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BOSCALID + PYRACLOTROBIN RESISTANCE AND EXPRESSION OF *SUCCINATE DEHYDROGENASE* GENES (*sdhA* AND *sdhB*) IN CHESTNUT BLIGHT FUNGUS *Cryphonectria parasitica*

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Abstract: Sweet chestnut, *Castanea sativa* Mill., is a significant species in Türkiye. The tree has been encountered with numerous diseases. Chestnut blight, caused by *Cryphonectria parasitica* (Murrill) M. E. Barr, is one of the most common diseases having spread to every chestnut-growing region. In this study, the effects of Bellis® (25.2% Boscalid + 12.8% Pyraclotrobin), a fungicide commonly used in plant disease control, were evaluated at different doses (10 ppm and 50 ppm) against the chestnut blight agent *C. parasitica*, which had not been previously studied for its effects *in vitro*. The isolates included two virulent strains (Cp1_2023, Cp2_2023) and two hypovirulent strains (Cp7_2023, Cp9_2023). The effects of Boscalid + Pyraclotrobin on the mycelial growth of the pathogenic fungus were determined for the first time *in vitro*, and these effects were detected at the *sdhA* and *sdhB* genes expression level. The results showed that the mycelial growth and pathogenicity of *C. parasitica* isolates in the fungicide-treated medium were consistent with the gene expression profile. Specifically, the virulent Cp1_2023 isolate exhibited higher mycelial growth, pathogenicity, and gene expression levels compared to the other isolate. In conclusion, it is recommended to conduct more detailed studies before using fungicides and to employ them in controlled environments.

Keywords: Chestnut, *Cryphonectria parasit*ica, Fungicide, *Sdh* genes

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1. Introduction

The chestnut tree, *Castanea sativa* Mill., is not only a forest tree comprising 81.232 ha pure forest (OGM, 2021) but with the production of 80.200 tons (FAOSTAT, 2024) is also an important fruit tree as well in Türkiye. Chestnut blight, known as chestnut canker, poses a significant threat to chestnut fruit production. This disease is caused by the Ascomyceteous fungus *Cryphonectria parasitica* (Murrill) M.E. Barr. first documented in Türkiye in 1967 (Delen, 1975), the blight has persisted across all regions of the country ever since (Çeliker and Onoğur, 2001; Erincik et al., 2008; Akıllı et al., 2009; FAO, 2014), affecting chestnut orchards throughout Türkiye.

In the latter part of the twentieth century, the healing of cankers was initially reported in Europe. This phenomenon was attributed to a viral infection of the fungus, specifically identified as Cryphonectria hypovirus (CHV), owing to its capability to diminish the virulence of the pathogen (Grente, 1965; Choi and Nuss, 1992; Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004). Cryphonectria hypovirus 1 (CHV-1), like other mycoviruses in the *Hypoviridae* family, is a positive strand-RNA virus that resides in the cytoplasm of its host, reducing the virulence of *C. parasitica*. This characteristic, known as hypovirulence, has been harnessed for the bio-control of chestnut blight in countries where chestnuts are cultivated.

The most effective method in this agent is the implementation of biological control studies. Biological control involves using natural enemies to suppress pathogens and insects. The objective of this approach is based on the transfer of the virus from an infected fungal host to a non-infected one through hyphal fusion (anastomosis), ultimately leading to the overcoming of chestnut disease (MacDonald and Fulbright, 1991; Bisiach et al., 1995).

The most effective approach for controlling chestnut blight appears to be through biological control efforts. Alongside biological control, numerous fungicidal and fungistatic chemicals have been utilized in combatting this fungus. Methyl-2-benzimidazole carbamate, carbendazim, copper oxychloride, benomyl, and azaconazole, along with imazalil, are among the chemicals employed to manage chestnut blight. It has been demonstrated that the efficacy of certain chemicals

is not sustained in long-term therapeutic treatments (Jaynes and Van Alfen, 1977; Anagnostakis, 1982; Canciani et al., 1995; Aksoy and Serdar, 2004; Delen 1980, González-Varela and González, 2007). The feasibility of therapeutic treatments with chemicals does not appear to be a promising option for controlling chestnut blight (Rigling and Prospero, 2018). It has been reported that the application of these fungicides may induce phytotoxic effects and trigger the development of resistance in the fungus (Dias, 2012).

