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ORIGINAL ARTICLE

Unraveling the fungicide resistance in *Alternaria alternata* through integrated enzymatic, phenotypic, and colony growth analysis

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Abstract

Alternaria alternata causes black spots in a variety of fruits and vegetables. There are major post-harvest losses due to a hidden fungus that develops when fruits are kept in the low temperature and appears during the fruit marketing season. This study investigated how *A. alternata* develops resistance to fungicides and how growth rates are affected by various growth media. According to the results, the resistant strain grew more slowly in the EC_{100} medium than in the control media, which showed significant differences in radial growth across media. On the other hand, the wild strain in resistant media (WS-RM) showed less development, whereas the resistant strain in wild media (RS-WM) showed more growth. Wild strains multiplied, whereas resistant strains showed decreased mycelial growth. Biochemical assays revealed significant variations between resistant and wild strains. These distinctions are highlighted by the linear correlation (R^2 = 99.38%) between protein concentrations and absorbance variation. The wild strains' control protein ratio (CPr) was 0.192, whereas the resistant strains were 0.187. The mean values of MDA (360.89 nmol · mg^{-1} protein), CAT (35.54 U · ml^{-1} protein), SOD (179.60 U · ml^{-1} protein), and tyrosinase (52.18 U · ml^{-1} protein) in resistant strains were significantly higher than those in wild strains (MDA: 179.19, CAT: 11.91, SOD: 161.36, tyrosinase: 23.90). Standard deviations for all enzymes were more significant in resistant strain, indicating increased variability. According to the pathogenicity test conducted on *Populus nigra* leaves, the resistant strain's enzymatic reactions were demonstrated by the CK leaves' continued health, the RS plants' negligible symptoms, and the WS leaves' severe necrosis. These results highlight the necessity of further investigation into the molecular pathways underpinning interactions between plants and pathogens to create focused defense strategies. Improving crop tolerance to fungus infections and environmental stressors may result in more efficient treatments, lower agricultural losses, and forest protection.

Keywords: fungicide, leaf blight fungus, *p*oplar pathogenicity, resistant strain, wild strain

Introduction

The genus *Alternaria* was first described in 1816 by Nees von Esenbeck. It belongs to the phylum *Ascomycota*, class *Dothideomycetes*, order *Pleosporales*, and family *Pleosporaceae* (Woudenberg *et al.* 2013; Waqas *et al.* 2023). Since then, 275 species of the genera *Alternaria* have been discovered, and more than 1100 identities have been published (Gou *et al.* 2022). The ideal temperature range for the growth of *Alternaria* is 22 to 30°C, whereas it may flourish in temperatures as low as 2.5 to 6.5°C, and in regions with colder temperatures as low as between 0 to –5°C (Escrivá *et al.* 2017). The fungus *Alternaria* is widely distributed and comprises pathogenic, endophytic, and saprophytic species (Gul *et al.* 2022; Li *et al.* 2023). One of the most prevalent diseases affecting a wide range of commercial crops, including crucifers (such as cabbage, cauliflower, and broccoli), beans, cotton, citrus, and tomatoes, is a leaf spot and blight disease that is brought on by the

filamentous fungus *A. alternata* (Esfahani 2019; Golian *et al*. 2023; Ahmad *et al*. 2024).

Alternaria alternata is a fungal pathogen that causes rots, blights, and leaf spots on different plant sections. It affects about 380 plant host species. Severe yield losses may result from this infection (Budziszewska and Bereś 2024; Mmbaga *et al*. 2011). For instance, early blight damage to tomato plants has been recorded to cause output losses of up to 79% in Canada. This damage can lead to crop losses of up to 30% and postharvest losses of up to 10%. Disease management strategies including cleaning and crop rotation with non-host crops, are not completely effective because of the fungus's wide host range within the *Solanaceae* family, its long-life period in plant waste, and predominant airborne transmission (Tozlu *et al*. 2018).

The fast-growing, high-yielding tree genus Poplar (*Populus* spp) is essential for the ecological and economic health of the planet. In China, the widespread *A. alternate*-induced poplar leaf blight disease has had significant adverse economic effects (Wang *et al*. 2015; Bagherabadi and Zafari 2022). Poplar, an important timber forest species in China, faces numerous pests and diseases due to its single-stranded structure. Common diseases include leaf spots, ulcers, leaf rust, leaf blight, and branch and stem rot (Duong *et al*. 2024; Yang *et al*. 2024). In the field of agriculture, during the pre- and post-harvest phases, *Alternaria* species cause considerable losses. They can even survive under low temperature storage conditions. Thus, various physiological factors, such as pH, substrate, temperature, water activity, and so on, affect mycelial growth, sporulation, and virulence either directly or indirectly (Łukaszewicz *et al*. 2021; Ahmad *et al*. 2024).

Phytopathogenic fungi have developed a variety of reactive oxygen species (ROS) producing and scavenging mechanisms for maintaining the equilibrium of the oxidative status. Fungi have developed a complex antioxidant defense system consisting of various enzyme components like peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), which are essential for surviving in various stressful environments. Reactive oxygen species (ROS) are efficiently reduced, buffered, and scavenged by these enzymes, which guarantees their survival in a variety of situations (Zhang *et al*. 2020; Gul *et al*. 2022). Cellular components and unsaturated membrane lipids are protected from free radicals by the antioxidant defense mechanism (El--Beltagi and Mohamed 2013). Another crucial element of plant defense mechanisms is malondialdehyde (MDA), particularly when the plants are reacting to various environmental stimuli. Chemically speaking, MDA is a small, reactive chemical molecule that is present in many eukaryotic organisms (Morales and Munné-Bosch 2019; Muñoz-Pérez *et al*. 2024). SOD, an important enzyme for aerobic cells, transforms harmful superoxide radicals into oxygen and hydrogen peroxide. Hydrogen peroxide is further processed by potentially dangerous enzymes like peroxidase and catalase. SOD is the first defense against reactive oxygen species (Alam *et al*. 2021).

