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Biofumigation by Mustard Plants as an Application for Controlling Postharvest Gray Mold in Apple Fruits

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Abstract: Gray mold caused by *Botrytis cinerea* is a critical disease that results in severe postharvest losses for the apple industry. In recent years, biological control has become an increasingly effective approach for controlling postharvest diseases in fruits. *Brassica* plants contain abundant natural compounds with known antimicrobial activity against numerous plant pathogens. In this study, a large-scale screening of 90 mustard cultivars was conducted to evaluate their biofumigation effects against *B. cinerea*. Among these, one mustard cultivar named Dilong-1, displayed the highest inhibitory effect against *B. cinerea*, and was able to completely inhibit mycelial growth. Further investigations showed that fumigation with Dilong-1 inhibited mycelial growth, sporulation, and spore germination of *B. cinerea* in vitro. In addition, fumigation using Dilong-1 showed a wide antifungal spectrum, including other fruit postharvest pathogens such as *Phytophthora litchii*. Furthermore, apple gray mold disease severity was significantly reduced by biofumigation using Dilong-1. Importantly, fumigation with Dilong-1 did not negatively impact final apple qualities, including weight loss, firmness, and total soluble solids. These results suggested that Dilong-1 significantly inhibited gray mold decay caused by *B. cinerea* without affecting the quality of apple fruits. In conclusion, biological fumigation of apple fruits with the mustard cultivar Dilong-1 is a promising eco-friendly approach for controlling apple gray mold during storage and shipment.

Keywords: biological fumigation; volatile organic compounds; antifungal activity; mustard; postharvest disease; *Botrytis cinerea*

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1. Introduction

Fruits such as apple are widely planted and highly consumed due to their rich nutrient content and significant health benefits. However, postharvest diseases caused by microbial pathogens result in tremendous economic losses during storage, shipment, and post-consumer purchase [1]. Among these diseases, gray mold caused by *Botrytis cinerea* is one of the most severe postharvest diseases of apple fruits [2]. *B. cinerea* has a wide range of hosts, including cucumbers, tomatoes, strawberries and grapes. It can infect crops at multiple stages, causing serious economic losses [3]. *B. cinerea* is a necrotrophic pathogen, and its mycelia appear gray and produce a large number of conidia [4]. The process of host infestation by *B. cinerea* consists of several stages: conidia attach to the host surface, germinate and produce appressorium to penetrate the host surface, kill host tissues, form primary lesions, expand lesions and macerate tissue [5]. Due to its scientific and economic significance, *B. cinerea* ranks second in the "world top 10 fungal pathogens in molecular plant pathology" [6]. This pathogen can cause rot in apples through two pathways: first, as a wound pathogen via wounds that occur during harvest and, secondly, from infections that

arise at the blossom and remain symptomless but subsequently develop in stored apples [7]. This unique infection pattern exacerbates the difficulty of controlling postharvest disease. Therefore, developing suitable and effective strategies to control postharvest diseases is crucial for improving the postharvest quality and extending the shelf-life of the apple fruits.

The application of synthetic fungicides has been the most effective method to control fruit postharvest diseases in production. It is also the traditional approach to controlling gray mold rot in the apple fruits [3]. However, the persistent use of commercial fungicides has led to the emergence of fungicide-resistant strains and has generated a series of negative impacts, including food security and environment pollution [8,9]. Therefore, the exploration of safer and eco-friendly alternative strategies has become an emerging demand, especially given the increasing regulatory restrictions on the use of chemical fungicides [10]. Some promising measures have been suggested to increase the postharvest life of fruits, such as cold or controlled atmosphere storage [7], ozone [11], heating [12], salts [13], UV-C [14] and other physiological treatments [15]. Resistance inducers [16] and biological control measures [17] have also been studied extensively and recognized as effective strategies to control postharvest diseases. Among these approaches, biological control has gained widespread attention as a promising strategy for controlling postharvest diseases.

Biofumigation is a promising biological control method that involves the use of volatile organic compounds (VOCs) from natural sources to inhibit a wide range of storage pathogens and control fungal decay [18]. This new approach to postharvest disease control offers several potential advantages [19]. Firstly, the volatiles used for fumigation are natural ingredients that are generally regarded as safe. Secondly, VOCs can inhibit pathogenic microorganisms without directly contacting the fruit [20]. Finally, the continuous and slow emission of active substances has a long-term inhibitory effect on latent postharvest pathogens [21]. These properties of biofumigation can greatly increase the shelf-life of fruits. Recently, biofumigation has emerged as a more suitable strategy for controlling postharvest diseases and has garnered increased interest [22]. However, the effectiveness of fumigation largely depends on the material used [23]. Therefore, selecting safe and effective fumigation materials is crucial for ensuring efficacy.

Many plants of the family Brassiceae (brassica) have been reported to have a proven antimicrobial effect against numerous plant soil and food-borne pathogens. Biofumigation effects of brassica plants are associated with glucosinolates (GSLs) present in the tissues. When brassica crop tissues are damaged, GSLs are hydrolyzed by myrosinase to form volatile isothiocyanates (ITCs), which are thought to be the primary active ingredient in biofumigation [24]. Many different brassica-derived materials have been used to study aspects of biofumigation, such as fresh plant tissues, rape seed meal, freeze-dried plant tissues, and oil extracts [25,26]. Glucosinolate profiles vary due to species, tissue types and plant age, and determine the type and quantity of ITC release which determines the overall biofumigation effect. Highest ITC release potential occurs at 50% flowering growth stage across species [27]. Currently, few studies on the effect of brassicaceous crops on fruit postharvest diseases have been carried out.

