

Article

Cell-Free Supernatant of *Bacillus subtilis* Suppresses the Mycelial Growth of *Penicillium expansum* by Destroying the Cell Membrane Integrity and Provoking Cell Death

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Simple Summary

Penicillium expansum causes rot in fruits such as grapes and citrus, accompanied by the production of harmful mycotoxins, leading to substantial economic losses. At present, synthetic chemical agents are widely applied to control this pathogen, yet they exert adverse impacts on the environment and human health. Accordingly, this study investigated a natural and eco-friendly control strategy, namely, the utilization of cell-free supernatant derived from *Bacillus subtilis* to combat *P. expansum*. Assays demonstrated that the cell-free supernatant of *B. subtilis* effectively inhibited the germination and spread of spores on postharvest fruits. The underlying antifungal mechanism involves disrupting the protective outer membrane of the fungus and triggering severe intracellular stress responses, which ultimately result in the mortality of *P. expansum*.

Abstract

Penicillium expansum inflicts significant economic damage in the fruit and vegetable industry due to its wide distribution and ability to infect a diverse range of hosts. Therefore, developing safe and environmentally sustainable strategies to suppress the growth of this pathogen is of critical importance. *Bacillus subtilis*, recognized for its broad antimicrobial activity and widespread occurrence, has been widely utilized in the biological management of plant diseases. This research seeks to assess the inhibitory potential of *B. subtilis* against *P. expansum*. The cell-free supernatant (CFS) derived from *B. subtilis* significantly suppresses the germination of spores, germ tube extension, and hyphal development of *P. expansum*. It also reduces disease incidence in grapes and citrus and suppresses the expansion of lesions. Further investigation had shown that it induced mycelium reactive oxygen species (ROS) accumulation, destroyed the cell membrane integrity, led to leakage of cytoplasmic contents and induced membrane lipid peroxidation. Moreover, exposure to high concentrations of CFS results in mycelial contraction and morphological abnormalities, triggering the disintegration of intracellular organelles and markedly upregulating the expression of apoptosis-like programmed cell death genes. The self-protective response elicited by 5% CFS is insufficient to counteract the extent of cellular damage, ultimately driving cells toward a dynamic, multistage, and disintegrative form of cell death. The findings of this study offer a theoretical foundation for managing *P. expansum* after harvest.



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1. Introduction

Penicillium expansum is a ubiquitous necrotrophic fungus found in various natural habitats [1]. It thrives at low temperatures and produces abundant light conidia, which endows it with a strong ability to spread. It has a wide host spectrum and can infect numerous kinds of berries, pomes, and drupes [2,3], including grapes [4], apples, pears, peaches, and cherries [5,6]. The disease caused by *P. expansum*, commonly referred to as blue mold decay, is considered one of the most widespread causes of fruit deterioration after harvest globally [3]. It causes significant economic losses in the fruit industry during storage, transportation, and marketing because of its characteristics of low-temperature resistance and easy spreadability. During fruit infection, *P. expansum* is capable of producing patulin, a toxic fungal metabolite that can be harmful to human health, which is primarily synthesized in the necrotic regions of the infected tissue, and can also migrate to other parts of the fruit [7]. Managing the growth and progression of *P. expansum* continues to be a major challenge in maintaining the safety and quality of fruit-based products.

Traditionally, chemical agents like myclobutanil (MYC), imazalil (IMA), and pyrimethanil (PYR) have been extensively used to control *Penicillium* spp. [8]. Nevertheless, their extensive use has led to the development of pathogen resistance and caused environmental contamination. Growing public concern about reducing fungicide use and ensuring the production of healthy, safe and environmentally friendly strategies should be developed to suppress the growth of *P. expansum*. In recent years, there has been increasing interest in biological methods for controlling *P. expansum*, as they pose fewer risks to human health and the environment. The application of microbial antagonists to suppress pathogenic organisms has emerged as a central area of research. Commonly used biological control agents include yeast [9] and bacteria [10–12]. Among these, *Bacillus* has garnered significant attention due to its widespread distribution in soil and broad-spectrum inhibitory activity, leading to its successful application in various commercial and industrial settings. The primary *Bacillus* species that have been utilized in biological control research and practical applications include *Bacillus subtilis* [13], *B. cereus* [14,15], *B. megaterium* [16], *B. pumilus* [17], *B. siamensis* [18], *B. amyloliquefaciens* [19] and *B. velezensis* [20]. Numerous studies have demonstrated that *Bacillus* spp. can inhibit various plant pathogenic fungi including but not limited to *Aspergillus flavus* [21], *Fusarium verticillioides* [22], *Botrytis cinerea* [23], and *Botryosphaeria dothidea* [24]. The cell-free supernatant (CFS), which contains a diverse array of enzymes and secondary metabolites, facilitates the proliferation of beneficial soil microorganisms and provides a sustainable, efficient, and environmentally friendly solution to the challenges related to microbial inoculation. Its advantages, including low required concentrations, better storage stability than live microbial cells, easy application, and reduction in the biosafety risks inherent in viable microorganisms, make it highly applicable in fields such as ecological conservation and post-harvest disease management [25]. In this study, the CFS of *B. subtilis* was expanded to inhibit *P. expansum* and manage soft rot in grape and citrus fruits. We primarily sought to investigate the inhibitory effects of CFS on *P. expansum*, assessed by mycelial growth, spore germination, mycelial activity, while also delving into the mechanisms by which CFS damages *P. expansum* characterized by cell membrane integrity, ROS production and gene expression. This research aims to contribute to the development of an effective, environmentally friendly, and safe biological strategy for controlling *P. expansum*.