The radial development of *A. alternata* isolates on potato dextrose agar (PDA) plates was significantly inhibited by fungicides. The systemic triazole fungicide propiconazole is used to manage a variety of fungal diseases in a range of crops, such as cereals, fruits, vegetables, and ornamental plants. With increasing fungicide doses, mycelial growth inhibition increased significantly, with the higher fungicide dose resulting in total inhibition of the examined fungus (El-Ghany 2019; Nira *et al*. 2022). "Switch" is a noteworthy fungicide combination that can be used in a blueberry management program to stop *Alternaria* rot. This fungicide has two active ingredients: fludioxonil and cyprodinil. Whereas fludioxonil belongs to the phenylpyrrole fungicide class, cyprodinil is a member of the anilinopyrimidines fungicide class (Wang *et al*. 2022; Ziedan 2022). Fludioxonil was obtained from *Pseudomonas*; fludioxonil is a chemical derivative of pyrrolnitrin, a naturally occurring substance (Oiki *et al*. 2022). Fludioxonil is classified as moderately harmful by the World Health Organization (WHO) but toxic to aquatic species. According to European classification, it has long-term negative consequences (Elskus 2012; Haegerbaeumer *et al*. 2019).

In the present investigation, *A. alternata* stress responses to environmental stressors and the mechanisms underlying their antifungal resistance were examined. Microscopy and growth media tests were among the methods used to examine the phenotypic characteristics, growth patterns, pathogenicity on poplar leaves (*Populus nigra*), and biochemical reactions of fungal strains. Antioxidative defense mechanisms, such as soluble protein levels, catalase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD), were the subject of biochemical tests. The research showed how environmental factors and fungal growth dynamics are related, highlighting the need for specialized disease management strategies and more investigation into molecular pathways to improve food security and sustainable farming.

Materials and Methods

Alternaria alternata preparation of conidia suspension

The PDA medium was prepared and preserved *A. alternate* strains (AaNEFU1) were activated. These strains had been isolated from infected poplar leaves at

Northeast Forestry University, provided by the Laboratory of Forest Pathology (Harbin, China). This strain can be found in GenBank with accession number PQ605768, although it is not a part of any official microbiological collection. After this, they were incubated at 25°C for about 15 days to allow for sporulation. After sporulation, 1 mL of sterile water was added to the *A. alternata* culture on the PDA plate. A sterile coating rod was used to gently scrape the mycelium and collect the spores. The spore suspension was transferred to a centrifuge tube and centrifuged to remove the supernatant, leaving behind the concentrated spore suspension for storage. Ten µl of spore suspension was evenly distributed on a PDA culture medium and incubated for 1 day or several days. At least 10 single colonies were cultured.

Comparative analysis of *Alternaria alternata* growth on fungicide-treated media

The objective of the present experiment was to observe the responses of fungus *Alternaria alternata* to various growth media types and environmental factors. For this experiment, four different media types were used. The CK media – PDA was used as the control and the wild strain of *A. alternata* was allowed to grow without any further treatments. Since the media EC_{100} had resistance, a fungicide was applied to the PDA. On the treated media, subsequently a strain of *A. alternata* (AaNEFU1) was inoculated that had previously been identified as resistant to this fungicide (Fludioxonil). For resistance strains, wild media (RS-WM) were used. As in the control media, PDA was used. To create an environment where the resistant strain could grow under usual conditions, it was inoculated with the same fungicide-resistant strain. The wild strain of *A. alternata* was isolated and subsequently inoculated on a wild strain's resistance media (WS-RM) medium that had been treated with the same fungicide as the EC_{100} media. Using this media setup and strain inoculation, there were three replicates of each culture medium. Every plate was incubated for 5 days. Throughout the incubation period, the colony diameter of each strain was measured regularly to monitor its growth. Each measurement was entered into a spreadsheet for further analysis after compiling all the data.

Phenotype analysis of resistant strains

Spore suspensions were examined from a resistant strain of *A. alternata* treated with an EC100 dosage of fludioxonil under a microscope. The spore germination rates were observed and recorded for both the wild and resistant strains. A PDA medium was used that contained potatoes, dextrose, and agar. For 7 days, these cultures were incubated at 25°C in an incubator. Using a fluorescent microscope, conidia size, color, septation, branching of the conidiophore, diameters of the catenulate conidia, spore germination rate, and structure were examined to characterize *Alternaria* spp. The resistant strain and the wild strain were compared in order to understand the differences between them (Saleem and El-Shahir 2022).

Determination of soluble protein content

The Coomassie brilliant blue G-250 protein staining method was used to determine the soluble protein content. A mycelium sample (0.1 g) was weighed, 1 ml of 0.9% physiological saline was added to an ice bath, ground into a homogenate, and centrifuged at 8000 × g at 4°C for 10 minutes. 150 µL of the supernatant was transferred to an enzyme-linked immunosorbent assay (ELISA) plate. Then, 200 µL of 1× G250 staining solution was added to each well of the plate (Beijing Solar Bio Science & Technology Co., Ltd., Beijing, China). The staining solution was allowed to stand at room temperature for 5 minutes, and the absorbance value at A595 nm was measured using a microplate reader. Protein concentration was calculated from a standard curve prepared under the same conditions, and three biological replicates were performed for each treatment.

Determination of malondialdehyde (MDA) content

The content of malondialdehyde (MDA) in *A. alternata* was determined using a malondialdehyde kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), in accordance with the manufacturer's instructions.

Determination of catalase (CAT) activity

Catalase (CAT) activity was measured using a catalase kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), in accordance with the manufacturer's instructions.

Determination of superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity was measured using a SOD assay kit provided by Beijing Solar Bio Science & Technology Co., Ltd. (Beijing China), following the manufacturer's instructions.

Determination of tyrosinase activity

The tyrosinase activity of *Alternaria alternata* was measured using a kit provided by Beijing Solar Bio

Science & Technology Co., Ltd. (Beijing China), following the manufacturer's instructions.

Alternaria alternata pathogenicity on poplar leaves under stress from fludioxonil

To activate the strain of *A. alternata*, a PDA plate was inoculated with the purified strain that had been kept on a PDA slant. Before using, the plate was kept at 25°C for 5 days. Young, healthy poplar leaves were collected. The leaves were rinsed with fresh water, then with 70% ethanol their surfaces were wiped gently to disinfect them. Then, the leaves were cleaned with sterile water and reserved for later use. The inoculation needles were sterilized and the poplar leaves were carefully punctured to make tiny punctures. To retain humidity, the injured leaves were placed in a sterile container lined with moist, sterile non-woven cloth and kept ready for inoculation. Fungal discs were extracted from the prepared plates of the resistant *A. alternata* strain and the wild-type *A. alternata* strain using a sterile hole punch (5 mm in diameter). The control group was consisted of resistant *A. alternata* strain + wild-type *A. alternata* strain + uninoculated, healthy leaves. The fungal discs were introduced onto the wounds made on the poplar leaves. For 5 days, the inoculated leaves were kept in an incubator set at 25°C with adequate moisture. To prevent further fungal growth and disease progression, the fungal discs were removed from all treatment groups (control, wild-type, and resistant strains) on Day 5. Following this removal, photos were taken, and measurements and documentation of the disease lesions' sizes were made. The lesions shown on Day 5 indicated the maximum disease severity the pathogen had produced during the incubation period up to that point because the removal of the fungal inoculum stopped additional fungal infection. The size of the disease lesions that formed on the leaves during incubation was measured and photographs were taken for documentation (Verma *et al.* 2020; Dreischhoff *et al.* 2023; Roy *et al.* 2023).