In this study, we evaluated the antifungal activities of 90 mustard cultivars against *B. cinerea*, and identified one mustard cultivar, Dilong-1, as having the highest inhibitory effect. We then investigated the biofumigation effects of Dilong-1 on mycelial growth, mycelial morphology, spore production, and spore germination of *B. cinerea* in vitro. Additionally, we investigated the antifungal spectrum of Dilong-1 against other postharvest fruit pathogens. Finally, we assessed the effects of Dilong-1 fumigation on gray mold decay and fruit quality in apple fruits.

2. Materials and Methods

2.1. Fruit Pathogens

Botrytis cinerea was previously isolated from decayed apple tissue exhibiting gray mold symptoms and was preserved at 13 °C in our lab. Other plant pathogens including *Fusarium oxysporum* [28], *Fusarium solani* [29], *Colletotrichum gloeosporioides* [30], *Thielaviopsis basicola* [31],

Phytophthora litchii [32], and *Pythium aphanidermatum* [33], were also isolated from diseased fruits and preserved. The fungi and oomycetes were stored on potato dextrose agar (PDA) and V8 slants, respectively, at 13 °C. When needed, they were transferred to fresh PDA or V8 plates and cultured in darkness at 25 °C for 5 to 7 days. *B. cinerea* conidia were obtained by flooding the surface of the culture with sterile distilled water after 10 days of culturing on PDA plates. The resulting conidia suspension was filtered through a double layer, and the concentration was determined using a hemocytometer.

2.2. Mustard Cultivars and Apple Fruits

We collected a total of 90 *Brassica* cultivars from different regions in China and sowed their seeds in an experimental field at Nanjing Agriculture University. At the 50% flowering growth stage, the whole plant (without roots) of each cultivar was harvested to prepare fumigant material for subsequent experiments. Individual samples of each cultivar were sealed in zip-lock bags and temporarily stored at −20 °C for later use. The plant material was briefly soaked in liquid nitrogen and then ground for 0.5–1.0 min into a powder using a blender.

Apple fruits were harvested at a commercial mature stage from an orchard in Yantai, China. We selected fruits with uniform maturity, shape, size, and free of any physical injuries. Before treatment, the fruits were soaked in 0.1% (*v*/*v*) sodium hypochlorite for 2 min to eliminate surface microbes, washed with sterile water, and then air-dried [34].

2.3. Screening of Mustard Cultivars as Biofumigants with High Antifungal Activity against *B. cinerea*

Ninety mustard cultivars were planted and harvested at full flowering stage for the antifungal activity assay. The harvested samples were stored at −20 °C until use. Based on the preliminary experiment, 25 g/L (grams per liter of volume) of mustard powder was chosen to evaluate the antifungal activity of all mustard cultivars against *B. cinerea*. To test the inhibitory effect of ITCs derived from the crushed tissues, a bioassay system was set up by placing a 5 mm diameter plug from an actively growing culture in the center of a 90 mm diameter petri dish. Fresh mustard powder was placed on the lids of the upside-down petri dish, and the plates were sealed with parafilm and incubated in the dark at 25 °C. Control plants were inoculated with pathogen but treated with distilled water instead of mustard. The experiment was repeated three times with three replicates per treatment, and mycelial growth inhibition was calculated accordingly [35]. The antifungal ability of mustard was evaluated based on the inhibition rate.

2.4. Inhibitory Efficacy of Dilong-1 on Mycelial Growth and Morphology of *B. cinerea*

To further explore the antifungal effects of Dilong-1, *B. cinerea* was treated with Dilong-1 at concentrations of 5, 10, 15, 20, and 25 g/L. The inhibitory effects on mycelial growth of *B. cinerea* were monitored daily for three days. An equal volume of distilled water instead of mustard was used as the control. The experiment was repeated three times with three replicates for each treatment. The mycelial growth inhibition was calculated accordingly [35]. Additionally, the mycelial morphology of *B. cinerea* was observed using a light microscope at 20× magnification.

2.5. Inhibitory Efficacy of Dilong-1 on Spore Production and Germination of *B. cinerea*

The effects of Dilong-1 on spore production and germination of *B. cinerea* were investigated in 90 mm-diameter petri dishes, following a previously reported method with slight modifications [10]. The colony diameter was recorded and colony morphology was captured as described above.

The effect of Dilong-1 on spore production was evaluated following the above treatment using the concentrations of 10 and 20 g/L after incubation in the dark at 25 °C for 14 days. The spores produced were collected from each plate by adding 10 mL sterile water onto each plate, and the suspension was filtered through a double layer cheese cloth. Spores

were counted using a microscope and a hemocytometer. Three plates for each treatment were washed to calculate the spore count, and the experiment was repeated three times.

For evaluation of the effect of Dilong-1 on spore germination, the spore suspension was prepared in sterile water with a concentration of 1×10^5 spores mL^{-1}. Fifty microliters of suspension was evenly spread onto the center of the water agar (WA) plate, and Dilong-1 fresh powder was added to the lids of petri dishes (90 mm in diameter) at concentrations of 10 and 20 g/L. The criterion for spore germination was set as a germ tube length greater than half the maximum diameter of the spore [36]. Germination rates were calculated as the percentage of germinated spores out of the total number of evaluated spores. Once the spore germination rate of the WA plate without Dilong-1 reached more than 90%, the number of germinated spores in the treatment group was counted using a hemocytometer and a microscope. The experiment was performed three times.

