CHARACTERIZATION OF CYLINDROCARPON-LIKE ANAMORPHS CAUSING ROOT AND BASAL ROT OF APRICOT AND *IN VITRO* ACTIVITIES OF SOME FUNGICIDES

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ABSTRACT

Four apricot nurseries were surveyed in Hatay province in Turkey to evaluate the phytosanitary status of the nursery plant material. Endophytic and potential pathogenic fungi were identified in plants and 12 Cylindrocarpon-like anamorph isolates were detected in the root system and basal stems of analyzed rootstocks. Based on partial sequencing ITS, three different Cylindrocarpon-like anamorph species were identified as *Dactylonectria torresensis* (6 isolates), *Dactylonectria novozelandica* (3 isolates) and *Neonectria candida* (3 isolates). Pathogenicity tests were conducted under greenhouse conditions which showed that all three Cylindrocarpon-like anamorph species, were identified as pathogens. ADt12 (*D. torresensis*) isolate, obtained from the survey area, have been tested *in vitro* for its sensitivity to several fungicides (thiophanate-methyl (70%), fluazinam (500g/L), fludioxonil (230g/L), and boscalid (26.7%)+pyraclostrobin (6.7%)). It was determined that ADt12 isolate was highly sensitive to fludioxonil and fluazinam, and sensitive to thiophanate-methyl and boscalid+pyraclostrobin as a result of probit analysis of EC_{50} values.

Key words: Apricot, characterization, Cylindrocarpon-like, fungicides, *in vitro.*

INTRODUCTION

Apricot (*Prunus armeniaca*) stands first in the world with a production area of 132.748 hectares and a total yield of 833.398 tonnes Turkey (FAOSTAT, 2020). Turkey ranks first in the world's total apricot production with 847 thousand tons, Uzbekistan ranks second with 537 thousand tons and Iran ranks third with 330 thousand tons (Turkey apricot product report, 2021). Apricot cultivation and consumption has increased greatly in recent years due to profitability. Nowadays, in the areas where stone fruit is grown, it is not possible to grow without management with monilia during the flowering period and freckle diseases after the flowering period. In addition, fungal disease factors that cause tree losses cause significant economic losses. One of these losses is the problems caused by soil-borne fungal disease agents.

Cylindrocarpon Wollenw are common and are isolated on dead plant material as root colonizers or pathogens or pathogens of a variety of weak herbaceous and woody plants (Brayford, 1993). And decay of many herbaceous and woody plants with high economic importance, including grapevine, olive, loquat, kiwifruit, apple, peach, avocado, strawberry and raspberry, and forest trees (Capote et al., 2022). This group of fungi has been commonly associated with wilting, root rot or bark necrosis in nurseries. (Mora-Sala et al., 2018). The genus Cylindrocarpon (Sordariomycetes, Hypocreales, Nectriaceae) was first described by Wollenweber in 1913 as the asexual form of Neonectria (Rossman et al., 1999). Its taxonomy,

based on morphological and phylogenetic analysis, has undergone many changes over the years. Currently, Cylindrocarpon-like anamorphs are considered a group that includes species of the genera *Campylocarpon, Cylindrocladiella, Cylindrodendrum, Dactylonectria, Ilyonectria, Neonectria, Pleiocarpo*n and *Thelonectria* (Marek et al., 2013; Capote et al., 2022). Teleomorphs with Cylindrocarpon anamorphs were traditionally classified in Nectria (Fr.) Fr., but are now considered to belong to Neonectria Wollenw (Rossman et al., 1999, Mantiri et al., 2001, Brayford et al., 2004). Wollenweber based this name on *Neonectria ramulariae* Wollenw (1916). The re-introduction of Neonectria resulted from the realization that Nectria was too broadly defined and that its segregation into numerous teleomorphic genera could be corroborated by anamorphic, phylogenetic, and ecological character patterns (Rossman et al., 1999).