Some fungicide applications in forest areas have been prohibited in many countries. However, alongside these restrictions, some *in vitro* studies have investigated the effects of fungicides on the mycelial growth of the pathogenic fungus. González-Varela and González (2007) explored the impact of six agrochemicals (v-captan, epoxiconazole, azoxystrobin, folpet in combination with cymoxanil and ofurace, carbendazim plus flutriafol, and flusilazole plus carbendazim) on *C. parasitica* in an *in vitro*. Epoxiconazole was identified as the most effective product in *in vitro* studies, inhibiting fungal growth even at the lowest concentrations. Trapiello et al. (2015) demonstrated that epoxiconazole, in particular, is the most effective fungicide in controlling chestnut blight. Cheradil et al. (2022) tested the *in vitro* efficacy of four fungicides (Pictor-dimoxystrobin+boscalid, Amistar Sun-azoxystrobin+difenocanazole, Score 250 EC-difenocanazole, Cupraxat FW-tribasic-copper-sulphate) against *C. parasitica*. They indicated that Score 250 EC and Amistar Sun chemicals, which inhibited fungal mycelial growth, were the most effective fungicides. Azoxystrobin, pyraclostrobin, and boscalid are primary fungal respiration inhibitors widely used in plant diseases control. They have been reported to exhibit both therapeutic and eradicative effects (Rohel et al., 2001; Wong and Wilcox, 2002). The fungicidal activity of boscalid + pyraclostrobin compounds against various diseases is well-documented in numerous studies (Ritchie and Pollard, 2003; Schnabel and Powell, 2003). Pyraclostrobin is categorized as a strobilurin fungicide and belongs to the group of quinone outside inhibitor (QoI) fungicides. QoI fungicides hinder mitochondrial respiration by binding to the Qo site of cytochrome b, thereby interrupting the electron transfer between cytochrome b and cytochrome c1. This disruption leads to a disturbance in the energy cycle of the fungus (Andrade et al., 2022). Boscalid is known as a new fungicide group of succinate dehydrogenase inhibitor (SDHI) (Stammler et al., 2007). Succinate dehydrogenase (SDH), known as respiratory chain complex II, is one of the components of mitochondrial respiration, playing a crucial role in the tricarboxylic acid cycle (TCA cycle) and the electron transport chain (ETC). It is composed of hydrophilic subunits flavoprotein (FP, chian sdhA), iron-sulfur protein (Ip, chain sdhB), and two membrane-anchor proteins (CybL, chain sdhC; CybS, chain sdhD) (Zhang et al., 2019).

SDHI fungicides are crucial for protecting plants from a variety of phytopathogenic fungi. By attaching particularly to the mitochondrial complex II's ubiquinonebinding site (Q-site), these chemicals prevent fungal respiration (Russel, 2004). Newer active components (such boscalid, penthiopyrad, and fluopyram) exhibit broad-spectrum action against a variety of fungal species, in contrast to the first generation of SDHI fungicides (like carboxin), which was extremely active against fungi. But frequent use of site-specific fungicides, like SDHIs, might cause resistant fungal genotypes to be selected, which could eventually cause a sharp drop in fungicide efficacy (Stammler et al., 2007). As previously mentioned, research on fungicides indicates a reduced sensitivity in various pathogens, particularly observed against compounds like those in the benzimidazole group, phenylamide, EBI, dicarboximide, and dithiocarbamate. The response of pathogens to fungicides is reportedly unaffected by variations in isolate virulence. Another important aspect is that resistance emerges through mutations. Once a fungus gains resistance to a specific fungicide, it can also develop automatic resistance to other fungicides sharing the same mechanism of action. Although effective chemicals against chestnut blight have been identified *in vitro*, their application in forested areas is challenging. Additionally, the resistance that fungi develop over time against these chemicals should be taken into account. However, from a producer's perspective, biological control efforts are a long-term and challenging method of combat, especially in areas such as nurseries and reforestation sites. This highlights the necessity for a swift approach in combating *C. parasitica*, at least in regions where application is more feasible. Delen (1980) found chemical control efforts effective but did not recommend them due to concerns about resistance acquired by virulent isolates and potential adverse effects on hypovirulent isolates.

Previous studies have reported variations in the virulence among *C. parasitica* isolates. The effectiveness of the pathogenicity has been tested by researchers in biological control studies. Therefore, in the efficacy study of Bellis® fungicide, the impact on the mycelial growth of virulent *C. parasitica* with varying virulence and hypovirulent isolates was investigated *in vitro*. Controlling pathogens using chemical methods is highly challenging. However, in contemporary times, especially in plantation areas, the use of pesticides is widespread. To this end, the objective was to determine whether Bellis® fungicide (25.2% Boscalid + 12.8% Pyraclotrobin), commonly used in plant diseases caused by fungi, would affect *C. parasitica* in an *in vitro* setting. The aim was to assess its impact on the mycelial growth of the fungus and determine the expression levels of the fungus's *sdhA* and *sdhB* genes following fungicide applications. Two gene regions have been selected to determine fungicide sensitivity.

2. Materials and Methods

2.1. Collection of Bark Samples

Ten bark samples from the chestnut cankers were collected at Ordu province at Saraycık village in 2023. The samples were taken from the peripheries of the active cankers near the intact tissue by removing the bark, 1.0 cm in diameter, with a cork-borer from the two end points up and below and from the centre (OGM, 2014). The samples were placed between paper towels, kept in cool boxes, and brought to the laboratory, with the relevant information on them.

2.2. Isolation of *Cryphonectria parasitica*

Bark tissues were disinfected in 1% sodium hypochloride for 3 min and dried between paper towels. The bark disks were cut into two pieces transversely, about 2×2 mm were dissected from the adjacent tissue of the intact and diseased portions and they were plated on potato dextrose agar amended methionine and biotin (PDAmb; Difco, Sparks, MD, USA, 40 g, methionin 100 mg, biotin 1 mg, distilled water 1000 mL) medium. The plates were incubated at 25±2 °C. The white growth of the hypovirulent isolates on PDA and was used to distinguish them from the orange growth of the virulent isolates, as reported by Anagnostakis (1977).