2.6. Antimicrobial Spectrum Analysis of Dilong-1

Based on the above results, the mustard cultivar Dilong-1 demonstrated the most effective inhibitory effect and exhibited favorable biological characteristics. Consequently, we conducted additional assessments to determine its antifungal activity against the five other postharvest pathogens listed earlier, using the same methodology as described previously. Following three days of fumigation, the extent of mycelial growth inhibition for each pathogen was subjected to statistical analysis.

2.7. Effects of Dilong-1 in Controlling Postharvest Gray Mold in Apple Fruits

The effects of fumigation by Dilong-1 on controlling postharvest disease in vivo were evaluated as described previously with slight modifications [35]. Each apple was subjected to three uniform wounds, each measuring 5 mm wide and 3 mm deep, at the equator, before inoculation. Mycelial plugs, measuring 5 mm in diameter and obtained from the actively growing edge of the *B. cinerea* colony, were placed with the mycelium facing outwards into each wound. Six inoculated apples were then placed into a plastic box measuring 30.5 cm × 23.5 cm × 11.5 cm. Fresh Dilong-1 powder at concentrations of 10 and 20 g/L were placed in a plastic cup in the center of the box, which was immediately sealed. The enclosed boxes were stored at room temperature with a high relative humidity of 90–95%. A control group was established by placing water in a plastic cup. Disease incidence was calculated as the percentage of infected wounds, and lesion diameter was measured for infected wounds at 3 days post-inoculation (dpi). Each treatment comprised three replicates, with six fruits per replicate. The experiment was repeated three times.

2.8. Effects of Dilong-1 on Storage Quality of Apples

We conducted assays to evaluate the effects of Dilong-1 on the postharvest quality of apple fruits. The apples were placed individually in a plastic box measuring 17.5 cm × 12.7 cm × 10 cm with a sealed cover. A plastic cup containing 20 g/L of Dilong-1 fresh powder was placed in each box, while an equal volume of water served as the control. The apples were then stored for 20 days at 20 °C, with each replicate consisting of six apples. The whole experiment was performed three times. Natural decay rate and quality parameters were determined after storage accordingly [34]. The storage quality traits of the treated apples were detected. Weight loss was measured before and 20 days after storage, and calculated using the formula (M1-M2)/M1, where M1 is the weight before storage and M2 is the weight after storage. Fruit firmness was determined before and 20 days after storage by a penetrometer (Fruit Pressure Tester FT011, Italy), with a 10 mm tip, and expressed as Newton (1 N = 9.8 kg). All analyses were performed three times. The total soluble solid (TSS) content of the apples was measured for each treatment using the method described [37]. The TSS content was expressed as a percentage (g TSS per 100 g fresh weight). The malic acid content was detected for each treatment according to the method described [38]. Titration was performed with 0.1 N NaOH to pH 8.1. Four grams of fresh apple juice diluted with 20 mL of distilled water was evaluated for each replicate. The

results were expressed in terms of the percentage of malic acid. The total phenolic content was determined using the tungsten molybdenum acid reduction method with a Plant Total Phenolic Content Assay Kit AKPL016C (Beijing Boxbio Science & Technology Institute, Beijing, China), following the protocol provided by the manufacturer [38]. The absorbance of samples was recorded at 760 nm using a spectrophotometer, and total phenolic content was expressed as mg/g FW. Three biological replicates were used in each treatment, and the experiment was repeated three times.

2.9. Statistical Analysis

Statistical analyses were conducted using the software package SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). The statistical significance of variances was determined by an independent samples T-test, and significance was defined as $p < 0.05$.

3. Results

3.1. Large-Scale Screening of Mustard Cultivars as Biofumigants with High Antifungal Activity against B. cinerea

Many *Brassica* plants have a proven antimicrobial effect against various plant soil and food-borne pathogens. To screen for *Brassica* plants with high antifungal effects against *B. cinerea*, a total of 90 collected mustard cultivars were collected from different regions in China (Table S1). These cultivars were planted in Nanjing and harvested at the 50% flowering growth stage, which has been reported to result in higher ITC release [27]. Using the widely used double petri dish dual-culture method (Figure 1A), fresh mustard powder was placed on the lids of the upside-down petri dish, and the colony diameters of *B. cinerea* were measured after three days of fumigation with 25 g/L of each mustard cultivar. In contrast to the control treatment of fumigation with an equal volume of water, the majority of tested mustard cultivars affected mycelial growth of *B. cinerea* at different levels (Table S1). Out of the 90, 21 mustard cultivars showed more than 40% mycelial growth inhibition (Figure 1B), indicating that they possessed potent antifungal activity against *B. cinerea*. On the other hand, the remaining cultivars had either no or weak antifungal activity against *B. cinerea* (Table S1). Interestingly, two mustard cultivars, Dilong-1 and Jing-6, exhibited the highest mycelial inhibition ratios of 90% and 86%, respectively, indicating that they were highly effective in suppressing the growth of *B. cinerea*. Given that Dilong-1 not only demonstrated the highest antifungal activity against *B. cinerea*, but also grew higher with potential for abundant biomass (Figure 1C), it was selected for further investigations.

