- Water Dispersible Granule.*

During the testing, different concentrations of fungicides taken for experiments, in the form of variants were added to nutrient media and prepared for the intended concentration to application before pouring dissolved PGA (Potato glucose agar) into sterile Petri dishes (Table 1). When agar nutrient media in Petri dishes solidified, the isolates of *B. cinerea* extracted from different parts of tomato plant were inoculated by variants in laminar clean bench by keeping sterile condition. Petri dishes with inoculated isolates, were placed in thermostat at 24-26 ℃, and the identification and recording of the growth of fungi on the surface of nutrient media were conducted on the 5th and 7th days of experiment.

The resistance index of *B. cinerea* isolates which were isolated from different infected parts (organ) of tomato was determined by comparing experimental and control variants. The diameter of the colonies of *B. cinerea* isolates of the experimental variants with fungicide treatment was compared to control variant without fungicide (Control (No. Fungicide)).

III. RESULTS AND DISCUSSION

Graymold disease pathogen *B. cinerea* infects all aboveground parts of tomato plant. In order to isolate *B. cinerea* isolates, the tomato plants were grown in the greenhouse of "Innovative work and extension center" state unitary enterprise at Tashkent state agrarian university (Fig. 1).

Fig. 1. Disease patterns (above) and their growth in Czapek agar nutrient media (below left) and wort agar nutrient media (below right).

Fungicide effects on the growth and development of *B. cinerea* fungus isolates were recorded in the 5th and 7th days.

In experiments, it was noted that fungicide resistance of *B. cinerea* strains isolated from different parts of tomato plant occurred differently. Intensive development of isolates was observed on the surface of nutrient media, and its isolates were found to be resistant to Chorus, 750 g/kg WDG (Cyprodinil) fungicide. Tomato stem isolates of 29.6%, leave isolates of 20.4%, fruit isolates of 18.8% relatively formed resistance to 0.07% concentration of this fungicide.

In the variant where 0.13% concentration of Chorus, 750 g/kg WDG fungicide used, these indications were equal to 17.5%, 12.5%, 11.3% relatively, and the resistance wasn't observed in the strains isolated from the flower (Table 2). When 0,07% and 0,13% concentrations of fungicide Chorus, 750 g/kg WDG were tested in added nutrient media, the diameter of colonies was formed by 29 isolates out of 32, in the nutrient media added with 0,07% concentration it was not more than 1-34 mm within 7 days (Fig. 2).

While in experiment variants where Difen Super, 55% WP fungicide was used, fungicide-resistant isolates were observed in the variants with 0.07% fungicide concentration, that is, the index was 1.4% in the strains isolated from the plant stem, 1.3% for the leaves and 1.1% for the fruit (table-2). Herein, under the effect of 0,.07% concentration of fungicide Difen Super, 55% WP only three isolates formed colonies of 1-3 mm in the nutrient media. In the experiment variants where 0,08% concentration of this fungicide used, no any fungi strains were observed.

All flower isolates were recorded to be non-resistant to this fungicide. There were no any resistant isolates to the 0.08% concentration of Difen Super, 55% WP fungicide and, 0.05% and 0.07% concentration of Skor, 250 g/l EC fungicide, that is, the mycelium growth was not noted in nutrient media.

In fungicide-free control variant the diameter of all isolates formed colonies which reached 60 mm within 7 days (Fig. 2).

The most used medium was PDA (potato dextrose agar). Conform to the literature, this medium is the most suitable for the development of this fungus, the growth of the mycelium, the conidiation and the development of sclerotia [28-30, 42]. It was also identified in our studies that the used PGA-Potato glucose agar and Czapek dox agar nutrient media was favorable for the growth of *B. cinerea* mycelium.

Table 2: Fungicide resistance of *Botrytis cinerea*strains isolated from tomato plants.