2. Materials and Methods

2.1. *P. expansum* and Preparation of Spore Suspension

P. expansum, provided by Professor Liu Yongsheng of Anhui Agricultural University, was cultured on potato dextrose agar (PDA) plates for 2 to 3 weeks at 25 °C temperature incubator. Spores were collected and resuspended in distilled water, then filtered through four layers of sterile cheesecloth to remove residual mycelium. Spore concentration was counted using a hemocytometer and then adjusted to 1×10^6 cfu/mL.

2.2. Resuscitation of *Bacillus subtilis* and Preparation of Cell-Free Supernatant

Refer to Fan et al. [24]. The *Bacillus subtilis* strain BS-1 was cryopreserved in the microbial culture collection. The strain was activated on a Luria–Bertani (LB) agar plate and incubated at 28 °C for 16 h. Subsequently, a single colony was transferred to a liquid medium and cultured in a shaking flask at 28 °C until the optical density at 600 nm (OD₆₀₀) reached 0.6. Thereafter, the culture was inoculated into a deodorizing fermentation medium (LB supplemented with 2% glucose) at an inoculum size of 0.5% and incubated at 28 °C for 72 h under agitation at 180 rpm to maximize its antifungal activity. After incubation, the bacterial suspension was centrifuged at 4 °C and 13,000 rpm for 20 min. The supernatant was filtered through a 0.22 µm membrane, supplemented with 50 mg/L streptomycin, and stored at 4 °C for later use.

2.3. Fruit Preparation and the Effect of CFS on Blue Mold Disease of Grape and Citrus Fruits

The grape (*Vitis vinifera* L. cv. Kyoho) and citrus (*Citrus sinensis* cv. Navel) were harvested from the orchards in Yinchuan at commercial maturity stage without any treatments and immediately transferred to the laboratory. Fruits free from physical damage or microbial contamination were treated with 2% (v/v) sodium hypochlorite solution for 2 min, rinsed three times with sterile water, and then allowed to dry naturally. Disinfected fruit was injured at two evenly spaced locations in the equatorial area with a sterile stainless-steel probe. Each injury measured 2 mm in diameter and extended 3 mm in depth. A 5 µL spore suspension of *P. expansum* and 5 µL 5% CFS were mixed and co-inoculated into each wound. LB medium was treated as control. The processed fruits were stored in plastic containers and kept in a temperature-controlled incubation chamber set at 25 °C, with relative humidity maintained between 80% and 90%. The incidence rate was statistically analyzed, and lesion diameters were recorded after 72 h of inoculation using a vernier caliper with the cross-crossing method.

2.4. Spore Germination, Germ Tube Elongation Test

For the spore germination and germ tube elongation assay, a 40 µL aliquot of *P. expansum* was uniformly spread on 1.5 × 3 cm sections cut from PDA plates containing 0, 1%, 2%, and 5% CFS. These sections were placed on sterile glass slides and incubated at 28 °C. After 8 and 10 h of incubation, spore germination and the extension of germ tubes were observed, respectively. For each treatment, 50 spores were chosen at random to determine the germination percentage, and the lengths of the germ tubes were assessed using ImageJ (version 1.54g) software.

2.5. Mycelial Growth and Dry Weight

The mycelial development of *P. expansum* was evaluated by inoculating 20 µL of the fungal suspension into the center of PDA plates supplemented with 0, 1%, 2%, and 5% CFS, using an Oxford cup as the delivery method. The inverted plates were placed in a constant-temperature incubator at 28 °C for incubation. The colony diameter was measured every 24 h using the cross-method measurement technique to evaluate the inhibitory effect.

A 1 mL sample of the spore suspension was inoculated into 20 mL of PDB medium and incubated at 28 °C with shaking at 180 rpm for 24 h. Subsequently, CFS were added to the cultures, adjusting the concentration to 0, 1%, 2%, and 5%, which were further incubated for 48 h. In order to determine the dry weight, the mycelia were harvested and dried in an oven at 55 °C until a constant weight was achieved.