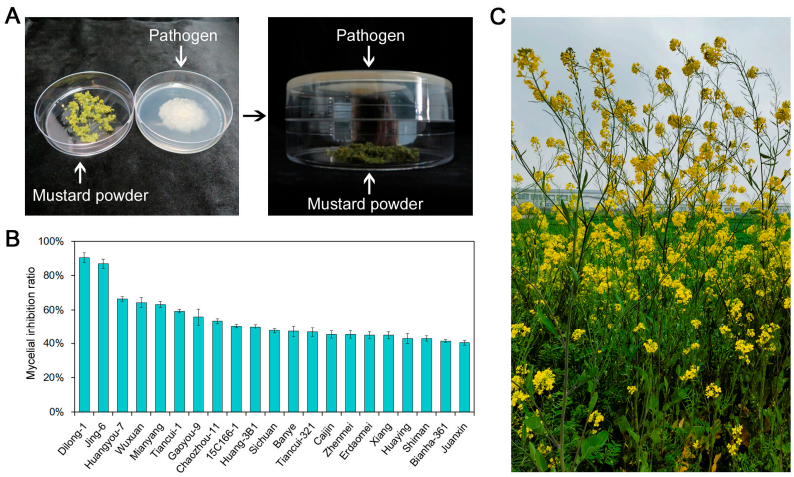

Figure 1. Screening of mustard cultivars as biofumigants with high antifungal activity against *B. cinerea*. (**A**) Experimental design used in this study. A plate with fresh powder of each mustard cultivar was on the bottom, and *B. cinerea* culture was placed on the top plate with the culture facing down. (**B**) Mycelial inhibition ratio of *B. cinerea* after three days of fumigation using 25 g/L of different mustard cultivars. (**C**) Dilong-1 plants at the 50% flowering growth stage.

3.2. Dilong-1 Suppresses Mycelial Growth and Morphology of *B. cinerea*

To investigate the antifungal effects of Dilong-1 in greater detail, different concentrations of Dilong-1 were used to treat *B. cinerea*, and the inhibitory effects on the mycelial growth were monitored daily for three days. In the control, where fumigation was carried out using water, *B. cinerea* grew rapidly and colonized almost the entire plate after three days of incubation (Figure 2A). However, in the Dilong-1 treatments, the radial mycelial growths of *B. cinerea* were inhibited to varying degrees by treating with 5 g/L to 25 g/L of Dilong-1. As the concentration of Dilong-1 increased, the inhibition of mycelial growth became more evident. After fumigation for three days, the inhibition ratio of mycelial growth was found to be 21%, 46%, 61%, 75%, and 90% when treated with 5, 10, 15, 20, and 25 g/L of Dilong-1, respectively (Figure 2A,B). Dilong-1 showed an inhibitory effect on the mycelial growth of *B. cinerea* with the IC_{50} (half maximal inhibitory concentration) value at 11.3 g/L. Notably, when treated with 25 g/L of Dilong-1, *B. cinerea* hardly grew during the fumigation process.