Statistical analysis

A one-way analysis of variance (ANOVA) was used to evaluate the differences in growth rates between different strains and types of medium. With a significance level set at $p < 0.05$, the Tukey test for comparisons was used to establish statistical significance. Each enzyme activity measurement performed in three biological replicates for each treatment. Data were analyzed using Microsoft Excel and Origin Pro, and the results are expressed as means ± standard deviations (SD). The OriginPro Software was used to create the graphic representations (Kumar *et al.* 2019).

Results

Effects of different fungicide-treated media on *Alternaria alternata* growth rate

The results demonstrated that *A. alternata* growth rates were influenced by several media types. Figure 1 illustrates how various media types support the growth of the fungal strain. The mean radial growth of *A. alternata* varied considerably across all studied media. With a mean of 5.97 (SD = 0.07), the fungus exhibited the highest level of radial growth on potato dextrose agar (PDA) among the various treatments (media) examined. This observation is consistent with expectations because PDA is a standard medium that has no internal resistance to *A. alternata*. Conversely, media containing compounds that were resistant to *A. alternata* showed significantly reduced growth

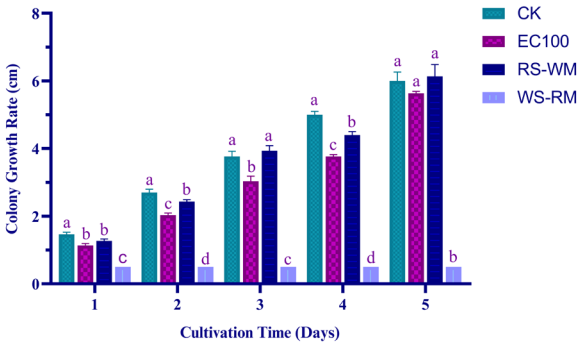

Fig. 1. Growth rate of *Alternaria alternata* on different media types over 5 days. CK – control, EC_{100} – resistant media, RS-WM – resistant strain wild media, and WS-RM – wild strain resistant media. Statistically significant differences are shown by different letters above the bars based on the Tukey test at $p < 0.05$. Bars with different letters are significantly different from ones with the same letter, which are not. The error bars represent the standard deviations (SD)

patterns. The resistant strain wild media (RS-WM) and the control media (CK) exhibited the highest radial growth rates when compared to other treated media; RS-WM's mean was 6.16 (SD = 0.14), while CK's was 5.0 (SD = 0.06). They demonstrate that under such circumstances, the growth conditions for fungal growth. With a mean of 5.63 (SD = 0.07), the EC_{100} medium supported less radial growth as well as the CK media, most likely due to the presence of resistance components. Additionally, RS-WM media grew higher than EC_{100} media, indicating that this strain may benefit from its resistant traits. In contrast to other tested media, the wild strain resistant media (WS-RM) showed restricted growth, with a mean of 0.5 (SD = 0), suggesting that the

inoculation of the wild strain on resistant media may have had inhibitory effects. In conclusion, the growth rates in CK media were almost 33%, in EC_{100} media they were 29.06%, in RS-WM media they were 33.43%, and in WS-RM media they were just 4%.

Moreover, ANOVA analysis showed that the calculated *F* value was greater than the critical *F* value (as shown in Table 1). The significant differences in the radial growth rates of *A. alternata* on different media are further illustrated in Fig. 2. Therefore, the null hypothesis was rejected and the alternative hypothesis was accepted. In ANOVA, the null hypothesis (H_0) assumes that there are no significant differences among the group means. However, if the calculated *F* value exceeds the critical *F* value, the null hypothesis is rejected. As for the alternative hypothesis (H_1), it

Table 1. ANOVA results indicating significant differences among group means ($F = 8.625594 > F\ crit = 2.539689$, $p = 1.77E-05$)

Source of Variation	SS	Df	MS	F	P-value	F crit
Between Groups	85.22767	4	21.30692	8.625594	1.77E-05	2.539689
Within Groups	135.8608	55	2.470197			
Total	221.0885	59				