2.6. Mycelial Activity Measurement

Following Patel's protocol [26] mycelia were harvested using the same method as described above, and their wet weight was recorded. The fragments were washed twice with PBS buffer (1 M, pH 7.0), then resuspended in 900 µL of PBS with 100 µL of MTT solution (5 mg/L). The samples were incubated in the dark at 28 °C on a shaker for 90 min, then centrifuged at $8000 \times g$ for 3 min. In total, 800 µL of DMSO was added to each sample, and the mixtures were gently shaken at 30 °C for 10 min to dissolve the formazan crystals completely. The absorbance was then recorded at a wavelength of 570 nm.

2.7. Morphology and Microstructure Examination

The mycelium of *P. expansum* was treated with 5% CFS for 72 h, rinsed two times using 0.1 M PBS buffer (pH 7.4) and subsequently fixed in 2.5% glutaraldehyde at 4 °C for 12 h. Following fixation, the sample underwent three 15 min rinses with the same PBS buffer. Dehydration was performed using an ascending concentration gradient of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%), after which the specimen was immersed in isopentyl acetate for 15 min and subsequently dried using a critical point drying method. Finally, the dried sample was attached to conductive carbon film with double-sided tape, mounted on an ion sputtering instrument, and coated with gold for about 30 s to make it electrically conductive. Mycelium morphology of five samples was observed using a scanning electron microscope (SEM) (HITACHI, SU8100, Hitachi High-Tech Corporation, Tokyo, Japan).

For transmission electron microscope (TEM) (HITACHI, HT780, Hitachi High-Tech Corporation, Tokyo, Japan) observation, mycelium samples were fixed and dehydrated as previously described. The samples were infiltrated and embedded in EMBed 812 resin using acetone as a transitional solvent. Fresh EMBed 812 solution was added every 2 h to gradually increase resin concentration. After overnight infiltration at 37 °C, the samples were polymerized in molds at 65 °C for over 48 h. Ultrathin sections of 60–80 nm were cut from the resin-embedded samples using an ultramicrotome, and these sections were subsequently mounted on 150-mesh copper grids pre-coated with a formvar support film. Sections were stained with 2% uranyl acetate in alcohol avoid light and 2.6% lead citrate CO₂-free (each 8 min), then five samples were observed and imaged under the TEM.

2.8. ROS Detection

Referring to the method of Li et al. [27], the mycelium was collected 16 h after treatment with 0, 1%, 2%, and 5% CFS. After removing the medium and being rinsed three times with 0.05 M PBS buffer (pH 7.0), the mycelium was stained with 25 µM 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and incubated for 60 min in a water bath maintained at 30 °C. The mycelium was resuspended, rinsed using 0.05 M PBS buffer (pH 7.0) and subjected to centrifugation at 10,000 rpm for 2 min until the dye solution was completely eliminated. ROS accumulation was examined on a laser confocal microscope (LSM710, Zeiss, Oberkochen, Germany) at 488 nm excitation and 520 nm emission. Fluorescence intensity was quantified with ImageJ (version 1.54g) software across five images with comparable mycelial density.

2.9. Cell Leakage Detection

Referring to the method of Zhao et al. [23], cell membrane permeability and leakage were assessed by measuring conductivity, nucleic acid content, protein content and malondialdehyde (MDA) content. A 0.1 mL sample of *P. expansum* spore suspension was added to 5 mL of PDB medium and mixed uniformly. The cultures were incubated in a rotary shaker at 28 °C and 80 rpm for three days. Fungal mycelia (0.5 g) were dispersed in 0, 1%, 2%, and 5% CFS and then cultured in a shaker at 25 °C for 0, 3, 6, and 9 h. The obtained cultures were filtered and subsequently utilized for measuring the electrolyte, nucleic acids and soluble protein content. The concentration of electrolytes in the solution was assessed using a conductivity meter, while protein levels were quantified using the Coomassie Brilliant Blue assay with Ultraviolet spectrophotometer (P4, Mapada, Shanghai, China) [28], and the extent of nucleic acid leakage was evaluated by determining the absorbance at a wavelength of 260 nm. The mycelial samples were thoroughly homogenized into a fine powder with the aid of liquid nitrogen prior to conducting the MDA analysis. The MDA level was measured using the thiobarbituric acid (TBA) assay [29].

2.10. Cell Membrane Integrity Detection

The sample for detecting cell membrane integrity was prepared as described in Section 2.9. Mycelium was collected by centrifugation at 8000 r/min for 2 min following 24 h of treatment. Following the method of Wang et al. [30], the mycelium was treated with a 5 mM propidium iodide (PI) solution, followed by incubation at 30 °C for 20 min in a water bath, and subsequently rinsed using 0.05 M PBS buffer (pH 7.0). The integrity of the mycelial cell membrane was assessed using laser confocal microscopy (LSM710, Zeiss, Oberkochen, Germany) at 535 nm excitation emission.