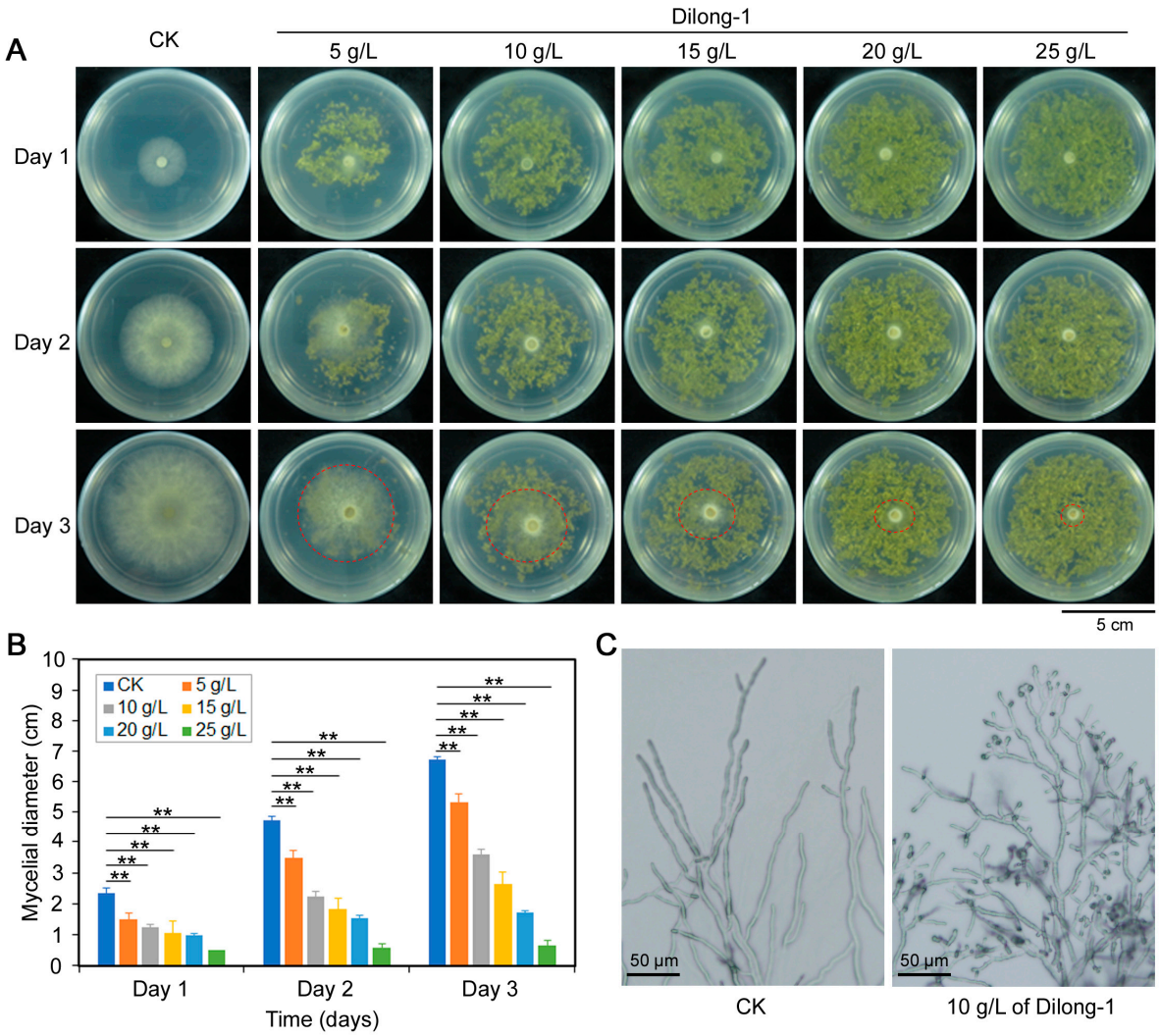

Figure 2. Antifungal activity of Dilong-1 against *B. cinerea*. Colony morphology (**A**) and mycelial diameter (**B**) of *B. cinerea* on the culture medium with Dilong-1 at indicated concentrations (5, 10, 15, 20, and 25 g/L) were measured every 24 h. The red circle marks the edge of the colony. Asterisks indicate a significant difference according to Student's *t* test; ** $p < 0.01$. (**C**) Fumigation by Dilong-1 changed the mycelial morphology of *B. cinerea*.

In addition to the effect on mycelial growth, the effects of Dilong-1 on the mycelial morphology of *B. cinerea* were also observed. The mycelial morphology of *B. cinerea* appeared normal in the control group, with a typical uniform, branching structure (Figure 2C). However, some striking abnormalities were observed after fumigation with 10 g/L of Dilong-1. The fumigated mycelia of *B. cinerea* became contorted and swollen, and the branches increased with stunted tips (Figure 2C). The observed changes in morphology suggested that Dilong-1 disrupted the internal structure of *B. cinerea* mycelia. Taken together, these observations indicated that Dilong-1 exerted its antifungal activity not only by inhibiting mycelial growth but also by altering the mycelial morphology of *B. cinerea*.

3.3. Dilong-1 Inhibits Spore Production and Germination of B. cinerea

The impact of Dilong-1 on the sporulation and spore germination of *B. cinerea* was investigated in further detail to determine the full extent of its antifungal activity (Table 1). The production of conidia was assessed by quantifying the number of spores/mL in control and Dilong-1 treated groups. Following fumigation with 10 g/L of Dilong-1, the production of conidia was 2.4×10^3 spores/mL, which was substantially lower than the control's 7.0×10^4 spores/mL. This represented a remarkable reduction in conidia production, indicating a potent inhibitory effect of Dilong-1 on the reproductive growth of *B. cinerea*. At a higher concentration of 20 g/L of Dilong-1, no spores were produced by *B. cinerea* (Table 1), indicating a potent inhibition of its sporulation. This suggested that Dilong-1 could effectively prevent the formation of new fungal colonies.

Further analysis revealed that the germination of *B. cinerea* spores occurred at a rate of approximately 91.9% in the control group, with an average germ tube length of 228 µm. However, in the treatment of 10 g/L of Dilong-1, the percentage of germinated spores decreased significantly to 31.2%, with an average germ tube length of 64 µm (Table 1), indicating a significant inhibition of spore germination by Dilong-1. This suggested that Dilong-1 might interfere with the metabolic processes essential for spore germination, such as nutrient uptake or energy metabolism. Hence, our findings suggested that Dilong-1 could effectively inhibit both the vegetative and reproductive growth of *B. cinerea*.

Table 1. Effect of Dilong-1 fumigation on sporulation production and germination.

	Sporulation (Spores/mL)	Spore Germination (%)	Length of Germ Tubes (µm)
CK	7.0×10^4 a	91.9 ± 2.1 a	228 ± 23 a
10 g/L	2.4×10^3 b	31.2 ± 1.8 b	64 ± 14 b
20 g/L	0 c	NA	NA

NA = not applicable. Different letters indicate a significant difference according to Student's *t*-test at $p < 0.05$.

3.4. Antifungal Activity of Dilong-1 against Various Postharvest Pathogens

To assess the broad-spectrum antifungal activity of Dilong-1 against a diverse range of postharvest pathogens, six additional pathogens, i.e., *Phytophthora litchii*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Fusarium solani*, *Colletotrichum gloeosporioides*, and *Thielaviopsis basicola*, were evaluated for their sensitivity to Dilong-1. After a 3-day fumigation treatment, all the tested pathogens exhibited significantly slower mycelial growth compared to the control (Figure 3A,B). Of these pathogens, *Py. aphanidermatum*, *F. oxysporum*, *F. solani*, *C. gloeosporioides*, and *T. basicola* showed moderate sensitivity to Dilong-1, with colony size reduction ranging from 40.3 to 50.8% compared to the control. These results indicated that Dilong-1 had the potential to be used as an effective alternative to traditional fungicides for controlling a wide range of postharvest pathogens.

Of particular interest was the high sensitivity of *P. litchii* to Dilong-1. This oomycete is the causal agent of litchi downy blight disease, which has become a significant threat to litchi production worldwide. Complete growth suppression of *P. litchii* was observed after fumigation with Dilong-1 (Figure 3A,B), highlighting its potential as a highly effective treatment for controlling this devastating disease.