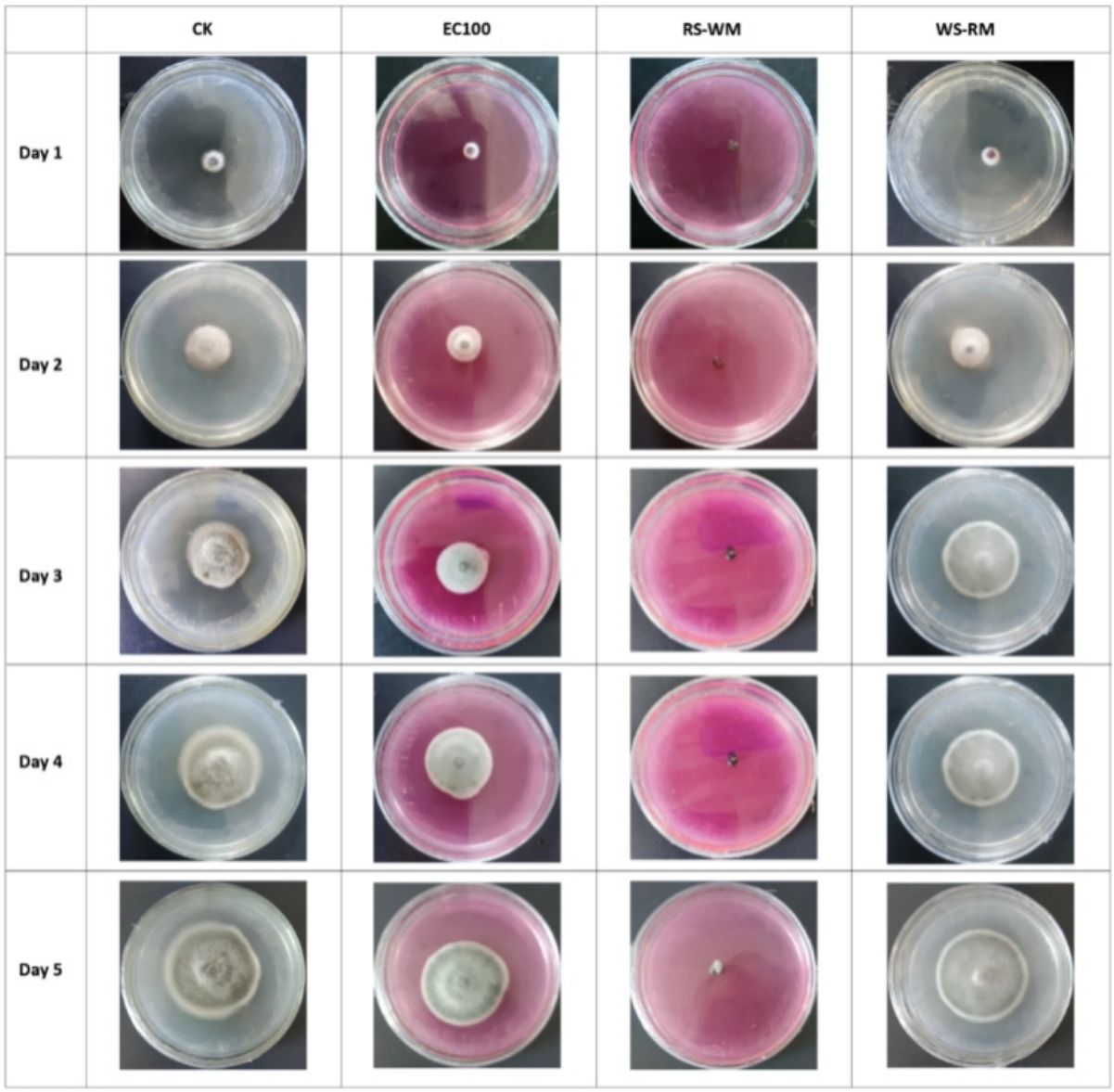

Fig. 2. Variation in *Alternaria alternata* growth pattern in different media types. Control (CK), resistant media (EC_{100}), resistant strain wild media (RS-WM), and wild strain resistant media (WS-RM)

was accepted that there was a significant difference in the group means. This result indicate that the various media types and their respective growth rates differ significantly from one another. The statistical analysis essentially highlights the variation seen in the fungal strain's ability to grow in the various growing conditions supplied by the various types of media that were used in the experiment.

Phenotype analysis of resistant and wild strains of *Alternaria alternata*

Beginning microscopic observations at a relatively small magnification – typically less than 20x – is a standard procedure to help locate the object of interest and obtain a general overview of the object. A higher power magnification is used after the object has been located and placed usually more than 60x, to enable a more thorough inspection and detailed examination of fine structural features or defining features. Investigating the phenotypic traits of *A. alternata* strains, notable differences were observed between the wild type and the resistant EC_{100} strain exposed to fludioxonil (as shown in Fig. 3). The EC_{100} strain displayed reduced mycelial growth, collapse of vertical mycelium, and uneven pigmentation due to the fungicide's interference with osmotic regulation and cell wall synthesis. This led to inhibited or slowed mycelial growth and structural abnormalities in fungal hyphae. Conversely, the wild strain exhibited typical mycelial development patterns with vigorous growth and homogeneous pigmentation under optimal conditions. It displayed minimal signs of stress and maintained healthy hyphal structures with well-defined morphology. The wild strain demonstrated tolerance to fludioxonil and environmental stressors by retaining consistent pigment distribution and growth patterns across fungal colonies. The distinctions between the two strains highlight how crucial it is to understand how each strain reacts differently to environmental disturbances and antifungal medications. The wild strain maintained consistent growth and pigment synthesis, whereas the EC_{100} strain showed vulnerability to fludioxonil-induced stress, leading to altered morphology and disrupted pigmentation.

Alternaria alternata soluble protein content

The investigation aimed to examine the relationship between protein concentration and absorbance values by constructing a standard curve between the two variables. The relationship between the protein standard concentration (x-axis) and the corresponding absorbance values (y-axis) derived from the spectrophotometric measurements is described by the equation $y = 2.9233x + 0.0129$ (as shown in Fig. 4). The line's slope, or coefficient 2.9233, indicates the rate at which absorbance increases with increasing protein concentration. This slope value indicates a high degree of assay sensitivity, meaning that even minor changes in protein concentration cause detectable changes in absorbance measurements. At zero protein concentration, the absorbance value is represented by the y-intercept of 0.01290, which is probably caused by background noise or non-specific absorption.

Furthermore, the linear relationship between the protein concentrations and absorbance values accounts for 99.38% of the variance in the absorbance values, according to the coefficient of determination R^2 value. Across every range of protein concentrations examined, the high R^2 value indicated a good fit of the linear regression model to the data points, indicating excellent agreement between the observed and predicted absorbance values. As a result, the protein quantification assay's accuracy and reliability were confirmed by the linear equation and high R^2 value, demonstrating in the assay's capacity to accurately determine protein concentrations in unknown samples within the tested range. Additionally, the control protein ratios (CPr) of the wild and resistant strains were quantified. They were 0.192 and 0.187, respectively.

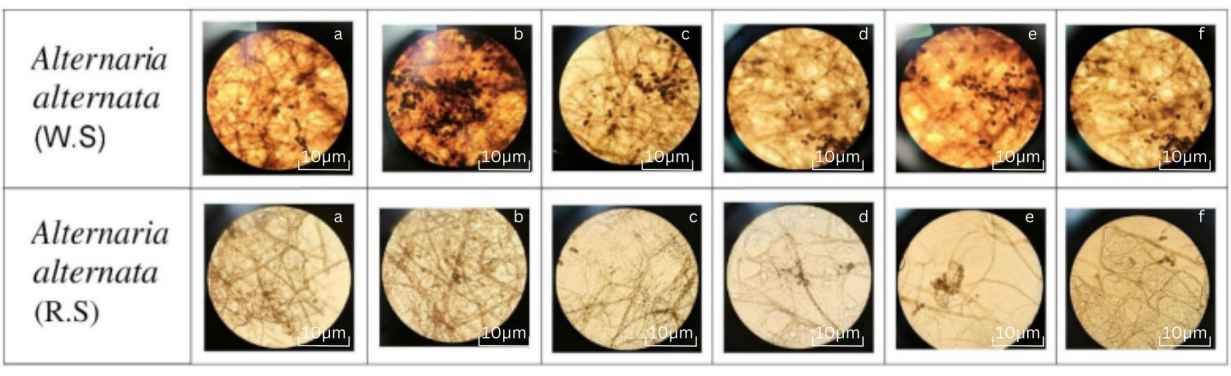

Fig. 3. The morphological differences between the wild strain (WS) and resistant strain (RS) of *Alternaria alternata*