To date, *Neonectria ramulariae* (anamorph: *Cylindrocarpon obtusiusculum*) has been associated with canker. In California, cold storage fruit and nut tree seedlings and *D. macrodidyma* have been associated with almond trees in California with no pathogenic assessment (Marek et al., 2013). In hazelnut products, *Cylindrocarpon destructans* (renamed *Ilyonectria destructans*) has been found (Lawrence et al., 2018; 2019). It has been reported as a pathogen on English walnuts in Italy (Montecchio et al., 1995) and *D. macrodidyma, D. novozelandica, D. torresensis, N. californica* and *Thelonectria aurea* have been associated with root rot symptoms in pistachio trees, but their pathogenicity has not been confirmed (Lawrence et al., 2019). *Cylindrocarpon macrodidymum* has been identified in vineyards in Turkey and Canada (Özben et al., 2012; Petit et al., 2011). The first report of a Cylindrocarpon-like anamorph species causing root and basal rot of almonds in Spain was recorded by Capote et al. (2022). There are currently a few specific recommendations for the control of root rot disease caused by Cylindrocarpon-like anamorph species. Further investigation of soil treatment and preparation is recommended to reduce soil pathogen populations as a way to manage disease and reduce possible infection and spread (Fourie & Halleen, 2002; Halleen et al., 2006). In addition, hot water treatment to seedlings, use of biological preparations (*Trichoderma* spp. mycorrhizae, bacteria), resistant varieties, rotation, soil fumigation and fungicides are used for disease control (Bleach, 2013).

The aim of this study is to characterize the anamorphs like Cylindrocarpon that cause root rot in apricot and to determine the *in vitro* activities of some fungicides.

MATERIALS AND METHODS

Disease surveys and fungal isolation

A survey was conducted in May-June 2019 in four apricot nurseries in Hatay, Turkey. The nurseries are located in the town of Samandag, which is an important sapling production center of Hatay. Rootstocks and cultivar mother plants were independently brought to laboratories for analysis for the detection of pathogenic fungi. There are domestic and foreign varieties such as 'Ninfa, orange ruby, şekerpare, aurora, Borsalino, roxana, mikado, magador, flopria' in the nurseries. During the survey, 30 samples were collected per rootstock (myrobolan) and per cultivar. Plant pieces of 3-4 mm in size, containing necrotic and intact tissues resulting from the infection, were disinfected from the surface for 4 min. in 2% NaOCl, rinsed in sterile distilled water for 2 times and left to dry on sterile blotting papers. The disinfected plant tissues were transferred to potato dextrose agar (PDA) medium containing 50 µg/ml streptomycin sulfate. Petri dishes containing PDA were incubated at 25°C for 5 days in the dark, and the mycelial structures taken from the ends of the fungal colonies growing in the tissues were purified by transferring them to new media containing the same medium.

Molecular characterization

The DNeasy (250) Plant mini kit (QIAGEN) protocol was applied for the isolation of genomic DNA from fungal cultures in accordance with the manufacturer's recommendations. The quantity and quality of the extracted genomic DNAs were measured with the Qubit 3.0 fluorometer. Molecular identification of fungal isolates from cultivar sapling was performed by sequencing fragments of the internal transcribed spacer (ITS), including 5.8S of the nuclear ribosomal DNA using the primers ITS1 (CTT GGT CAT TTA GAG GAA GTA A) - ITS-4 (TCC TCC GCT TAT TGA TAT GC). The PCR was prepared with 25 µl reaction volume, 0.2 µl of Taq DNA polymerase (Invitrogen) 5µl of 10× buffer, 0.5 µl of 50 mM $MgCl_2$, 0.75 µl of 10 mM dNTP, 0.5 µl of 10 mM primer, 30-40 ng of genomic DNA and set to final volume by sterile distilled water. The PCR was carried out on a thermal cycler with an initial denaturation at 95ºC for 3 min, followed by 35 cycles: 30 s denaturation at 95ºC, 45 s annealing at 55ºC and 1 min elongation at 72ºC, and a final elongation of 10 min at 72ºC. After PCR, all amplification products were separated with high-resolution capillary electrophoresis using QIAxcel Advanced System (QIAGEN, and Hilden, Germany). All the PCR products were sequenced by MEDSANTEK, (Istanbul, Turkey).