2.3. Pathogenicity of *Cryphonectria parasitica* Isolates

Six virulent pure *C. parasitica* isolates namely Cp1_2023, Cp2_2023, Cp3_2023, Cp4_2023, Cp5_2023, Cp6_2023, respectively, were employed. Preliminary pathogenicity testing for virulent isolates was conducted using Golden Delicious apples (Fullbright, 1984). Initially, the apples were surface disinfected via 70% ethanol to cotton swabs. Each apple's surface was then punctured with 0.5 cm holes using a sterile cork borer with a diameter of 5 mm. The mycelial surfaces of seven-day-old *C. parasitica* isolates, cultivated on PDAmb medium, were placed on the apple surfaces with the mycelium facing inward. Sterile agar plugs were employed as controls. Inoculation points were sealed with moist cotton and wrapped with parafilm. The apples were individually bagged and incubated at 23±2 °C. Lesion assessment was conducted by measuring the necrotic area on apples bidirectionally on 15th days, following the method outlined by Fulbright (1984). The study was conducted with six replications, utilizing the Cp1_2023 and Cp2_2023 isolates.

2.4. *In Vitro* Test *Cryphonectria parasitica* Using Fungicide

In vitro studies were conducted to determine the impact of the Bellis® fungicide (25.2% Boscalid + 12.8% Pyraclotrobin) on the mycelial growth of *C. parasitica* isolates. Two virulent *C. parasitica* isolates (Cp1_2023 and Cp2_2023) and two hypovirulent *C. parasitica* isolates (Cp7_2023 and Cp9_2023) were utilized for this purpose. The poisoned food technique (Groover and Moore, 1962; Pundir et al., 2010) was employed to introduce the fungicide into the culture media at two different concentrations: 10 ppm and 50 ppm (Cheradil et al., 2022).

Potato dextrose agar media were prepared, allowed to cool to 60 °C after autoclaving, and then 1000 µL of stock fungicide solutions were added to 20 mL of PDA media. The mixtures were poured into sterile Petri dishes. Subsequently, mycelial plugs from seven-day-old cultures of *C. parasitica* were placed in the center of petri dishes with added fungicide, while similar plugs were placed in fungicide-free petri dishes as controls. All petri dishes were incubated in the dark at 25±2 °C. Fungal colony growth was measured on the third and sixth days of incubation (Cheradil et al., 2022). The percentage of inhibition in *C. parasitica* mycelial growth by the fungicide at different concentrations was determined using the following formula. (Mycelial growth inhibition (%)=(dc-dt/dc) × 100 (%); dc = average diameter of fungal colony in non-poisoned PDA (control), and dt= average diameter of fungal colony in poisoned PDA) (Shakeel et al., 2021). The mycelial growth diameter (MGD), expressed in mm, was calculated using the following equation (Owaid et al., 2018). Two perpendicular colony diameters were measured per plate. (MGD (mm) = (D1 + D2) /2; D1= Colony diameter 1, and D2=Colony diameter 2).

2.5. Bavendamm Test (Phenol Oxidase Test)

This test was conducted to assess whether the application of fungicides had an impact on the presence of mycovirus in hypovirulent isolates. The experiment involved cultivating hypovirulent isolates on treatment agar medium containing tannic acid (Bavendamm's medium), following the method outlined by Rigling et al. (1989). The Bavendamm test was conducted with two replications, and isolates exhibiting a weak color change were considered potential hypovirulent strains. As a virulent control, an EU-26 tester isolate free of CHV-1 was employed.

2.6. RNA Isolation and Complemantary (cDNA) Synthesis

For total RNA isolation, virulent *C. parasitica* isolates were cultivated on PDA medium at 25 °C for 7 days. Approximately 50 mg mycelium with conidia were scraped from the agar surface and treated with liquid nitrogen. RNA extraction was according to the manufacturer's guidelines (RNA Extracol; Eurx Ltd, Gdańsk, Poland). The resultant RNA measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA templates were stored at −80 °C till used.

The first strands cDNA synthesis kit (Eurx Ltd, Gdańsk, Poland) was employed to synthesize cDNA from 1 µg total RNA. Reverse transcription was carried out in 20 µl of reaction mixture containing 1 µg total RNA, 4 µL 5× cDNA Buffer, 1 µL mix RT (reverse transcriptase), 1 µL oligo (dT), 1 µL random hexamers, 12 µL nuclease-free water. The thermal cycling conditions of the reaction for cDNA were 10 min at 20 °C, 40 min at 50 °C, 5 min at 85 °C, and finally, 10 min at 4 °C.

2.7. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The actin was utilized as a housekeeping gene in the qRT-PCR study (Table 1), and the primers were constructed using the Primer3 v4.1.0 tool. qRT-PCR reactions carried out in accordance with manufacturer's instructions using SYBRTM Green PCR Master Mix in the CFX ConnectTM Real-Time PCR Detection System. 12.5 µL of 2 × real-time PCR Mix (SYBR Green I), 0.5 µL of primer, and appropriately diluted cDNA as a template were added to each 25 µL qRT-PCR experiment. The qRT-PCR settings were as follows: 20 seconds at 95 °C, 40 cycles of 30 seconds at 95 °C, 20 seconds at 54 °C, and 10 seconds at 72 °C. For each sample, three technical and biological duplicates were run in each qRT-PCR experiment. The data was analyzed using the $2^{-\Delta\Delta CT}$ technique, and statistics were computed using one-way ANOVA (Livak and Schmittgen, 2001).