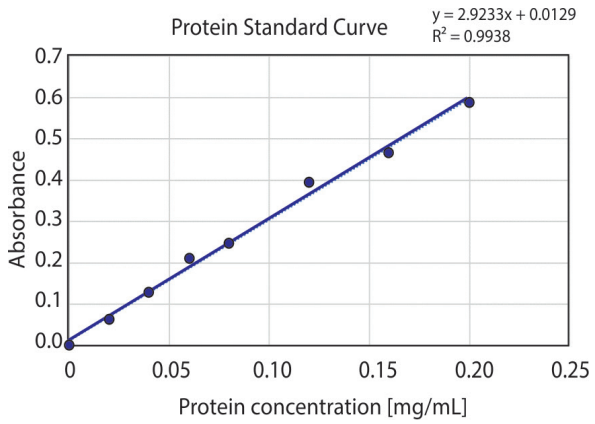

Fig. 4. The standard curve for the soluble protein assay showing the strong correlation between protein concentration and absorbance

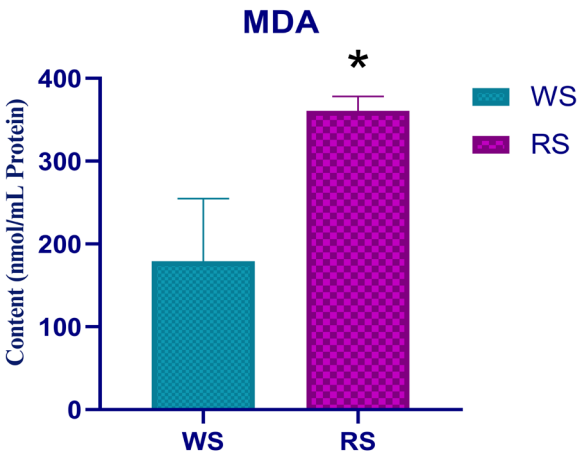

Fig. 5. Malondialdehyde (MDA) content of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Variations in protein expression levels, enzyme activity, or metabolic processes may be indicated by differences in CPr values among strains. The resistant strain's greater CPr value in comparison to the wild strain may indicate increased enzymatic activity or higher expression of relevant proteins.

Malondialdehyde (MDA) content

There was a significant difference in malondialdehyde (MDA) content between the wild and resistant strains. Specifically, the data indicated that the resistant strain had higher MDA content than the wild strain. This finding suggests a significant difference in the oxidative stress response mechanisms between the two strains, with the resistant strain exhibiting higher MDA content, indicating that the resistant strain may be more susceptible to lipid peroxidation (as shown in Fig. 5). The resistant strain exhibited greater overall lipid peroxidation than the wild strain, based on its higher MDA content. Lipid peroxidation produces MDA as a byproduct and elevated MDA content signifies more oxidative damage to cell membranes. The resistant strain's observed mean MDA content of 360.89 nmol · mg^{-1} stands in sharp contrast to the wild strain's 179.19 nmol mg^{-1}. The resistant strain's low standard deviation of 14.13 indicates closely clustered MDA values around its mean, which was indicated by a high degree of consistency. On the other hand, the wild strain's higher standard deviation of 61.82 indicated greater variability in MDA content values. Compared to the wild strain, the resistant strain consistently showed greater MDA content, indicating enhanced oxidative stress and lipid peroxidation. Greater MDA content in the resistant strain may indicate increased oxidative stress or a distinct response to external stress factors (as shown in Table 2).

Table 2. Comparison of antioxidant enzyme activity between different strain types (wild type, drug-resistant type)

Enzyme activity type	Strain type	Catalase (CAT) U · ml^{-1} protein	Superoxide dismutase (SOD) U · ml^{-1} protein	Malondialdehyde (MDA) nmol · mg^{-1} protein	Tyrosinase activity U · ml^{-1} protein
Biological Replicate 1	wild type	12.678	165.509	144.262	22.8952
	resistant type	36.811	175.473	361.144	51.21241
Biological Replicate 2	wild type	12.725	148.463	266.062	23.64586
	resistant type	34.535	176.004	378.058	51.94012
Biological Replicate 3	wild type	10.330	170.107	127.254	25.14718
	resistant type	35.261	187.330	343.455	53.39553
Mean ± Std. Dev.	wild type	11.91 ± 1.12	161.36 ± 9.31	179.19 ± 61.82	23.90 ± 1.15
	resistant type	35.54 ± 0.95	179.60 ± 5.47	360.89 ± 14.13	52.18 ± 1.11
P-value		<0.01	0.12	0.002	0.001

Catalase (CAT) activity

The findings of this investigation showed that the wild and resistant strains differed significantly in catalase (CAT) activity. Specifically, the results indicated that the resistant strain had higher CAT activity than the wild strain. This observable difference highlights a significant difference in the enzymatic antioxidative capabilities of the two strains, with the resistant strain displaying higher CAT activity. This finding points to a possible increase in the resistance strain's capacity to reduce oxidative stress in cells by improving the catalytic hydrolysis of hydrogen peroxide (as shown in Fig. 6). The resistant strain appeared to have more catalase enzyme activity than the wild strain, based on its increased CAT activity. Higher CAT activity is indicative of a more effective antioxidant defense system. Catalase is an enzyme that is essential in the decomposition of hydrogen peroxide into water and oxygen. The resistant strain had a mean CAT activity of 35.54 U · ml^{-1} protein, whereas the wild strain's was significantly lower at 11.91 U · ml^{-1} protein. Furthermore, the resistant strain had a lower standard deviation (0.95) than the wild strain, which displayed a greater standard deviation (1.12). This discrepancy shows that the resistance strain's CAT activity values were more densely clustered, while the wild strain's values were more widely distributed around the mean. A greater standard deviation signifies that the data points within a dataset are more widely distributed relative to the mean. These findings suggest that, by consistently exhibiting higher CAT activity than the wild strain, the resistant strain may have had a more effective antioxidative defense mechanism (as shown in Table 2).