Pathogenicity test

One-year twigs from the 'mikado' apricot cultivar were inoculated with five representative isolates of the Cylindrocarpon-like anamorph species detected in nursery plants. Pathogenicity tests were conducted under greenhouse conditions and 1-year-old potted rootstocks unheated glasshouse at the Agricultural Research Station of Hatay Mustafa Kemal University, Turkey. Just before inoculation, root epidermis was lightly scraped with a sterile scalpel. 20 ml of conidial suspensions (10^6 conidia/ml) per plant was given to the root regions of the plants. Control plants were similarly treated with sterile distilled water. 15 plants per isolate were used, with 3 plants per replication. Disease percentage value was calculated by the formula [(%) = (infected plants / inoculated plants) × 100 %].

Fungicides

The fungicides included in the experiment were used in commercial formulations, such as; thiophanate-methyl 70% (Sumitop WP, SUMI AGRO), fluazinam 500 g/l (Nando SC, NUFARM), fludioxonil 230 g/L (Mertect SC, SYNGENTA) and boscalid 26.7%+pyraclostrobin 6.7% (Signum WG, BASF).

***In vitro* Fungicidal Studies**

Mycelial growth trial

The rate of inhibition of mycelial growth was determined by measuring radial growth in PDA containing a series of concentrations. Fungicides included in the study were used in the following concentrations: thiophanate-methyl 0.1-5µg/ml, fluazinam 0.001-0.01 µg/ml, fludioxonil 0.005-0.1 µg/ml, boscalid +pyraclostrobin 0.25-5 µg/ml. Specific concentrations of each fungicide were mixed into PDA medium cooled to 50ºC and poured into Petri dishes. PDA without any fungicide was used as control. Five milimeter diameter mycelial discs from *Dactylonectria torresensis* (ADt12 isolate) were cut from the edges of actively developed colonies in PDA for 5 days and were placed in the middle of Petri dishes containing approximately 10 ml of PDA, with the mycelial side down. Cultures were incubated for 3-7 days at 20ºC in the dark. Daily radial growth was calculated by measuring average colony diameters during this period. The experiment for each isolate was repeated 3 times for each fungicide concentration. 0 values from the experiments were evaluated and the range of EC_{50}, effective rates (%) were determined for mycelial growth.

Conidial germination trial

To determine the effect of selected fungicides on *Dactylonectria torresensis* (ADt12 isolate) conidia germination, appropriate volumes of water agar (WA) were added to the medium at the following concentrations: thiophanate-methyl 0.1-5 µg/ml, fluazinam 0.001-0.01 µg/ml, fludioxonil 0.005-0.1 µg/ml, boscalid +pyraclostrobin 0.25-5 µg/ml. The spore suspension was prepared by scraping with the aid of spoon using sterile distilled water and filtered through a 2-layer cheesecloth. The spore suspension was adjusted to $1x10^6$ conidia/ml with the aid of a hemocytometer. 10 µl of conidia suspension was pipetted dropwise into Petri dishes, each containing 10 ml of WA. The experiment for each isolate was repeated 3 times for each fungicide concentration. Petri dishes were then kept in the dark at 20ºC for 14-18 hours to allow the conidia to germinate. The percentage of conidia germination (100 spores for each treatment) and the length of germ tube (the germtube length was about at least three times of the conidium length) were estimated under a microscope. Results were expressed as effective concentration (EC_{50}) which is the concentration causing 50% reduction in the length of germ tubes.

RESULTS AND DISCUSSION

Disease surveys and fungal isolation

Thirty diseased plant samples were taken from 4 different nurseries where saplings were grown in Samandag district of Hatay province where the study was carried out. The most frequent symptoms observed on plants in the sampled nurseries such as chlorosis , deformation, wilting, dieback (Figure 1).