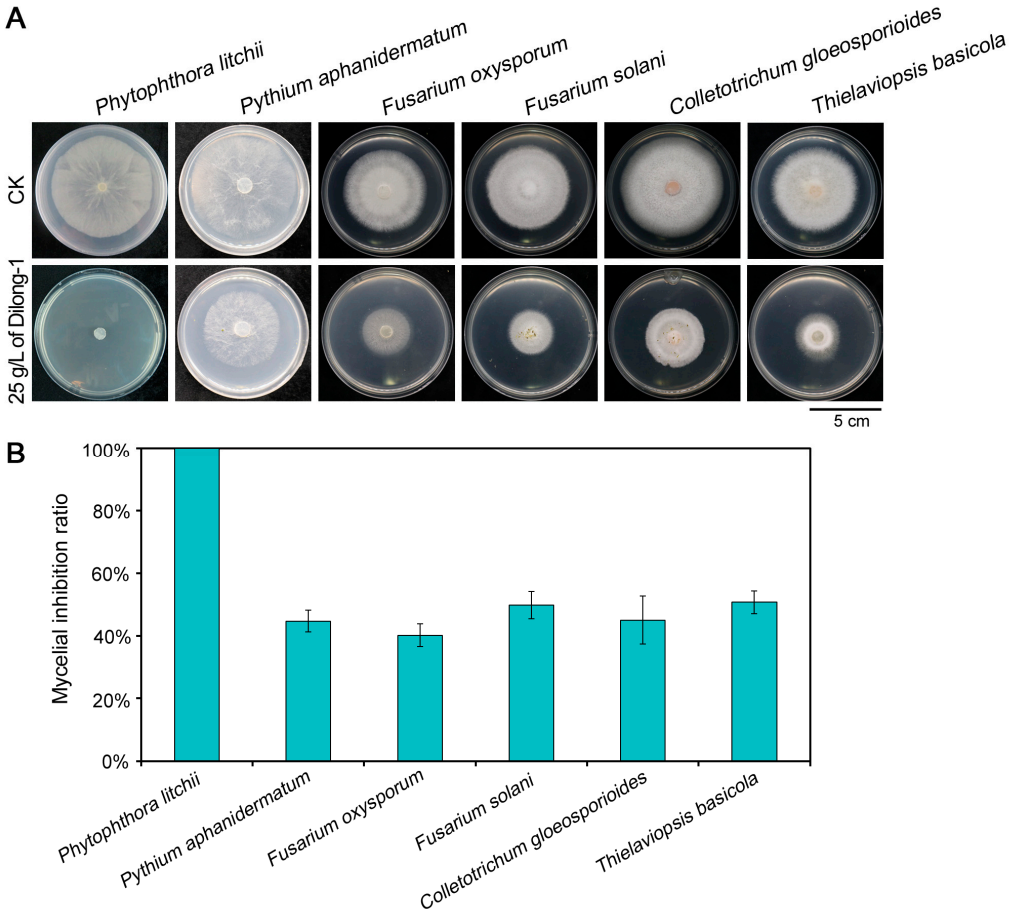

Figure 3. Antifungal spectrum of Dilong-1. Colony morphology (**A**) and mycelial inhibition ratio (**B**) of six postharvest pathogens on the culture medium after fumigation using 25 g/L of Dilong-1 were measured after 3 days.

3.5. Dilong-1 Suppresses Apple Gray Mold Decay

We further evaluated the effects of Dilong-1 in inhibiting gray mold decay in apples. The experiment was carried out by first inoculating all tested apple fruits with the mycelial block of *B. cinerea* and then placing them in an airtight box. Two treatment groups were established, with Dilong-1 used for fumigation in the first group, and water used for fumigation in the control group. After one day of incubation at 20 °C and 90% humidity, the apples in the control group displayed obvious gray mold symptoms, with a lesion diameter of 3.1 cm. In contrast, the apples in the treatment groups after fumigation with 10 g/L and 20 g/L of Dilong-1 showed much lower levels of gray mold decay, with lesion diameters of 2.1 cm and 1.4 cm, respectively (Figure 4A). Over the next two days, the gray mold symptoms spread rapidly, and the lesion diameter in the control group reached 5.4 cm. However, the severity of fruit rot was notably reduced in the groups treated with 10 g/L and 20 g/L of Dilong-1, and the extent of the decrease was positively correlated with the concentration of Dilong-1 used for fumigation (Figure 4A,B).

In the group treated with 20 g/L of Dilong-1 for three days, the lesion diameter was inhibited by 60% compared to that of the control group (Figure 4A,B). These results clearly indicated that Dilong-1 had a significant inhibitory effect on apple gray mold decay, and its efficacy was positively correlated with its concentration.