Superoxide dismutase (SOD) activity

The results of this investigation demonstrated that the wild and resistant strains differed significantly in terms of superoxide dismutase (SOD) activity. More specifically, the resistant strain had significantly higher SOD activity than the wild strain. This highlights a significant difference in the antioxidant defense systems functioning in the two strains, with the resistant strain exhibiting higher SOD activity. This finding suggests that the resistant strain possesses an enhanced ability to combat oxidative stress by effectively scavenging superoxide radicals, reflecting its enhanced enzymatic antioxidative response (as shown in Fig. 7). The resistant strain's increased SOD activity indicates that, on average, it had more superoxide dismutase enzyme activity than the wild strain. An effective antioxidant defense system was indicated by a higher level of SOD activity. SOD is an enzyme that is essential for neutralizing superoxide radicals. The resistant strain's mean SOD activity, measured at 179.60 U · ml^{-1} protein, significantly higher than that of the wild strain, which was measured at 161.36 U · ml^{-1} protein. The wild strain's greater variability standard deviation of 9.31 indicates greater variability in SOD activity measurements. The resistant strain, on the other hand, had a smaller standard deviation of 5.47, indicating that the SOD activity values were more closely clustered around the 179.60 U · ml^{-1} protein mean concentration. When compared to the wild strain, the resistant strain consistently exhibited greater SOD activity, suggesting a potentially more effective antioxidative defense mechanism (as shown in Table.2).

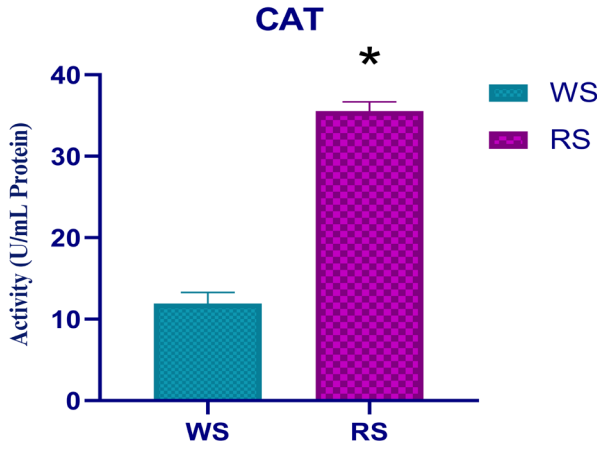

Fig. 6. Catalase (CAT) activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

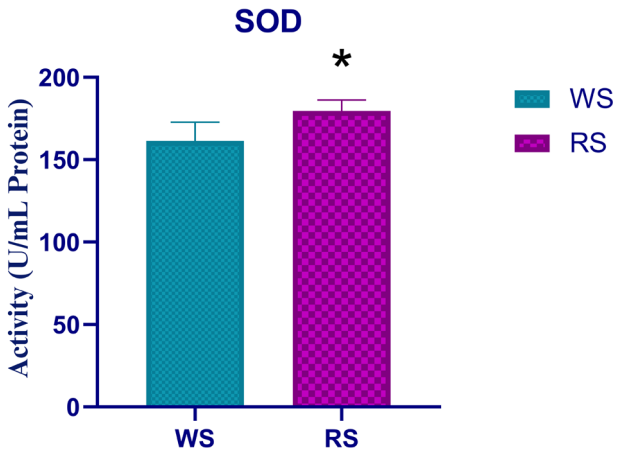

Fig. 7. Superoxide dismutase (SOD) activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Tyrosinase activity

The results demonstrated significant differences in the tyrosinase activity of *A. alternata* wild and resistant strains. Specifically the tyrosinase activity of the resistant strain was significantly higher than that of the wild strain. This notable variation suggests a major difference in the melanin biosynthesis pathways of the two strains, with the resistant strain exhibiting increased tyrosinase activity. This finding suggests that the resistant strain has a higher melanin production capacity, which may help explain why it is greater resilience to environmental stresses. The resistant strain has greater tyrosinase activity than the wild strain, which suggests that the mean enzyme activity is higher. The resistant strain had a significantly higher mean activity of 52.18 ± 1.11 tyrosinase than the wild strain, which had a mean activity of 23.90 ± 1.15. Elevated melanin production, which is involved in defense mechanisms against UV radiation and oxidative stress, is associated with this increased tyrosinase activity. The resistant strain's tyrosinase activity values were more closely clustered around the mean of 52.18 U · ml^{-1} protein, as reflected by its lower standard deviation of 1.11. On the other hand, the wild strain's 1.15 standard deviation indicates that its tyrosinase activity values were slightly greater variability. Compared to the wild strain, the resistant strain exhibited a more robust enzymatic response under stress conditions, as seen by its consistently higher and more stable tyrosinase activity (as shown in Fig. 8).

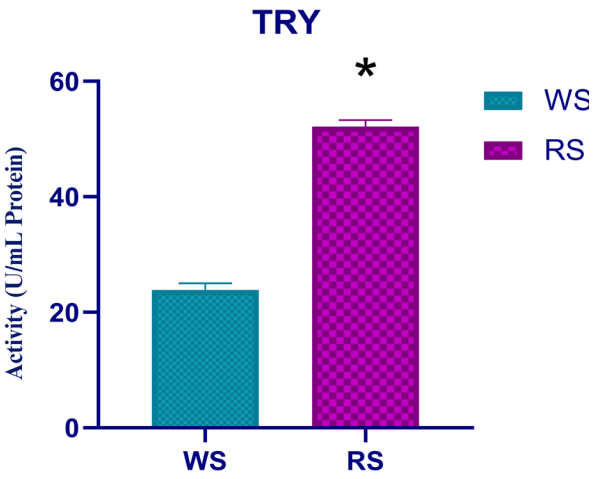

Fig. 8. Tyrosinase activity of different strains (wild type and resistant) of *Alternaria alternata*. The asterisk (*) represents a statistical difference ($p < 0.05$) between wild type (WS) and resistant strains (RS) of *Alternaria alternata*

Poplar pathogenicity

This experiment was conducted to evaluate the pathogenicity of poplar leaves (*Populus nigra*) with diseases, such as *A. alternata*. The treatment groups were as follows: CK (Control), which consisting of untreated leaves serving as a reference for comparison WS (Wild Strain), in which poplar leaves were inoculated with the wild-type strain of the pathogen, which is the naturally occurring form of the pathogen without any