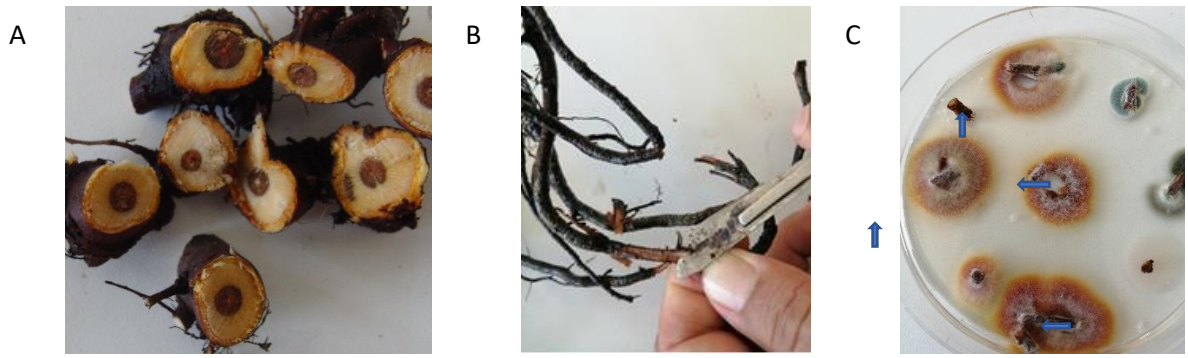

Figure 1. Internal (A) and black capillary roots (B) symptoms in ‘mikado’ apricot grafted onto ‘myrobolan’ and colony growth (C) on PDA of Cylindrocarpon-like anamorphs after isolation.

A total of 12 Cylindrocarpon-like anamorph isolates were obtained from the examined rootstocks. The incidence of Cylindrocarpon-like anamorphs in the four analyzed nurseries was 3%, 4%, 7% and 9%, respectively. The isolates were incubated at 24°C in the dark for 15 days, and slow-growing brownish, dense-buff colonies developed with luteous margins and a chestnut reverse-surface color.

Molecular characterization

As a result of fungal DNA isolations, genomic DNA was obtained in amounts ranging from 20-40 ng/µl. ITS (internal transcribed spacer), ~550-bp band were obtained from genomic DNA. BLAST program from the NCBI database, Cylindrocarpon-like anamorph species were identified as *Dactylonectria torresensis* (6 isolates), *Dactylonectria novozelandica* (3 isolates) and *Neonectria candida* (3 isolates). NCBI accession numbers were obtained from the selected isolates and deposited in the GenBank (Table 1).

Table 1. Representative isolates of Cylindrocarpon-like anamorph species obtained from apricot

Species	Isolates	Cultivar- Rootstock	Tissue	Accession No.
				ITS
Dactylonectria torresensis	ADt12	Flopria-Myrobolan	main root	ON303609
D actylonectria torresensis	ADt5	Mikado-Myrobolan	main root	ON303610
Neonectria candida	ANc2	Mikado-Myrobolan	main root	ON303611
Neonectria candida	ANc9	Magador-Myrobolan	main root	ON303612
Dactylonectria novozelandica	ADn4	Borsalino-Myrobolan	main root	ON303613

Dactylonectria torresensis was the most frequent Cylindrocarpon-like anamorph species detected in the rootstocks of nursery apricot (65.5%), followed by *Neonectria candida* (25%) and *Dactylonectria novozelandica* (10%).

Pathogenicity test

In the pathogenicity test performed on one-year-old 'mikado' potted apricot, all Cylindrocarpon-like anamorph species inoculated were able to cause necrotic lesions in the main and secondary roots and in the basal stem of the rootstocks, consisting of necrosis in sectorized areas. Inoculated plants showed sectorized necrosis in the main and secondary roots and the basal stem of the rootstock 3 months after inoculation. Representative five isolates of Cylindrocarpon-like anamorph species, were identified as pathogens. The inoculated isolates were re-isolated from the symptomatic tissues, fulfilling Koch's postulates (Table 2). No symptoms developed on the negative controls.