2.11. RNA Extracted and Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

After the mycelium was treated with 0, 5% CFS at 24 h and 48 h, the mycelium was collected and stored at −80 °C, respectively. Total RNA was extracted using the OMEGA R6840-01 kit (Omega Bio-tek, Norcross, Georgia). Reverse transcription of RNA was carried out using the HiScript[®] III RT SuperMix for qPCR kit (Vazyme Biotech, Nanjing, China), and fluorescence quantitative PCR was carried out with Vazyme ChanQTM SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China). The above experiments were conducted according to the kit instructions. The primer sequences and corresponding gene IDs used in this study are listed in Table 1. The expression level was normalized by the internal control gene *Isy1* using the $2^{-\Delta\Delta CT}$ method.

Table 1. Primer sequences employed in RT-qPCR assays.

Gene Name	Accession No.	Primer Name	Sequence of Primer (5'-3')
<i>PePRT</i> [31]	PEX2_027670	Pe-prtF Pe-prtR	CCGATGTTACCCCTAAGCAG AGGATCTGAGTGTAATTGGCG
<i>PeNoxA</i> [32]	PEX2_053880	Pe-NoxAF Pe-NoxAR	CATTAGATGAGTCGGCGTGG CAAGTTCTGGGCGGATATGG
<i>PeNoxR</i> [32]	PEX2_056490	Pe-NoxRF Pe-NoxRR	CTCTGAAGATGAAGGTGCAGG AACGCTCTTCCACCCATATC
<i>PeRacA</i> [32]	PEX2_019970	Pe-RacAF Pe-RacAR	GTACACAACGAATGCTTTCCC GATCGTAATCCTCTTGTCACG
<i>PeMetacaspased1</i>	XM_016744186.1	CASP1-F CASP1-R	TGATGTTTTTCAGGGTCCAAGG CATTTCCGGATCGTGTTTCAGC

Table 1. Cont.

Gene Name	Accession No.	Primer Name	Sequence of Primer (5'-3')
<i>PeMetacaspased2</i>	XM_016742860.1	CASP2-F CASP2-R	ACCAGCAAAACCCGATGAG TTCGTCACCATCCAAGTCG
<i>Isy1</i>	PEX2—072240	Isy1-F Isy1-R	CAAAGCCTGAGCGACTACCA CGCCCTTCATCGTCGTAAG

2.12. Data Statistics and Analysis

The experimental data were statistically analyzed and visualized using GraphPad Prism (version 8.0). A Duncan's multiple-range test was employed to compare the control and other treatment groups; a significant difference was observed when $p < 0.05$ and present with *, and an extremely significant difference was observed when $p < 0.01$ present with **. In the figures, samples that share the same letter are not significantly different from one another, whereas those labeled with different letters exhibit statistically significant differences.

3. Result

3.1. CFS Inhibited Spore Germination and Germ Tube Elongation of *P. expansum*

To evaluate the inhibitory effect of CFS on *P. expansum*, observations were conducted on spore germination and germ tube extension. As shown in Figure 1, the spores showed varying degrees of germination after 8 and 10 h of incubation. Various concentrations of CFS markedly suppressed spore germination and germ tube growth, with the observed reduction in activity showing a concentration-dependent trend. Treatment with 5% CFS could reduce the spore germination rate from 94.3% to 47.1% (Figure 1A,B), while the germ tube lengths decreased from 22.4 μm to 8.8 μm , reducing by 61.1% respectively (Figure 1A,C). At 10 h, while the control group showed a germination rate of 96.3%, the 5% CFS treatment group had a significantly lower rate of 84.8%. Germ tube elongation remained suppressed 10 h after treatment. Germ tube lengths were 33.34 μm , 25.91 μm , 19.47 μm , and 16.70 μm at concentrations of 0, 1%, 2%, and 5%, respectively. At a concentration of 5%, the germ tube length was reduced by 49.9% relative to the control group. Germ tube elongation remained suppressed 10 h after treatment. The results indicate that CFS markedly decreased the germination rate of *P. expansum* spores (Figure 1B) as well as the length of germ tubes (Figure 1C), with a more pronounced inhibitory effect observed at higher concentrations.

3.2. CFS Inhibits Mycelial Growth and Activity

When investigating the effect of CFS on *P. expansum* on PDA medium, it was observed that mycelial growth was inhibited to varying degrees on PDA medium supplemented with different concentrations of CFS. The diameter of the colony diminished as the concentration of CFS increased. Low-concentration CFS exhibited a limited inhibitory effect on mycelial growth; however, with the concentration of CFS increasing, 5% CFS significantly suppressed colony expansion (Figure 2A,B). Compared with the control group, in the group containing 5% CFS, the inhibition ratios from the second day to the sixth day were 25.1%, 26.8%, 23.1%, 21.9%, and 19.9%, respectively. The effect of CFS on mycelial growth was assessed by measuring the dry weight of the mycelium. After 3 days of incubation, the dry weights of mycelium were 3.38 g, 2.78 g, 2.05 g, and 1.28 g, respectively, under different concentrations of CFS (Figure 2C,D). Compared with the control group, the 1%, 2%, and 5% CFS reduced the dry weight by 18.2%, 39.3%, and 62.6%, respectively. The mycelium activity was measured using the MTT method. It was observed that the mycelium activity decreased significantly following treatment with varying concentrations of CFS, and the decrease

in activity showed a pattern dependent on concentration. After 48 h of treatment, the mycelium activity in the presence of 1%, 2%, and 5% CFS decreased by 0.08%, 27.7%, and 36.4%, respectively, while after 96 h, it decreased to 16.9%, 32%, and 41.2%, respectively (Figure 2E). The findings suggest that CFS is capable of markedly suppressing the mycelial development and physiological activity of *P. expansum*.