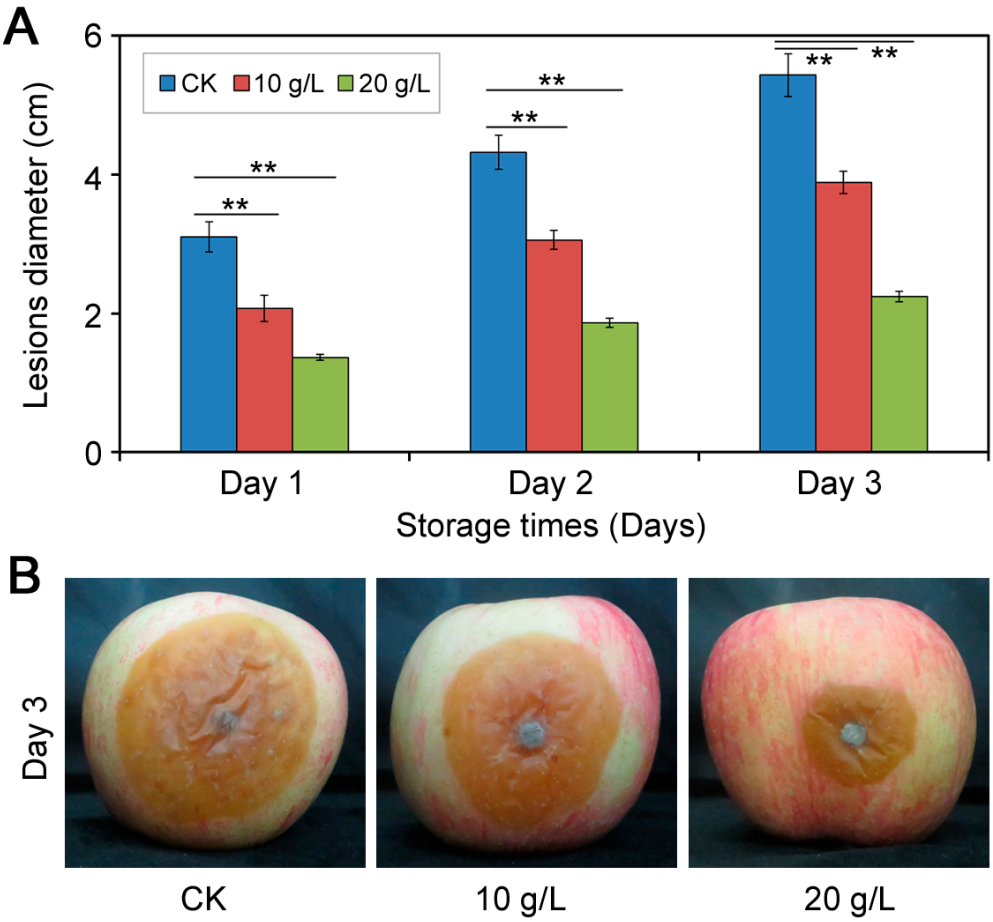

Figure 4. Effect of Dilong-1 on postharvest decay in apple fruits. All the tested apple fruits were first inoculated with mycelial block of *B. cinerea*, and then placed in an airtight box by fumigation with indicated concentrations (10 and 20 g/L) of Dilong-1, while water was used instead for the control treatment. (**A**) The lesion diameters of gray mold decay were assessed every 24 h after inoculation. Asterisks indicate a significant difference according to Student's *t* test; ** $p < 0.01$. (**B**) Disease symptoms on apples were photographed after inoculation for three days.

3.6. Dilong-1 Has No Harmful Effects on Apple Quality Traits

The effects of Dilong-1 fumigation on the quality traits of apple fruit were investigated. In this bioassay, apples were exposed to a concentration of 20 g/L of Dilong-1 for 20 days at 20 °C, while apples fumigated by water were used as the control. The weight loss of the apples was evaluated, and the results showed no significant difference between the Dilong-1 treated and control samples, indicating that Dilong-1 fumigation did not impact the water loss of the apples during storage (Figure 5A).

Moreover, we investigated the effect of Dilong-1 on various quality parameters of the apple fruit, such as firmness, total soluble solid content, malic acid content, and total phenol content. The results demonstrated that Dilong-1 fumigation significantly preserved the firmness of the apples compared to the control group (Figure 5B). In addition, Dilong-1 fumigation maintained the total soluble solid content (Figure 5C), malic acid content (Figure 5D), and total phenol content (Figure 5E) of the apples, which were important quality attributes that contribute to the sensory and nutritional value of the fruit. These findings suggested that Dilong-1 fumigation was a safe and effective treatment for controlling postharvest decay of apple fruit while maintaining their quality attributes.

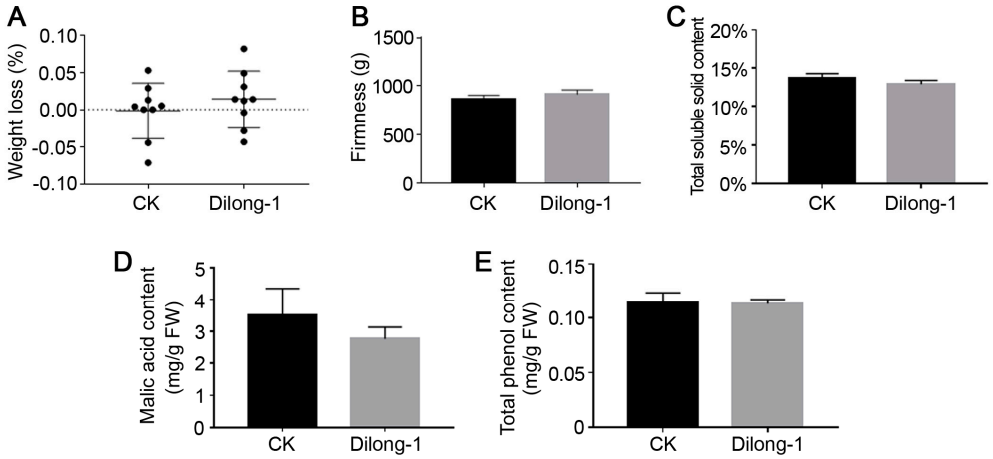

Figure 5. Effects on postharvest quality of apples. Apple fruits were exposed to 20 g/L of Dilong-1 for 20 days at 20 °C, while apples fumigated by water were used as the control. The apple quality traits including weight loss (**A**), firmness (**B**), total soluble solid content (**C**), malic acid content (**D**), and total phenol content (**E**) were assessed. All the experiments were performed with at least three independent replicates, and the data are presented as the mean ± SD.