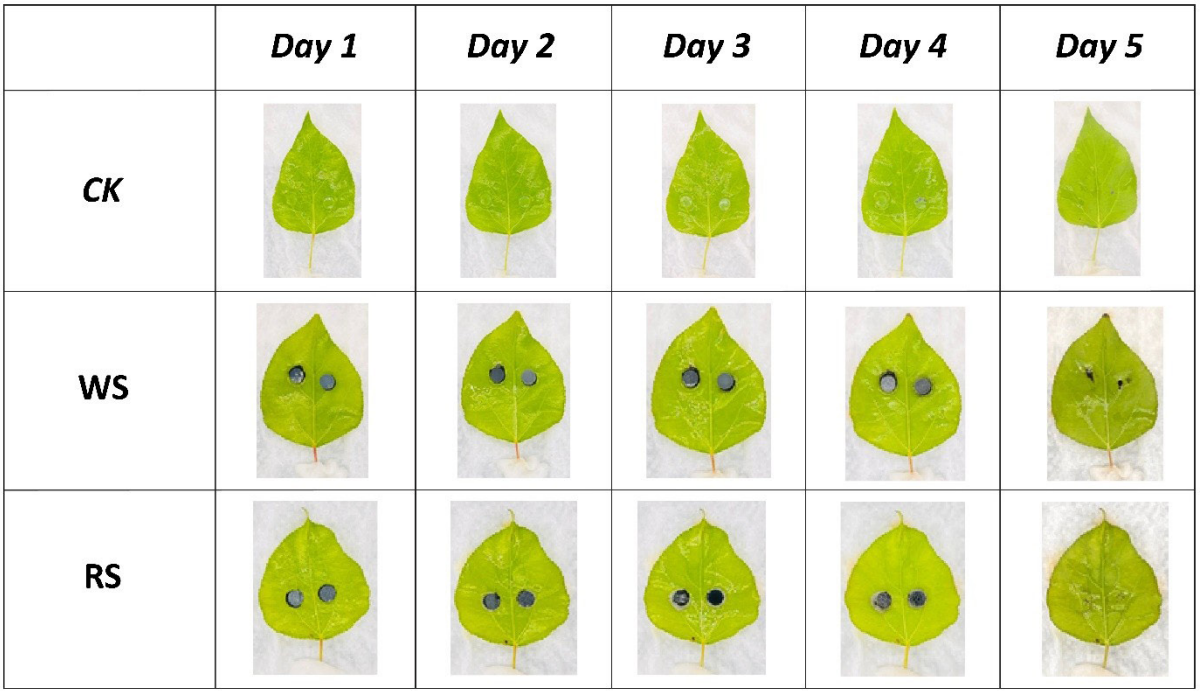

Fig. 9. Fludioxonil's effectiveness in reducing *Alternaria alternata* infection on poplar leaves, with minimal symptoms in resistant strain and disease progression in wild strain

alterations; and RS (Resistant Strain), in which poplar leaves were inoculated with a resistant strain of the pathogen that has become resistant to fludioxonil, potentially making it less virulent than the wild strain. The results showed that, throughout the incubation period, the CK leaves stayed healthy, suggesting that the pathogen was either absent or no disease developed in the absence of inoculation**.** Clear disease progression was observed in the WS leaves. Small lesions began to appear on Day 1. The lesions had increased in size and number on Day 2. On the third day, the lesions continued to expand and coalesce. The leaves showed significant damage with extensive necrotic patches by Days 4 and 5, suggesting that the infection was actively disease progression. There were few or no disease symptoms on the RS leaves. On Days 1 or 2, only a few minor lesions were observed, but they did not progress significantly. The leaves remained comparatively healthy by Days 4 and 5, suggesting that the resistant strain was exhibited reduced pathogenicity (as shown in Fig. 9).

Discussion

The growth of the *A. alternata* on the PDA plates was significantly inhibited by all fungicides (Feng and Zheng 2007) that have resistance against *A. alternata* at concentrations of 100 parts per million (ppm), 150 ppm, 200 ppm, 250 ppm, and 300 ppm. The fungicides and their concentrations had an impact on the rate of inhibition. The percentage of fungal inhibition increased as the increasing fungicide concentrations and the highest concentration tested resulted in the greatest degree of inhibition (Wang *et al.* 2023). The findings showed that different media types influenced *A. alternata* growth rates. As shown in Fig. 1, different types of media facilitated the fungal strain's growth. In all tested media, *A. alternata* mean radial growth differed significantly. Fungus showed the greatest degree of radial growth on PDA. Given that PDA is a conventional media with no internal resistance to *A. alternata*, this observation is consistent with predictions. On the other hand, different growth patterns were seen in media with resistance substances against *A. alternata*. In comparison to other treated media, RS-WM and CK showed the highest rates of growth. They showed that the growth conditions were ideal under such conditions. The existence of resistance elements is probably the reason why the EC_{100} medium did not grow as well as the CK media. Furthermore, RS-WM media showed greater growth than EC_{100} media, suggesting a possible benefit from the resistance characteristics in this strain. The wild strain's inoculation on resistant media may have had inhibitory effects, as the WS-RM exhibited limited growth in comparison to other tested media.

The most frequently isolated species from contaminated tomato fruits were *Alternaria* spp. (Zenelt *et al*. 2021; Schmey *et al*. 2023; Biswal and Das 2024). Using the PDA medium, 20 *Alternaria* isolates were described both macro- and microscopically, leading to the classification of these isolates into five species: *A. alternata, A. brassicicola, A. citri*, *A. radicina*, and *A. tenuissima*. The classification of the five isolated morphological types of *Alternaria* spp was made possible by varied colony morphological differentiation observed on the PDA and by microscopic inspection (El-Ganainy *et al*. 2021; Saleem and El-Shahir 2022). It was shown that the wild type and fludioxonil-resistant EC_{100} strain of *A. alternata* differed significantly. Because of fludioxonil interference, the EC_{100} strain exhibited reduced mycelial growth, collapsed mycelium, and uneven coloration. Under optimum conditions, the wild strain exhibits solid morphology, uniform pigmentation, rapid growth as well as hyphal resilience by maintaining uniform growth and pigment dispersion during stress or exposure to fludioxonil.

The soluble protein content of the mycelium was significantly reduced by both copper (II) hydroxide wettable powder (Cu $(OH)_2$ WP) and copper-based nanomaterials (Cu-based NMs), with dose-dependent differences being visible (Yuan *et al*. 2023; Parada *et al*. 2024). A common indicator of the degree of cell damage in eukaryotes is the concentration of specific soluble proteins, which can arise under a variety of stressful circumstances (Li Jiao *et al*. 2018). An *A. alternata* infection affected the malondialdehyde (MDA) level in mango fruit. The MDA contents of *A. alternata*-infected mango fruit were significantly higher than those of the untreated control fruit, which might be explained by the significant lipid oxidation of MDA that occurs in response to *A. alternata* infections (Li Jiao *et al*. 2018; Peić Tukuljac *et al*. 2023). Our results showed that a noteworthy distinction in MDA content between the resistant and wild strains. In more exact terms, the results suggest that the resistance strain exhibits more MDA contents than the wild strain. This result indicates a notable distinction in the two strains' oxidative stress response systems, with the resistant strain showing higher MDA contents and possibly being more vulnerable to lipid peroxidation (Fig. 5). Based on its greater MDA contents, the resistant strain appeared to exhibit more lipid peroxidation than the wild strain.