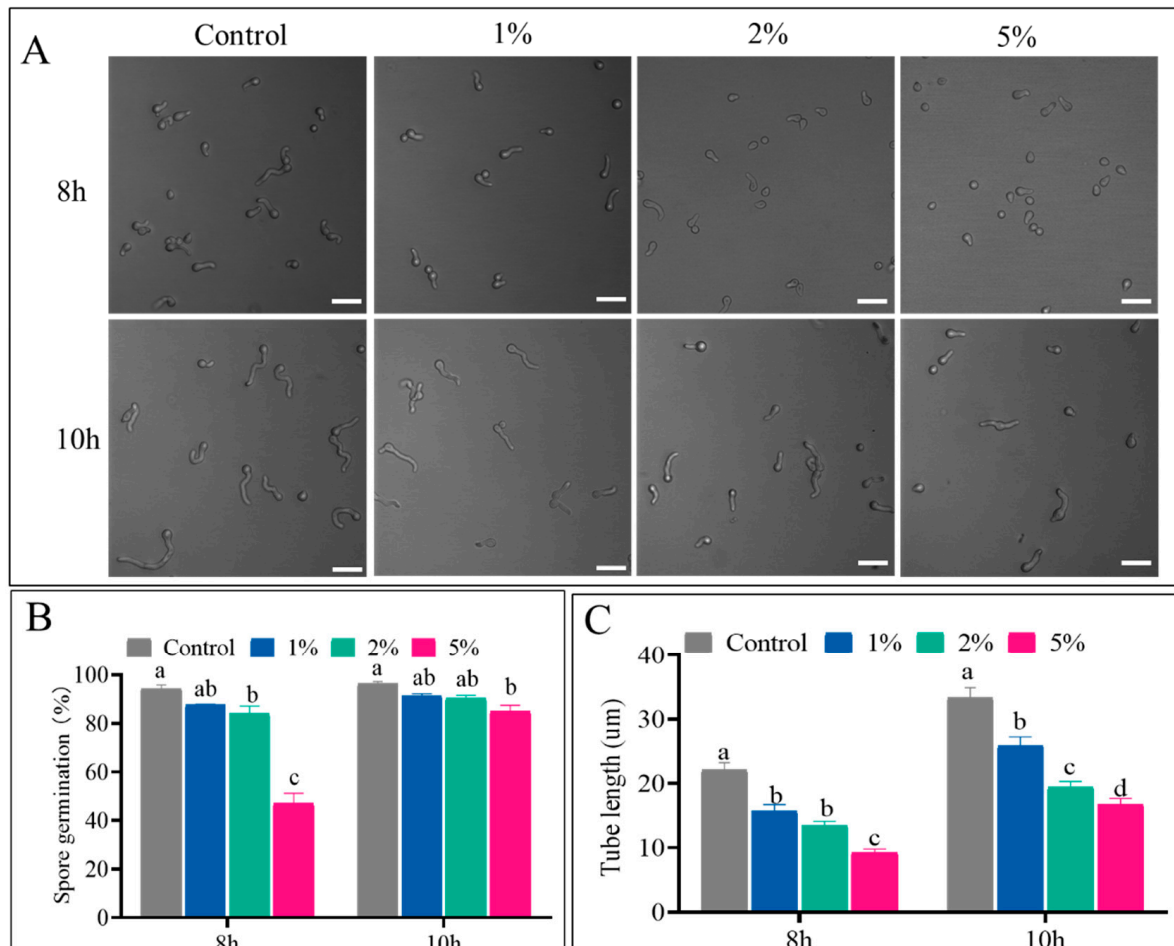


Figure 1. Effects of varying CFS concentrations on spore germination. (A) Effects of different concentrations of CFS on spore germination; (B) statistical chart of the effect of different concentrations of CFS on spore germination rate; (C) graph showing the statistical analysis of germ tube length influenced by varying concentrations of CFS; different letters indicated statistically significant differences between groups ($p < 0.05$).