4. Discussion

Postharvest diseases cause a significant number of apple fruits to be discarded annually, with gray mold caused by *B. cinerea* being one of the primary culprits. In recent years, except for the traditional application of synthetic fungicide, biological control has emerged as an effective and safe method for controlling postharvest decay in fruits. One promising alternative method for controlling fruit pathogens involves the use of natural products extracted from plants [39]. The GSL hydrolyzed products ITCs derived from *Brassica* plants have been reported to suppress numerous phytopathogens, especially soilborne pathogens [24]. However, their application in controlling postharvest diseases in fruits is rare. In this study, we performed a large-scale screening to evaluate the antifungal activities of 90 mustard cultivars against *B. cinerea*. Results revealed that these mustard cultivars exhibited varying antifungal effects, suggesting that mustard plants contained varying concentrations and chemical compositions of GSL compounds that may affect biofumigation efficacy. Of the 90 cultivars evaluated, 21 demonstrated more than 40% mycelial growth inhibition, suggesting that many mustard cultivars had a high efficacy in inhibiting *B. cinerea*.

In recent years, there has been increasing interest in using microbial antagonists and natural plant products for the biological control of fruit postharvest diseases [38–41]. Some natural plant products, such as citral, jasmonates, benzaldehyde, and ethanol, have been found to have potential antimicrobial activity against postharvest pathogens [42], but research on these is still limited. In this study, a mustard cultivar called Dilong-1 was found to greatly suppress the mycelial growth of *B. cinerea* compared to other cultivars. Further investigation revealed that Dilong-1 not only suppressed mycelial growth and morphology, but also inhibited spore production and germination of *B. cinerea*. Fumigation using Dilong-1 also significantly reduced gray mold decay in apple fruits, indicating that suppression of fungal development was one main mode of action of Dilong-1 in protecting the postharvest decay of apple fruits. Notably, fumigation using Dilong-1 displayed a wide antifungal spectrum including other fruit postharvest pathogens, especially for *Phytophthora litchii*, suggesting that Dilong-1 had a promising potential used as a biofumigant with a broad antifungal spectrum, and could be potentially used in fruit postharvest disease control. Moreover, fumigation with Dilong-1 did not negatively impact final apple qualities, including weight loss, firmness, and total soluble solids. Therefore, fumigation using Dilong-1 could be viewed as a safe and environment-friendly measure.

Many more questions regarding Dilong-1 need to be further studied. Firstly, mustard cultivars have varying concentrations and chemical compositions of GSL compounds in their tissues, and GSL hydrolyzed products ITCs are also present differentially among mustard cultivars. One previous study evaluated antifungal activities of eight ITCs against the pathogenic fungi in tomato, and found that AITC exhibited the lowest EC_{50} values against *B. cinerea* [43]. In the current study, we only used the fresh powder of Dilong-1 for a series of fumigation experiments. The quantities and chemical compositions of GSLs and ITCs in Dilong-1 need to be measured in the near future. Secondly, some natural products extracted from plants are known to induce plant resistance to protect the postharvest decay of fruits. Methanolic extracts of Acacia seyal and Withania somnifera promote the synthesis of cell wall-bound phenolics to increase citrus fruit resistance against *Penicillium digitatum* [44]. In another study, the combined treatment with chitosan and salicylic acid increased total phenolics compounds and activated the activity of defense enzymes in grapefruit fruits to control green molds caused by *P. digitatum* [45]. Therefore, it is worth exploring whether Dilong-1 could induce resistance in apple and other fruits. Finally, the efficacy of postharvest treatments of Dilong-1 on fruits may depend on application methods. A study has reported that *Brassicaceae* plants still contain a high level of GSLs and sufficient myrosinase to hydrolyze them even after drying, and the dried plants show good inhibitory activity against *Pythium* ssp. and *Rhizoctonia solani* after water addition [46]. Considering the similarity of Dilong-1, some industrial techniques can be used to dry Dilong-1 plants, allowing the dried plants to be stored for a longer time. When ready to use, the dried Dilong-1 plants can be placed in a breathable bag or container and a certain amount of water can be added to restore their inhibitory activity against fungi, thereby achieving the purpose of preventing fruit postharvest diseases. The application of fumigation with Dilong-1 needs to be further investigated.

5. Conclusions

In this study, we conducted a large-scale screening of mustard cultivars and identified Dilong-1 as having the highest inhibitory effect against *B. cinerea*. Fumigation with Dilong-1 demonstrated a suppressive effect on *B. cinerea* in both in vitro assays and in apples stored under controlled conditions. These results suggest that Dilong-1 has promising potential as a biofumigant with a broad antifungal spectrum, and could potentially be used in postharvest disease control for fruits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13061490/s1, Table S1: List of tested mustard cultivars as biofumigants with antifungal activity against *B. cinerea*.

Author Contributions: D.D. and D.S. conceived and designed the experiments. Y.T., Z.Y., W.S., H.Z. and Q.Y. carried out the experiments and analyzed the data. H.X. and B.H. provided valuable advice. Y.T., Z.Y. and D.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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