The plant defensive systems against many stresses, including pathogen invasion, are impacted by the enzymes ascorbate peroxidase (APX), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), POD, SOD, and CAT (He *et al*. 2017; Wiraswati *et al*.

2019). Superoxide and hydrogen peroxide radicals are changed into less dangerous and more stable forms by the detoxifying enzymes CAT, SOD, and peroxidases, which deal with ROS (Hossain *et al*. 2015). The first line of defense is CAT and peroxidases, which further SOD-catalyzed dismutation of superoxide anion into oxygen and hydrogen peroxide. Hydrogen peroxide removal involves members of a large class of enzymes known as peroxidases (Ihsanullah *et al*. 2024; Jomova *et al*. 2024). The primary distinction between them is the kind of reducing substrate that each one utilizes: pyrogallol peroxidase uses pyrogallol, guaiacol peroxidase uses guaiacol, and ascorbate peroxidase uses ascorbate (Hiner *et al*. 2000; Morales-Urrea *et al*. 2023). Increases in the activities of CAT, SOD, and peroxidase suggest that the initial infection stimulated these enzymes, which in turn activated the antioxidant defense system of the plant (Hernández *et al*. 2016). Compared to control fruits, the inoculated fruits of Una and Kurtovska kapia exhibited reduced CAT activity. This aligns with the findings of Li Jiao *et al*. (2018). CAT activity in mango fruits infected with *A. alternata* decreased drop-by-drop. After Kurtovska kapia fruits were injected with water, the level of CAT activity was maximum. Amfora's CAT activity was much lower than that of the control group when water was put into the fruits. There is a decrease in CAT and SOD levels in avocado fruit during wound stress (Jiao *et al*. 2018; Muhammad *et al*. 2024). The conclusions from this investigation demonstrate that there were notable differences in the CAT activity of the normal and resistant strains. Specifically, the investigation showed that the resistant strain exhibited greater CAT activity than the normal strain. The resistant strain had increased CAT activity, highlighting a discernible difference in the two strains' enzymatic antioxidative capabilities. This result suggests that the resistant strain may have been better able to catalyze the hydrolysis of hydrogen peroxide, thereby reducing oxidative stress in cells (Fig. 6). However, the results of this investigation conclusively demonstrated that there were notable differences in SOD activity between wild-type and resistant strains. More precisely, the findings indicated that the wild strain's SOD activity was substantially lower than that of the resistant strain. This notable distinction indicates that the two strains' antioxidant defense mechanisms operated very differently, with the resistant strain having greater SOD activity. This finding highlights the resistant strain's improved enzymatic antioxidative response (Fig. 7) and implies that it may be better able to fight oxidative stress by efficiently scavenging superoxide radicals.

Tyrosinase is a monooxygenase that contains copper and catalyzes the O-hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine and dopaquinone. In eukaryotes, this process is crucial for the formation of melanin (Maamoun *et al*. 2021). Tyrosinases are metalloenzymes with copper that are found in bacteria, fungi, plants, insects, and mammals. They are involved in a variety of biological activities (Khan *et al*. 2018; Beaumet *et al*. 2023). Tyrosinase is the main rate-limiting enzyme in the manufacture of L-DOPA melanin, which is widely found in microorganisms, plants, animals, and the human body (Rao *et al*. 2011; Ihsanullah *et al*. 2024). Although tyrosinase is a component of the L-DOPA melanin production pathway, its function in the pathophysiology of *M. oryzae* is still unclear. Biochemical, functional genetics, and cell biology methods were used to assess the role of the unidentified gene (MGG_14598) called tyrosinase (MoTyr) in the pathogenic and morphological development of the rice blast fungus, which is economically devastating. Many fungi have well-conserved orthologs of tyrosinase (Rao *et al*. 2011; Li *et al*. 2021a, b). Tyrosinase research in the medical, cosmetic, culinary, and other domains has been primarily focused in recent years; nevertheless, studies pertaining to tyrosinase in pathogenic fungi are lacking (Fan *et al*. 2023). Our finding showed that the tyrosinase activity of the resistant strain was significantly higher than that of the wild strain. This notable variation suggests a major difference in the routes by which the two strains produce melanin, with the resistant strain exhibiting increased tyrosinase activity (Fig. 8). This finding raises the possibility that the resistant strain has a higher melanin production capacity, which may help explain why it is more resilient to environmental stresses. The resistant strain had greater tyrosinase activity than the wild strain, which suggests that the mean enzyme activity was higher.

Neofusicoccum parvum and *Botryosphaeria dothodea* are both considered important fungal species in the Botryosphaeria genus of the Botryosphaeria family, according to research findings. *B. dothodea*, while previously thought of as a potentially dangerous fungus, is very common and cryptic in forestry, agricultural, and natural forest ecosystems, whereas *N. parvum* has been observed to cause cankers and diebacks in a variety of woody species worldwide (Marsberg *et al*. 2017; Khan *et al*. 2018; Hassan *et al*. 2021; Ihsanullah *et al*. 2024). The findings of our experiment research demonstrated that the absence of *A. alternata* illness and the continued health of the CK leaves. Lesions in the WS leaves started to be visible on Day 1 and grew by Day 3, resulting in significant damage by Day 5. By Day 5, the RS leaves looked largely healthy and displayed minor symptoms, including a few lesions that did not spread. This suggests that the sickness was less severe.

Conclusions

The resistance mechanisms of *A. alternata* to environmental stressors and fungicide resistance were investigated in this study. The main conclusions were that the resistant strain exhibited reduced mycelial development, altered pigmentation, and higher antioxidative enzyme activity (SOD, CAT, tyrosinase, and MDA) than the wild strain; fungal development was influenced by growth media, with notable variances in growth rates between media types. A significant challenge was the wild strain's inhibited growth on resistant media, underscoring the intricacy of managing fungal resistance. The resistant strain was found to inhibit the fungal effects on *Populus nigra* leaves, indicating its potential to mitigate plant damage. In light of these results, we advise more research into the molecular processes that underlie these reactions in order to create focused interventions that will enhance disease control and advance methods for sustainable agriculture.

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