3.3. Inhibitory Effect of CFS on the Pathogenicity of *P. expansum* in Inoculated Grape and Citrus Fruits

To further investigate the effects of CFS on the pathogenicity of *P. expansum* in grape and citrus fruits, 5% CFS and *P. expansum* were co-inoculated into grape and citrus fruits. After 3 days of inoculation, the incidence rates were 83.5% and 61.0%, respectively, which were significantly decreased 22.5% than the control group on grape (Figure 3A,B). On citrus (Figure 3C–E), the incidence rates were 67.9% and 25.8%, respectively, significantly decreased 42.1% than the control group at 3 days. The lesion diameter was 7.03 mm and 5.25 mm, respectively, representing a reduction of 25.4% compared to the control group at 3 days. All fruits in the control group had developed complete disease symptoms, whereas the treated group exhibited an 83% disease incidence rate at 5 days. Moreover, neither physical nor morphological changes were observed in the grapes and citrus fruits

treated with 5% CFS. The results demonstrated that 5% CFS could significantly suppress the alleviated the pathogenicity of *P. expansum* on grape and citrus.

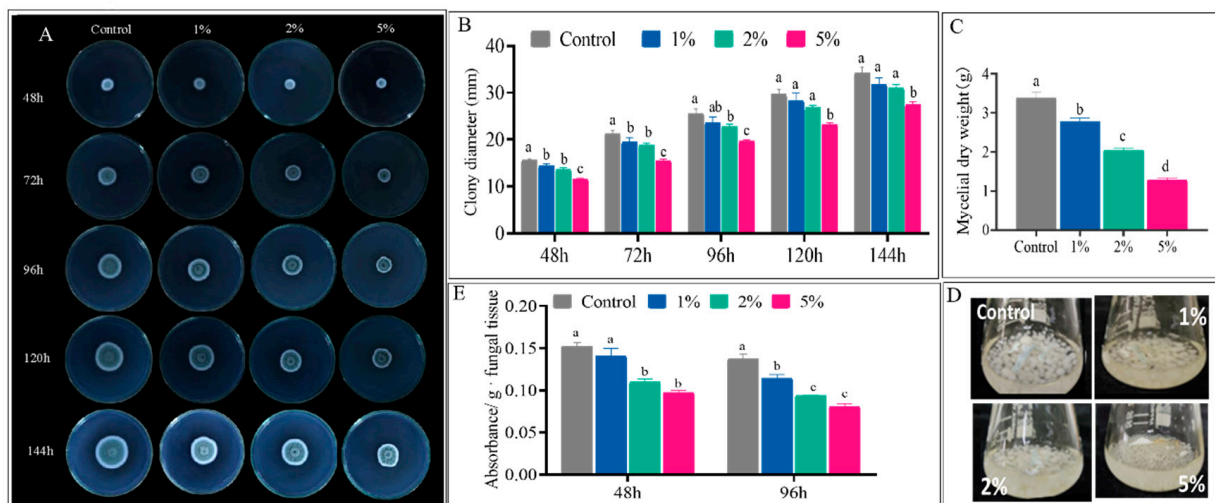


Figure 2. Effects of CFS on colony growth, dry weight and mycelial activity: (A) colony growth recorded at 48 h–144 h; (B) statistics analysis of colony diameter; (C,D) mycelium dry weight recorded and statistics analysis; (E) mycelial activity analysis; different letters indicated significant differences between groups ($p < 0.05$).