2.8. Statistical Analysis

In order to determine the significance of mean mycelial growth diameter (mm) of the isolates, one-way ANOVA was applied on the percent disease values by using IBM SPSS Statistics 22 package programme and the significance was calculated by Tukey multiple comparison test ($P<0.05$). Statistical significance was determined by comparing the non-treated control with the treated samples. To compare the mycelial growth diameter (mm) of all isolates, an initial examination of data normality was conducted, and due to the non-normal distribution of the data, Kruskal-Wallis test was applied in some analysis.

3. Results

3.1 Isolation and Their Identification

Ten isolates were identified as *C. parasitica* based on their morphological aspects. Six isolates were identified as virulent, whereas four isolates were identified as hypovirulent.

3.2 Virulence in Apple

In the study, the pathogenicity of six virulent *C. parasitica* isolates was evaluated on Golden Delicious apples. In the experiment, conducted with six replications, the lesion area on the 10th day for the Cp1_2023 isolate measured 12.1 cm^2, while for the Cp2_2023 isolate, it was measured as 9.6 cm^2 (Figure 1). The measurements for other isolates are as follows: Cp3_2023 isolate recorded 9.3 cm^2, Cp4_2023 isolate measured 8.7 cm^2, Cp5_2023 isolate measured 5.6 cm^2, and Cp6 _2023 isolate measured 7.2 cm^2.

3.3 Inhibition of Mycelial Growth of *Cryphonectria parasitica* with Fungicidal Treatment

The mycelial growth of virulent *C. parasitica* isolates subjected to fungicide application at two different concentrations was measured on the third and sixth days. The results are presented in Table 2 and Table 3 (Figure 2). On the 3rd and 6th day measurements, it was determined that the two isolates differed in terms of mycelial growth. Percentages of inhibition of mycelial growth were calculated to assess the effects of fungicide on fungal mycelial development (Table 3). The percentage of inhibition of mycelial growth after the application of fungicide concentrations set at 10 ppm and 50 ppm is presented in Table 4. The mycelial growth of hypovirulent *C. parasitica* isolates was measured on the third and sixth days, and the results are presented in Table 4 and Table 5 (Figure 3). Even at both applied concentrations, the mycelial development of virulent *C. parasitica* continued beyond the sixth day, indicating that the fungus did not completely perish at these doses. In contrast, the growth of hypovirulent *C. parasitica* isolates treated with fungicide was slower, and the applied doses hindered the fungus mycelial growth.

Table 1. Primers sequences used for qRT-PCR experiment

Gene regions	Primers	Primer sequences (5'-3')	Product size (bp)
β-actin (housekeeping)	*β-actin*F	TGAGCAAGGAGATTACAGCATTGG	150
	*β-actin*R	CATACTCTGCCTTCGCAATCCAC	
sdhA	*SdhA*F	GCTAACTCCCTGCTCGATCT	207
	*sdhA*R	TTCCGTCTGCATGGTCTTCT	
sdhB	*sdhB*F	AGCATCTTCTCCGGTGTTGA	221
	*sdhB*R	AGATCGAGCAGGGAGTTAGC	

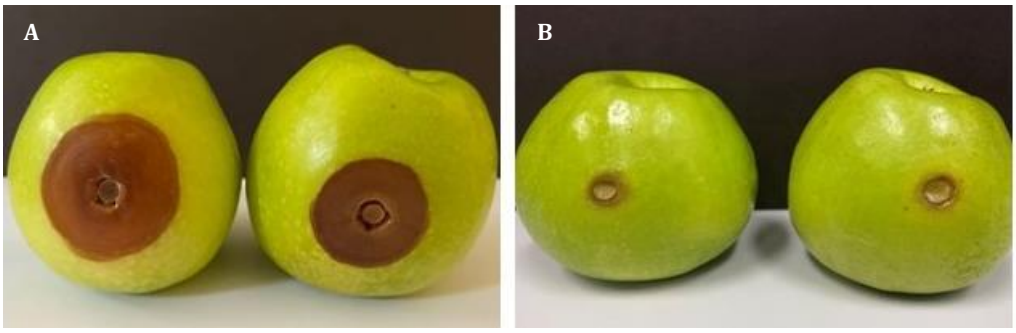

Figure 1. The lesion areas caused by *Cryphonectria parasitica* in Golden Apple test. Apples inoculated with Cp1_2023 and Cp2_2023 (A), control (B).

Table 2. Mean mycelial growth diameter (mm) obtained in third and sixth after treated fungicide

Isolate name	Fungicide concentration					
	Measuring the average size of fungal colony diameter (mm)					
	3th day			6th day		
	10 ppm	50 ppm	Control*	10 ppm	50 ppm	Control
Cp1_2023	12.3	9.9	17.4	25.9	23.1	48.1
Cp2_2023	14.1	12.8	26.7	27.3	26.8	58.2

*C= control petri, P<0.05 for 3th measuring; P<0.05 for 6th measuring.