Table 2. Pathogenicity testing of Cylindrocarpon-like anamorph species: percentage of infected plants

Species	Isolates	Infected plants (%)
Dactylonectria torresensis	ADt12	73.3
Dactylonectria torresensis	ADt5	60.0
Neonectria candida	ANc2	40.0
Neonectria candida	ANc9	46.6
Dactylonectria novozelandica	ADn4	66.6

In the pathogenicity, *Dactylonectria torresensis* ADt12 isolate (73.3%) reached highest disease rate among 5 isolates of Cylindrocarpon-like anamorph species detected in the apricot plants, followed by *Dactylonectria novozelandica* ADn4 isolate (66.6%) (Table 2).

***In vitro* Fungicidal Studies**

Mycelial growth trial

When the effects of different fungicides on the inhibition of mycelial growth of *Dactylonectria torresensis* ADt12 isolate were examined, fluazinam and fludioxonil (100%) were found to have the highest effect at the lowest concentrations (0.01 and 0.1 µg/ml) (Figure 2). At high concentration (5 µg/ml), 100% effect was determined in thiophanate-methyl and boscalid+pyraclostrobin fungicides.

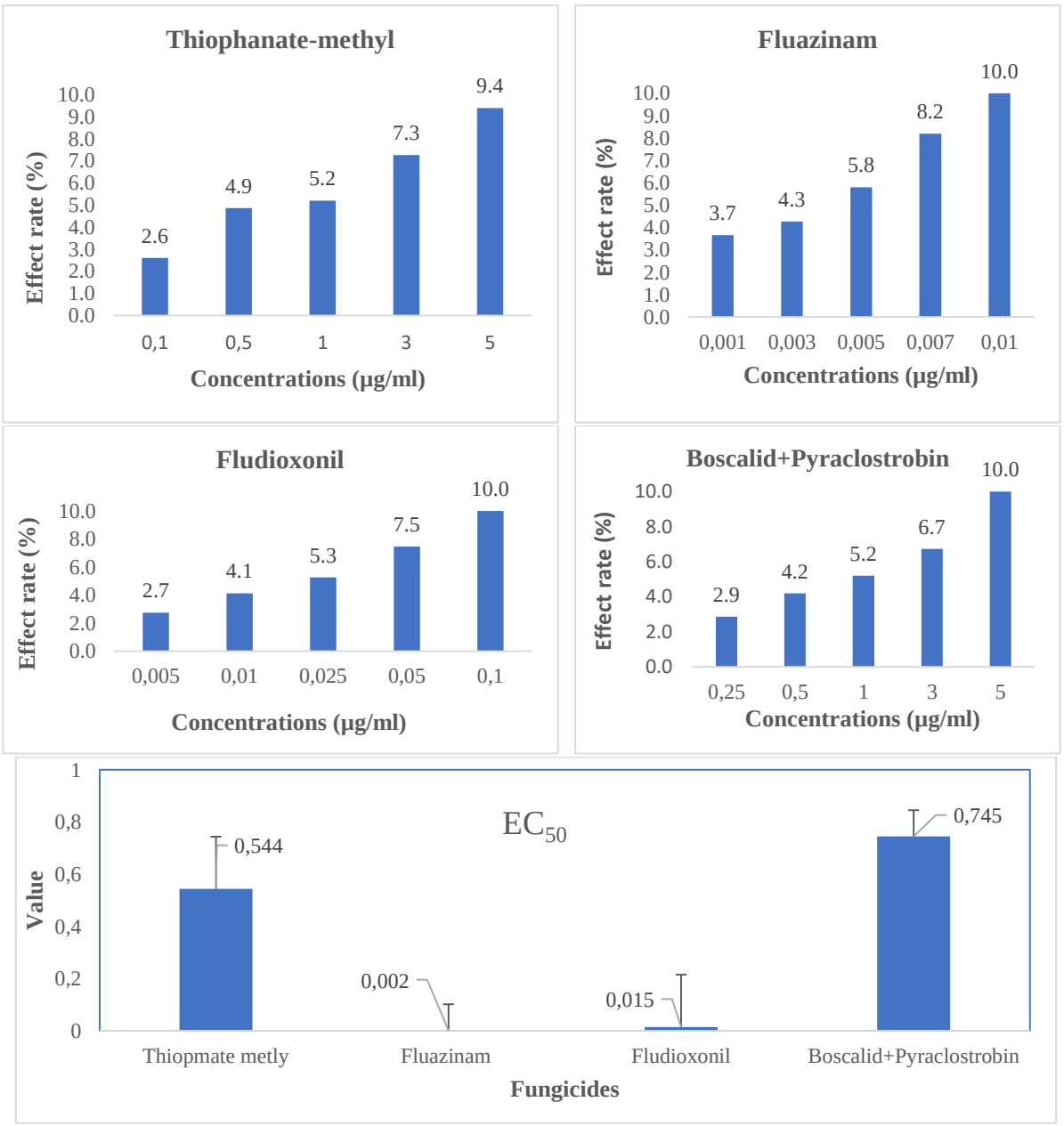

Figure 2. Effective rate (%) of thiophanate-methyl, fluazinam, fludioxonil, boscalid +pyraclostrobin fungicides on mycelial growth of ADt12 isolates

When the sensitivity of mycelial growth of *Dactylonectria torresensis* ADt12 isolate to fungicides was examined over EC_{50} values, it was determined that the fungicides fluazinam (0.002 µg/ml) and fludioxonil (0.015µg/ml) had the lowest EC_{50} values (Figure 2).

Conidial germination trial

When the effects of different fungicides on the inhibition of conidial germination of *Dactylonectria torresensis* ADt12 isolate were examined, fluazinam (96.7%) and fludioxonil (88.6%) were found to have the highest effect at the lowest concentrations (0.01 and 0.1 µg/ml). (Figure 2). High concentration (5 µg/ml) was determined in thiophanate-methyl (95.6%) and boscalid+pyraclostrobin (97.6%) fungicides.