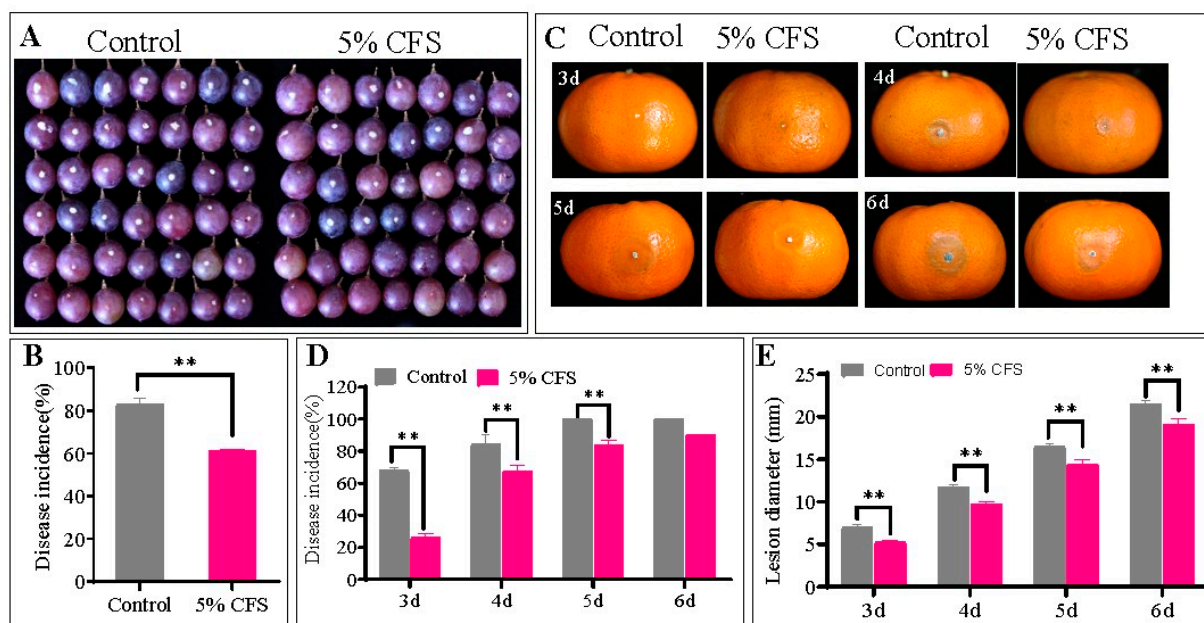


Figure 3. Effect of 5% CFS on pathogenicity of *P. expansum* on grape and citrus: (A) Disease symptoms on grape after 3 days; (B) statistics of grape incidence after 3 days treatment with 5% CFS; (C) disease symptoms on citrus after 3, 4, 5, 6 days; (D,E) disease incidence and lesion diameter statistics. ** indicated significant difference between groups ($p < 0.01$).

3.4. CFS Upregulated the Expression of Genes and Induced ROS Accumulation in the Mycelium of *P. expansum*

Reactive oxygen species (ROS), which encompass both oxygen-free radicals and non-radical reactive molecules, play a critical role in regulating essential biological processes, including growth, stress tolerance, and programmed cell death (PCD). To better understand the mechanism by which CFS induces hyphal inhibition, ROS levels in hyphal cells were monitored following exposure to CFS. As shown in Figure 4A, the intensity

of ROS-associated green fluorescence in hyphal cells rose progressively with increasing CFS concentration, suggesting sustained ROS buildup after CFS application. Pathogenic fungi generate ROS through the catalytic activity of NADPH oxidases (Nox). NoxA is the only one that contains a complete catalytic core domain among the three Nox catalytic subunits in filamentous fungi. The regulatory subunit NoxR and the small GTPase RacA are essential for activating the function of fungal NoxA [32,33]. The *PeNoxA*, *PeNoxR* and *PeRacA* genes related to ROS synthesis were detected. As shown in Figure 4C–E, *PeNoxA*, *PeNoxR*, and *PeRacA* were significantly upregulated by 64.18 ± 2.58 -fold, 30.91 ± 2.50 -fold, and 40.46 ± 2.41 -fold at 48 h post-CFS treatment, respectively.