S. No.	Fungicides	Fungicides concentration, %	Isolates, isolated from infected organs of tomato plants	Fungicide resistance of isolates, %
1.	Difen Super, 200 g/kg WP	0,07	leaf	1.3
			bud	1.4
			fruit	1.1
		0,08	leaf	0
			bud	0
			fruit	0
2.	Skor, 250 g/l EC	0,05	leaf	0
			bud	0
			fruit	0
		0,07	leaf	0
			bud	0
			fruit	0
3.	Chorus, 750 g/kg WDG	0,07	leaf	20.4
			bud	29.6
			fruit	18.8
		0,13	leaf	12.5
			bud	17.5
			fruit	11.3

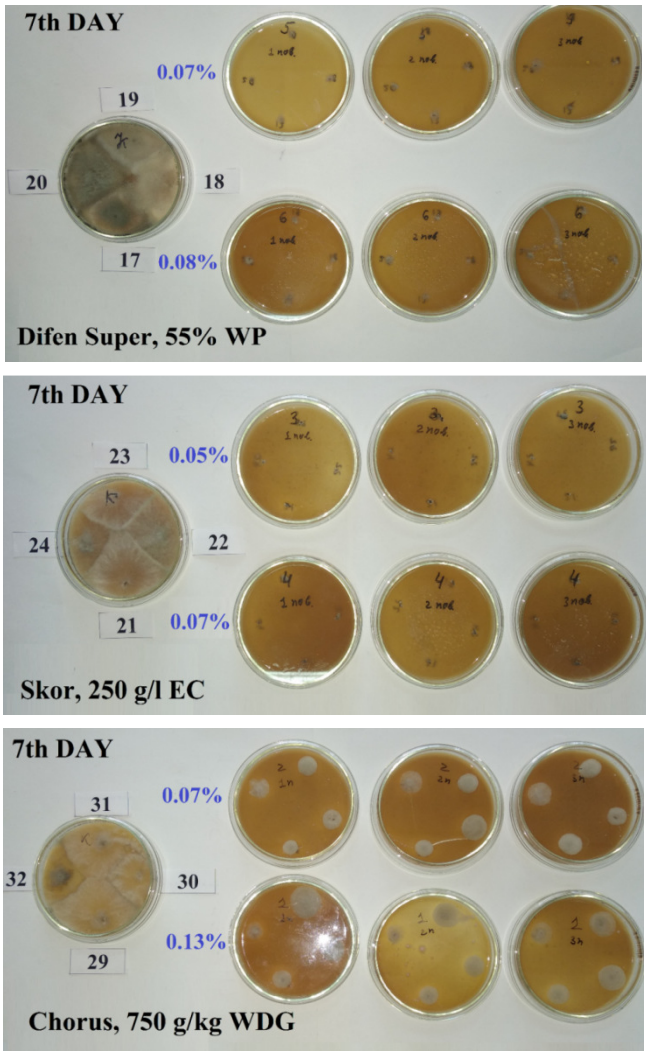

Fig. 2. The influence of different in vitro fungicides on the development of *B. cinerea* isolates (17, 18, 19, 20; 21, 22, 23, 24; 29, 30, 31, 32 – Control (without fungicide).

For the inoculation of the fungus the most used methods were the inoculation in a central point with *Botrytis cinerea* Pers. spore suspension in different concentrations [30, 31], but we didn't use central point inoculation method, since our purpose is to determine fungicide-resistance of the isolates of *B. cinerea* isolated from different parts (leaf, bud, fruit) of tomato plant to different concentrations of fungicides. Therefore, we carried out a testing of 4 types of *B.cinerea* isolates in one Petri dish (Fig. 2).

In this article, first reports have been presented on the study of fungicide-resistance of *B. cinerea* isolates obtained from tomato plant in Uzbekistan.

Overall, this study confirms the emergence of resistance of *B. cinerea* isolates to the fungicides (benzimidazoles-triazoles, anilinopyrimidines) that belong to different groups.

Multiple fungicide resistance of gray mold was previously reported in German, Chilean, and Italian vineyards [32, 33] and in other crops worldwide [34, 35]. Isolates resistant to both old and new botryticides have emerged over time in many crops worldwide [36-42].

IV. CONCLUSION

In accordance with the results of conducted experiments, it can be concluded that there were isolates with different resistance level in natural population of *B.cinerea* fungus type to the fungicides

No any resistant isolates were observed towards 0.08% concentration of Difen Super, 55% WP fungicide with active ingredient - Difenoconazole, and 0.05% and 0.07% concentrations of Skor, 250 g/l EC fungicide.

B.cinerea fungus isolates were found to be highly resistant to 0.07 and 0.13% concentration of Chorus, 750 g/kg WDG fungicide with active ingredient - Cyprodinil. In its turn, it shows that *B.cinerea* fungus is resistant to active ingredient Cyprodinil compared to Difenoconazole.

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Conflict of Interest. No conflict of interest declared.

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