Table 3. Percentages of inhibition of mycelial growth using fungicide on third and sixth days

Isolate Name	Percentages of inhibition of mycelial growth			
	3th day		6th day	
	10 ppm	50 ppm	10 ppm	50 ppm
Cp1_2023	29.3 %	43.1 %	46.1%	51.9%
Cp2_2023	47.2%	52.0%	53.0%	53.9%

Table 4. Mean mycelial growth (mm) obtained in third and sixth after treated fungicide

Isolate name	Fungicide concentration					
	Measuring the average size of fungal colony diameter (mm)					
	3th day			6th day		
	10 ppm	50 ppm	Control*	10 ppm	50 ppm	Control
Cp7_2023	10.4	8.6	23.6	20.2	17.0	33.0
Cp9_2023	11.7	10.2	27.9	22.9	20.2	50.4

*C= control petri, P<0.05 for 3th measuring; P<0.05 for 6th measuring.

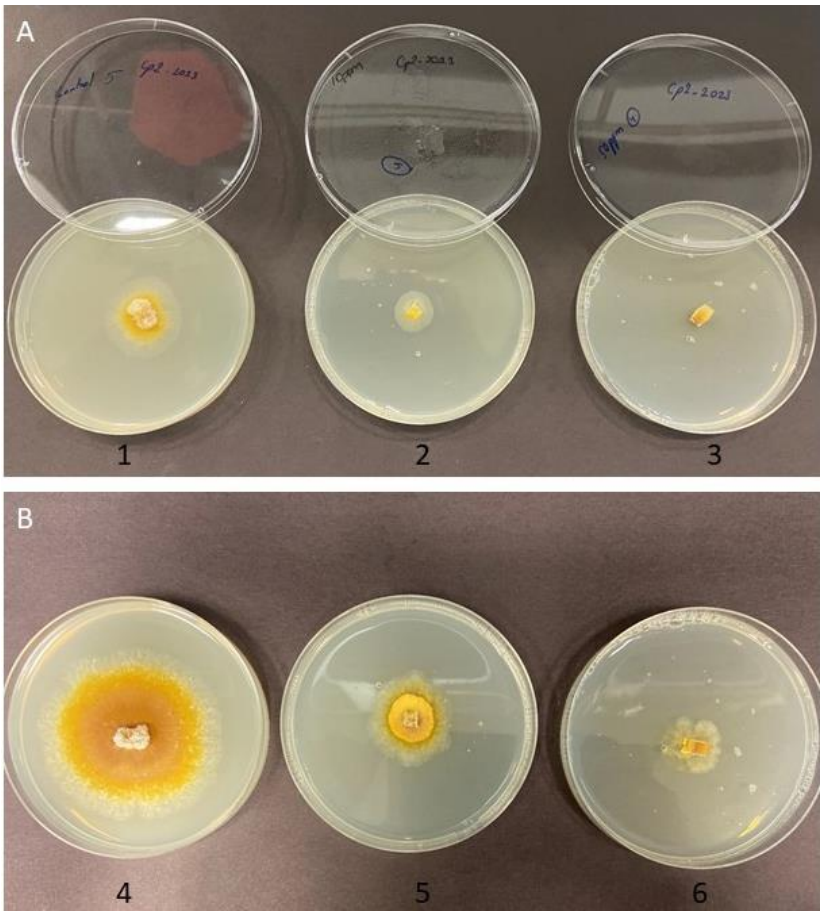

Figure 2. Mycelial growth of *Cryphonectria parasitica* (Cp2_2023) at 3th day on PDA treated with fungicide (A), mycelial growth of control petri (1), mycelial growth at 10 ppm (2), mycelial growth at 50 ppm (3); Mycelial growth of *Cryphonectria parasitica* (Cp2_2023) at 6th day (B), mycelial growth of control petri (4), mycelial growth at 10 ppm (5), mycelial growth at 50 ppm (6).

Table 5. Percentages of inhibition of mycelial growth using fungicide on third and sixth days

Isolate Name	Percentages of inhibition of mycelial growth			
	3th day		6th day	
	10 ppm	50 ppm	10 ppm	50 ppm
Cp7_2023	55.9%	63.5%	38.7%	48.5%
Cp9_2023	58%	63.4%	54.5%	59.9%