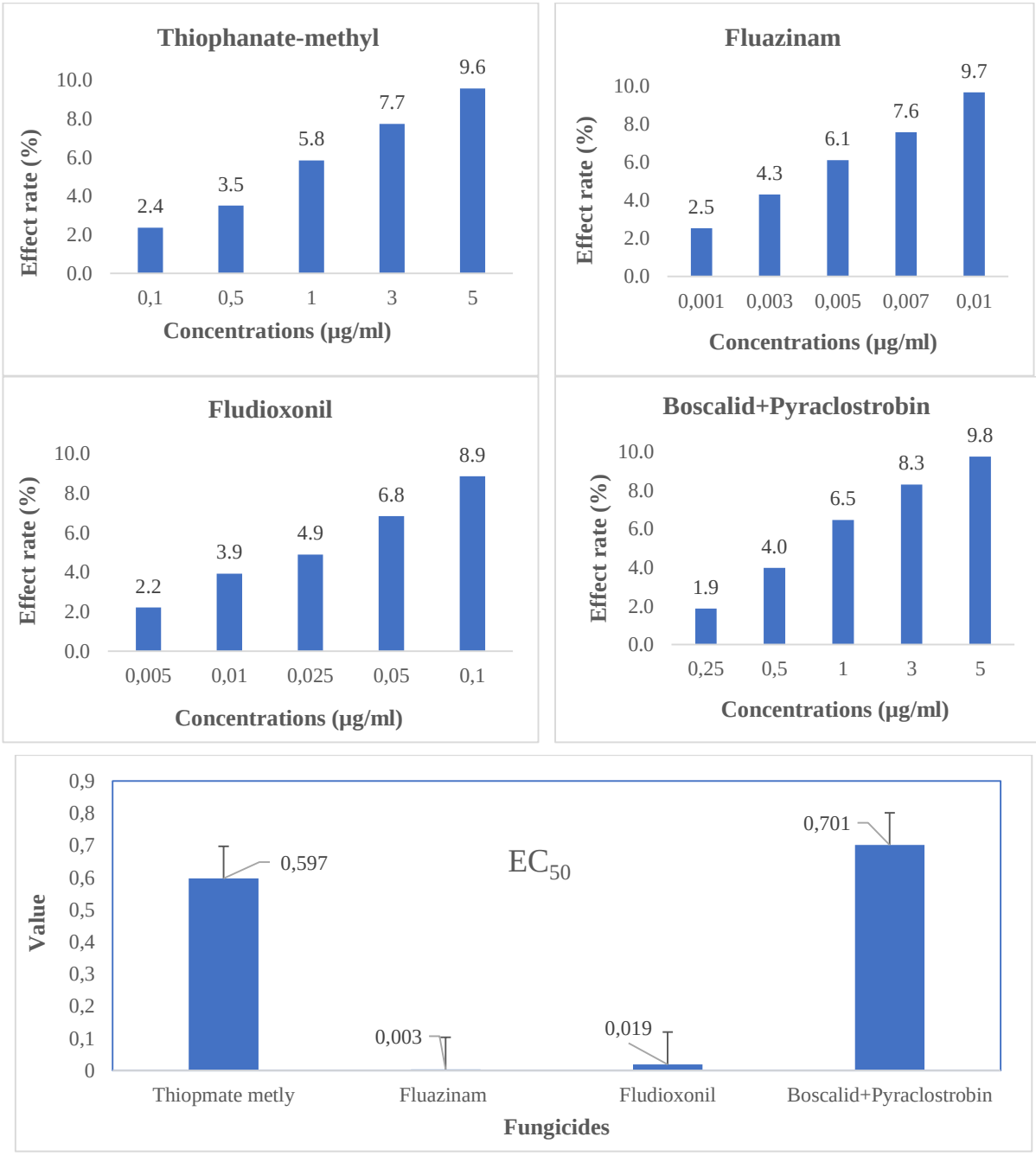

Figure 3. Effective rate (%) of thiophanate-methy, fluazinam, fludioxonil, boscalid +pyraclostrobin fungicides on conidial germination of ADt12 isolates

When the sensitivity of conidial germination of *Dactylonectria torresensis* ADt12 isolate to fungicides was examined over EC_{50} values, it was determined that the fungicides fluazinam (0.003 µg/ml) and fludioxonil (0.019 µg/ml) had the lowest EC_{50} values (Figure 3). It was observed that EC_{50} sensitivities in conidial germination were higher than in mycelial growth experiment. This study reveals the presence of Cylindrocarpon-like anamorph species that can cause root and crown rot in plant material collected from apricot nurseries in Samandağ district of Hatay province in Turkey. Three different Cylindrocarpon-like anamorphs have been isolated from the main and secondary roots of plants. Similar to other studies, these species were identified as *Dactylonectria torresensis*, *Dactylonectria novozelandica* and *Neonectria candida* (Mora-Sala et al., 2018; Capote et. al., 2022). In the pathogenicity tests, *Dactylonectria torresensis* ADt12 isolate (73.3%) reached highest disease rate Cylindrocarpon-like anamorph species detected in the apricot plants followed by *Dactylonectria novozelandica* Adn4 isolate