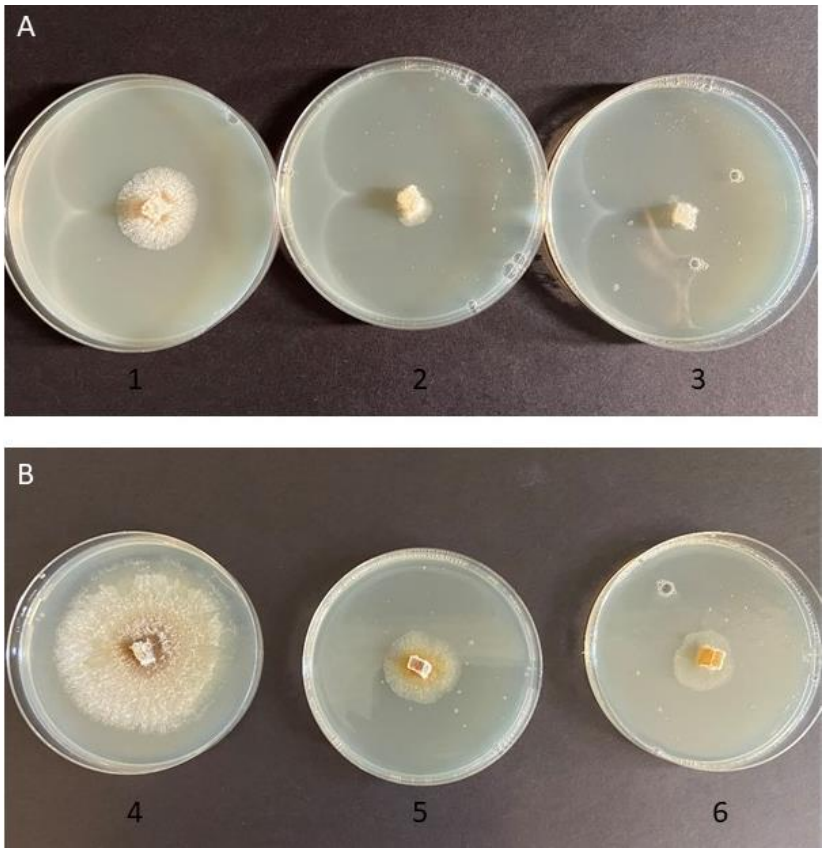

Figure 3. Mycelial growth of *Cryphonectria parasitica* (Cp9_2023) at 3th day on PDA treated with fungicide (A), mycelial growth of control petri (1), mycelial growth at 10 ppm (2), mycelial growth at 50 ppm (3); Mycelial growth of *Cryphonectria parasitica* (Cp9_2023) at 6th day (B), mycelial growth of control petri (4), mycelial growth at 10 ppm (5), mycelial growth at 50 ppm (6).

3.4 Bavendamm Test

Two (Cp7_2023 and Cp9_2023) of all hypovirulent isolates exhibited a weak color change on the Bavendamm test media, whereas the virulent control isolate, EU-26, displayed a dark color change (Figure 4).

3.5 Gene Expression

The molecular resistance of *C. parasitica* isolates to two concentrations of Bellis® was investigated in terms of *sdhA* and *sdhB* gene expression. When comparing the gene expression levels of Cp1_2023 and Cp2_2023 isolates of the highest virulence. It was observed that the expression levels of *sdhA* and *sdhB* genes in the Cp1_2023 isolate were higher than those in the Cp2_2023 isolate (Figure 5). Specifically, the expression levels for Cp1_2023 at 10 ppm and 50 ppm were calculated as 1.16 and 2.51, respectively, while for Cp2_2023, the corresponding expression levels were calculated as 0.72 and 1.55.

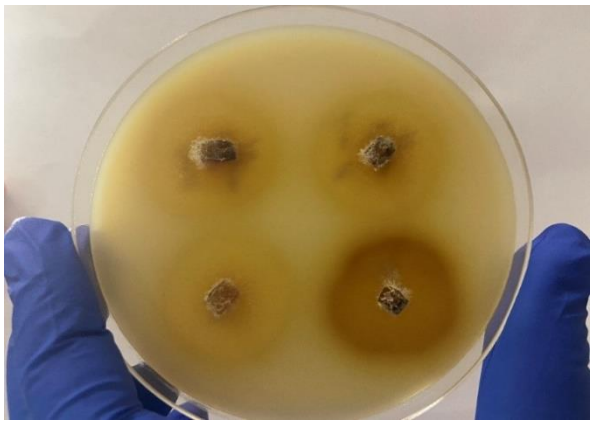

Figure 4. Growth of virulent (EU-26=dark colour) and hypovirulent (Cp9_2023=light colour) isolates in Bavendamm test.

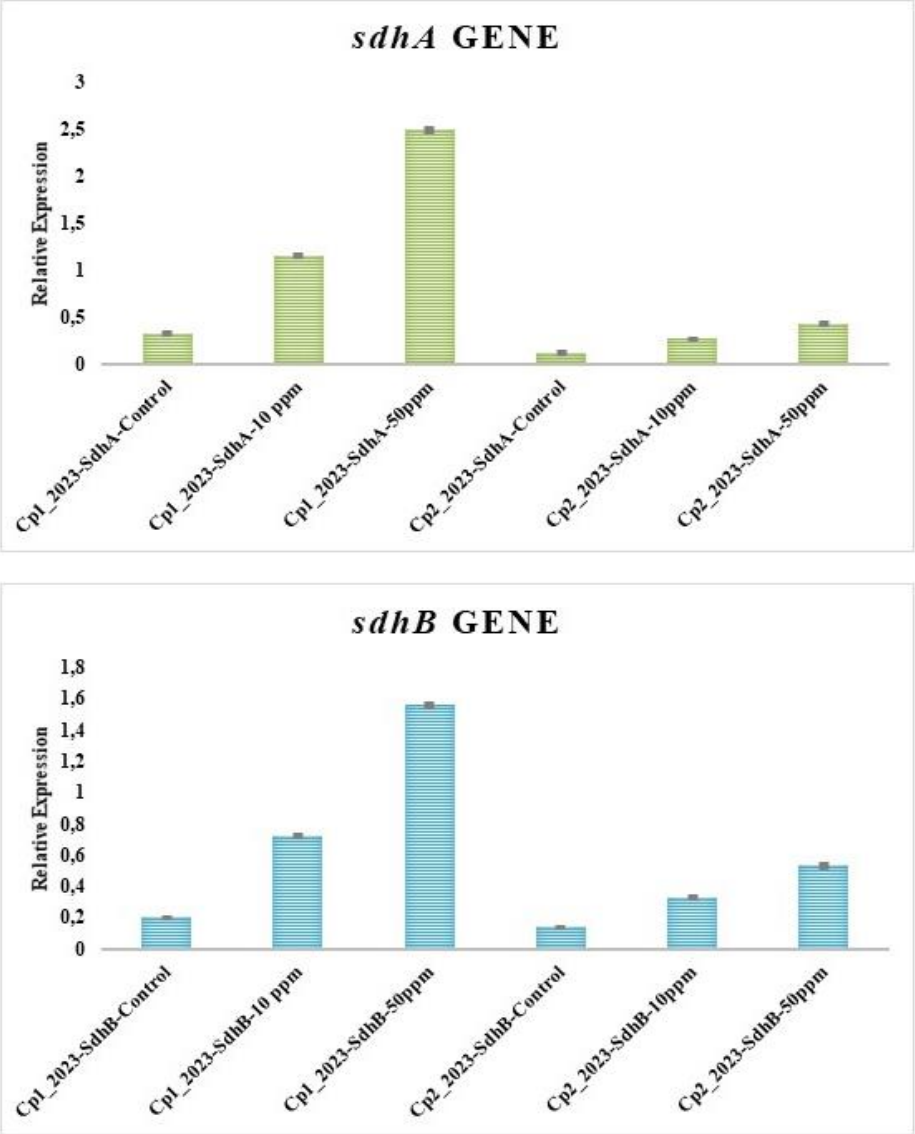

Figure 5. Effect of fungicide application against Cp1_2023 and Cp2_2023on *sdhA* and *sdhB* genes expression.

4. Discussion

The antifungal effect of the Bellis® fungicide, containing 25.2% Boscalid and 12.8% Pyraclotrobin active ingredients, against two virulent *C. parasitica* and two hypovirulent *C. parasitica* isolates, was determined *in vitro*. The percentage of inhibition caused by the fungicide on the mycelial growth of the fungus was calculated, and it was observed to prevent mycelial growth after colony measurements on the third and sixth days. The applied fungicide concentrations did not completely kill the fungus; however, they exhibited an antifungal effect on the fungus (Table 2, 3). In study conducted by Cheradil et al. (2022), the effects of four fungicides, namely Pictor (dimoxystrobin+boscalid), Amistar Sun (azoxystrobin+difenoconazole), Score 250 EC (difenoconazole), and Cuproxat FW (tribasic-copper-sulphate), on the mycelial growth of *C. parasitica* were investigated. They reported that the most effective fungicides were Score 250 EC and Amistar Sun, indicating their effectiveness on the fungus, even at the lowest doses, inhibiting the growth of the fungus. González-Varela and González (2007) reported that six agrochemicals (captan, epoxiconazole, azoxystrobin, folpet in combination with cymoxanil and ofurace, carbendazim plus flutriafol, and flusilazole plus carbendazim) exhibited varying degrees of inhibition on mycelial growth of *C. parasitica in vitro*. They noted a considerable variability in the percentage of inhibition across these chemicals. They identified epoxiconazole as the most effective chemical in their findings. However, in our study, the development of virulent *C. parasitica* was monitored after measurements on the sixth day, and it was observed that mycelial growth continued. The doses used were found to initially slow down the growth of the fungus but did not completely eradicate it. Some studies have reported encountering resistance of the fungus to Boscalid + Pyraclotrobin in fungal diseases (Kim and Xiao, 2010; Avenot et al., 2008). In our study as well, resistance of the fungus was encountered and the mycelial growth of the fungus continues after 3th and 6th days measurements.

The pathogenicity of virulent *C. parasitica* isolates test results indicated variations between virulent isolates. Differences in the pathogenicity of the fungus may have affected the rates of fungicide efficacy. The percentage of fungicide inhibition of mycelial growth of the fungus was found to be different between *C. parasitica*. We think that this difference may have been affected by the virulence of the fungus. Prospero and Rigling (2013) noted that none of the chemical alternative approaches have been systematically implemented on a large scale in field settings. González-Varela and González (2007) used chemicals in an *in vivo*, which were thought to have potential effects on fungus control, but these products are prohibited for use against *C. parasitica* in Spain. However, nowadays, nurseries and private garden owners use fungicides (Aiello et al., 2013; Gilardi et al., 2016). Due to the continued use of fungicides, various studies have been conducted to find the most suitable fungicide for controlling *C. parasitica* (González-Varela and González, 2007; Cheradil et al., 2022). Trapiello et al. (2015), in the context of chestnut blight control, evaluated agrochemicals against the pathogen in an *in vivo* environment. Researchers reported that Epoxiconazole was the most effective chemical in disease management. Researchers have suggested that chemicals could be recommended in enclosed spaces or under specific conditions, such as nurseries.