(66.6%). *Dactylonectria torresensis* was the most abundant Cylindrocarpon-like species found in the prospected nurseries. This species is the most frequent Cylindrocarpon-like anamorph associated with black-foot disease in grapevines in Italy (Carlucci et. al., 2017), Portugal (Reis et. al., 2013) and Spain (Berlanas et. al., 2017). In four different fungicide trials, all fungicides were found to be effective against Cylindrocarpon-like anamorphs. According to Lieten et al. (2021) out of 8 tested fungicides against *Cylindrocarpon destructans*, fluopyram combined with trifloxystrobin gave the highest efficacy. According to Alaniz et. al., (2011) the efficacy of 14 selected fungicides against *Cylindrocarpon liriodendri* and *Cylindrocarpon macrodidymum* was evaluated *in vitro* by testing their effect on mycelial growth and conidial germination. Carbendazim, hydroxyquinoline sulphate, imazalil, and prochloraz were the most effective fungicides in reducing mycelial growth in both Cylindrocarpon species. Captan, copper oxicloride, didecyldimethy- lammonium chloride and thiram were the most effective to inhibit conidial germination of both species. Accurate identification of cylindrocarpon-like anamorphs, their pathogenic characterization and determination of the effectiveness of some fungicides will help to prevent infections caused by these pathogens in commercial apricot nurseries or to establish the most accurate control method.

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