In the biological control studies against chestnut blight, this method is considered the most effective approach (Prospero and Rigling, 2018). In this study, the fungicidal effects were assessed on two hypovirulent isolates. It was determined that the application of fungicide at each dose inhibited the mycelial growth of the fungus. After the application of fungicide, the mycelial growth of hypovirulent isolates exhibited a slower rate compared to virulent isolates. It was observed that, following the sixth-day colony measurement, the hypovirulent isolates did not show any further development. Concluding that fungicide applications disproportionately impact hypovirulent isolates could have detrimental consequences for biological control studies, underscoring the importance of avoiding such misapplications. Additionally, it was observed that the applied fungicide dose did not affect the presence of the virus in hypovirulent isolates, and a Bawendamm test was conducted to confirm the absence of the virus.

Following fungicide application on the Petri dishes, there were variations in colony development among hypovirulent isolates, and a significant difference was identified according to statistical testing. The variation among isolates can be attributed to differences in virus concentration. The hypovirulent *C. parasitica* isolates, which developed in a fungicide-treated were subjected to the Bawendam test. In conclusion, it has been observed that, despite the cessation of mycelial growth in the fungus in a fungicide-treated, it did not lose its hypovirulent characteristic. In other words, as long as the fungicide does not kill *C. parasitica* isolates, the virus can persist and continue its life within the fungal cells. However, the duration of the virus's persistence can be determined through detailed research. Delen (1980) indicated that the application of carbendazim in combination could provide a long-lasting effect against *C. parasitica* but also mentioned the potential for the fungus to develop resistance. The development of resistance to fungicides by fungi has been reported in several studies (Avenot and Michailides, 2007; Miles et al., 2014).

Fungicide resistance may also be a valuable tool for advancing our understanding of the molecular mechanism of action of a certain class of compounds, even if resistance is a major role in lowering the effectiveness and life of vital, useful fungicides (Steffens et al., 1996). In strawberry fields, kiwifruit, and stored apples, resistance to a fungicide containing the active components boscalid + pyraclostrobin has been reported (Bardas et al., 2010; Kim and Xiao, 2010; Fernández-Ortuño et al., 2012; Fernández-Ortuño et al., 2014).

In our current study, results suggest that the *C. parasitica* isolates exhibit different fungicide phenotypes in response to different SDHI fungicide, Bellis®, concentration treatments and differ in their sensitivity to mycelial development and conidial germination. These results are consistent with the difference in *sdh* genes profile among isolates after fungicide treatment. The data show that the expression level of *sdhA* and *sdhB* genes significantly increased in Cp1_2023 isolate treated with fungicide at 10 ppm and 50 ppm concentration compared to the control. In Cp2_2023 isolate, an increase in the expression level of *sdhA* and *sdhB* genes was observed after fungicide application at two different concentrations compared to the control group. However, the gene profile increase observed in this isolate group was lower than Cp1_2023. In the fungicide experiments the mycelial growth of the two virulent isolates displayed variations. This difference was consistent with the gene expression profile, indicating that the isolate Cp1_2023, with a higher gene expression level, exhibited greater mycelial growth and pathogenicity compared to the other isolate. According to statistical test, the *P*-value for the 3th day was found to be 0.00, and for the 6th day, it was 0.00. This indicates a significant difference between virulent isolates the groups at a 95% confidence level.

Although there is no study on this subject in *C. parasitica*, it has been observed that *sdhA* and *sdhB* genes stand out as a result of boscalid application in *Alternaria solani* Sorauer, resulting in moderate or high-level resistance (Gudmestad et al., 2013). There are two mutations in the *AasdhB* gene sequence of *Alternaria alternata* (Fr.) Keissl. that confer high levels of resistance to boscalid (Avenot et al., 2008), while boscalid resistance in *Didymella bryoniae* (Fuckel) Rehm is the result of mutations in the *DbsdhB* protein in which histidine is replaced by tyrosine or arginine (Avenot et al., 2012). A similar situation exists in *Botrytis cinerea* Pers. and *Corynespora cassiicola* (Miyamoto et al., 2010; Yin et al., 2011).

5. Conclusion

In plant pathology, reducing the use of chemical fungicides is one of the key objectives. In this study, the effects of the previously untested Bellis® fungicide on the mycelial growth of the chestnut blight agent *C. parasitica* were examined *in vitro*, and the expression levels of the *sdhA* and *sdhB* genes were determined. According to these results, two different doses of the selected fungicide were found to be effective in inhibiting mycelial growth of virulent and hypovirulent isolates. However, it did not completely eliminate virulent *C. parasitica*, and based on the results of the gene expression profile, a resistance mechanism against the fungicide was determined to be present in the fungi. Additionally, for the two selected hypovirulent isolates, fungicide applied at the same doses indicated, according to the Bawendamm test results, that the presence of the virus persisted. It was observed that the development of hypovirulent isolates did not continue compared to virulent isolates. In future studies, a more detailed evaluation of the presence of mycoviruses by examining virus gene regions will provide more comprehensive information. We recommend conducting more detailed studies before the use of fungicides and suggesting for their use in controlled environments.

Author Contributions

The percentage of the author(s) contributions is presented below. All authors reviewed and approved the final version of the manuscript.

	E.D.T.	D.Ç.	S.A.Ş.
C	35	35	30
D	30	40	30
S			100
DCP	50	50	
DAI	50	50	
L	60	20	20
W	35	35	30
CR	30	30	40
SR	35	35	30

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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