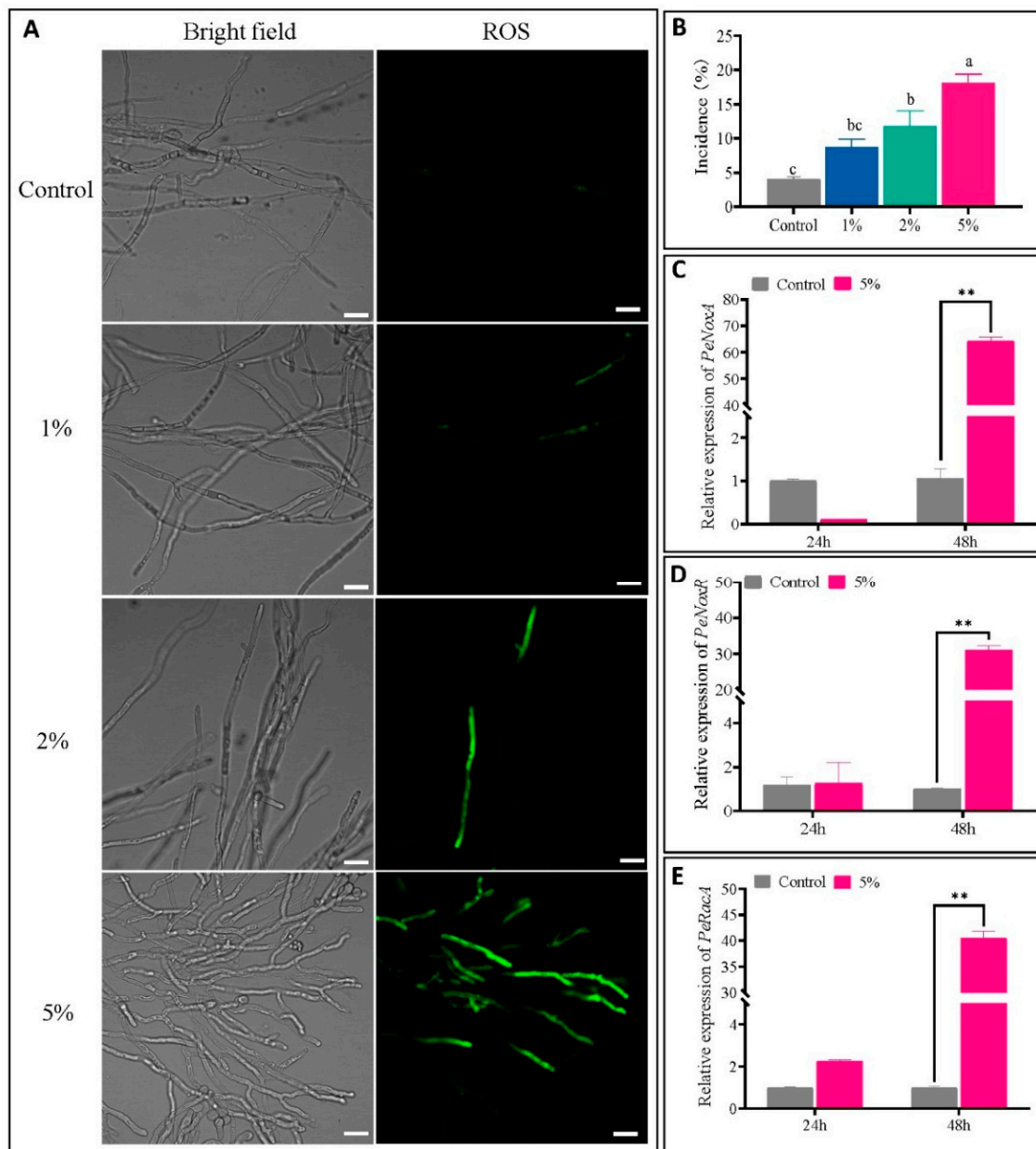


Figure 4. CFS on ROS accumulation (A), the relative intensity of fluorescence (B), and relative gene expression of *PeNoxA* (C), *PeNoxR* (D), *PeRacA* (E). Different letters indicated significant differences between groups ($p < 0.05$). ** indicates significant difference between groups ($p < 0.01$).

3.5. CFS Destroyed the Cell Membrane Integrity, Resulted in the Release of Cytoplasmic Components and Triggered Lipid Peroxidation in the Cell Membrane of *P. expansum*

To further investigate the impact of excessive ROS accumulation on the cell membrane, membrane integrity was assessed using PI staining, macromolecule secretion analysis, and measurement of MDA levels. Propidium iodide (PI) can bind to DNA and emit red fluorescence; however, because it cannot penetrate intact cell membranes, it is widely used as a reliable tool for assessing cell membrane integrity. As shown in Figure 5A, the control group displayed normal cellular morphology, with nearly negligible red fluorescence detected across the entire field of view. In the CFS treatment group, 1% and 2% CFS also produced faint red fluorescence; stronger red fluorescence was observed in 5% CFS treatment group. The intensity of red fluorescence gradually rose with increasing CFS concentration, further confirming a dose-dependent response. The alterations in cell membrane integrity were further demonstrated by measuring the conductivity, nucleic acid content, and protein concentration in the supernatant obtained from the co-culture of CFS and *P. expansum* following 3 h, 6 h, and 9 h of treatment with 5% CFS. The conductivity (Figure 5C), nucleic acid concentration (Figure 5D) and soluble protein content (Figure 5E) exhibited a sustained and statistically significant increase compared to the control group. MDA, resulting from oxidative degradation of lipids in the fungal hyphal membrane, also gradually accumulated with the treatment time and was significantly higher than the control group (Figure 5F).

3.6. CFS Causes Mycelial Shrinkage and Deformation, Leading to the Breakdown of Cellular Organelles

To gain deeper insights into the mechanisms through which CFS suppresses the growth of *P. expansum*, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses were performed. As shown in Figure 6A, in the control group, the mycelium was smooth, intact, with clearly visible septa. The mucopolysaccharide layer on the cell wall surface was evenly distributed (white arrow). The mycelia treated with 5% CFS showed mild damage, such as slight localized shrinkage (red arrow), partial distortion, uneven surface thickness, and sparse mucus distribution. The mycelium diaphragm also had uneven thickness (white arrow) (Figure 6B). The microstructure of the mycelium was analyzed using transmission electron microscopy. The results revealed no significant shrinkage of the cell body and preserved the normal morphology of cellular organelles in the control (Figure 6C). The overall damage to the bacteria treated with 5% CFS was relatively pronounced, characterized by sparse cytoplasm, significantly reduced electron density, shrunken cell bodies, and most organelles displaying evident swelling and vacuolation. The thickness of the cell wall (CW) was irregular, with sparse and unevenly distributed mucilaginous polysaccharides on the surface. The plasma membrane (PM) showed extensive damage and signs of disintegration. The nuclear membrane (N) was absent, and the chromatin appeared to have dissolved. Mitochondria (M) exhibited marked swelling, with a sparse and partially dissolved matrix, reduced or absent cristae, and vacuolar changes. The rough endoplasmic reticulum (RER) displayed a relatively reduced profile along with noticeable dilation. Autophagolysosomes (ASS) were observed as isolated structures (Figure 6D). In summary, treatment with 5% CFS induced notable structural damage to the mycelium, as evidenced by cytoplasmic rarefaction, a marked decrease in electron density, cellular atrophy, and pronounced swelling and vacuolation of most organelles.

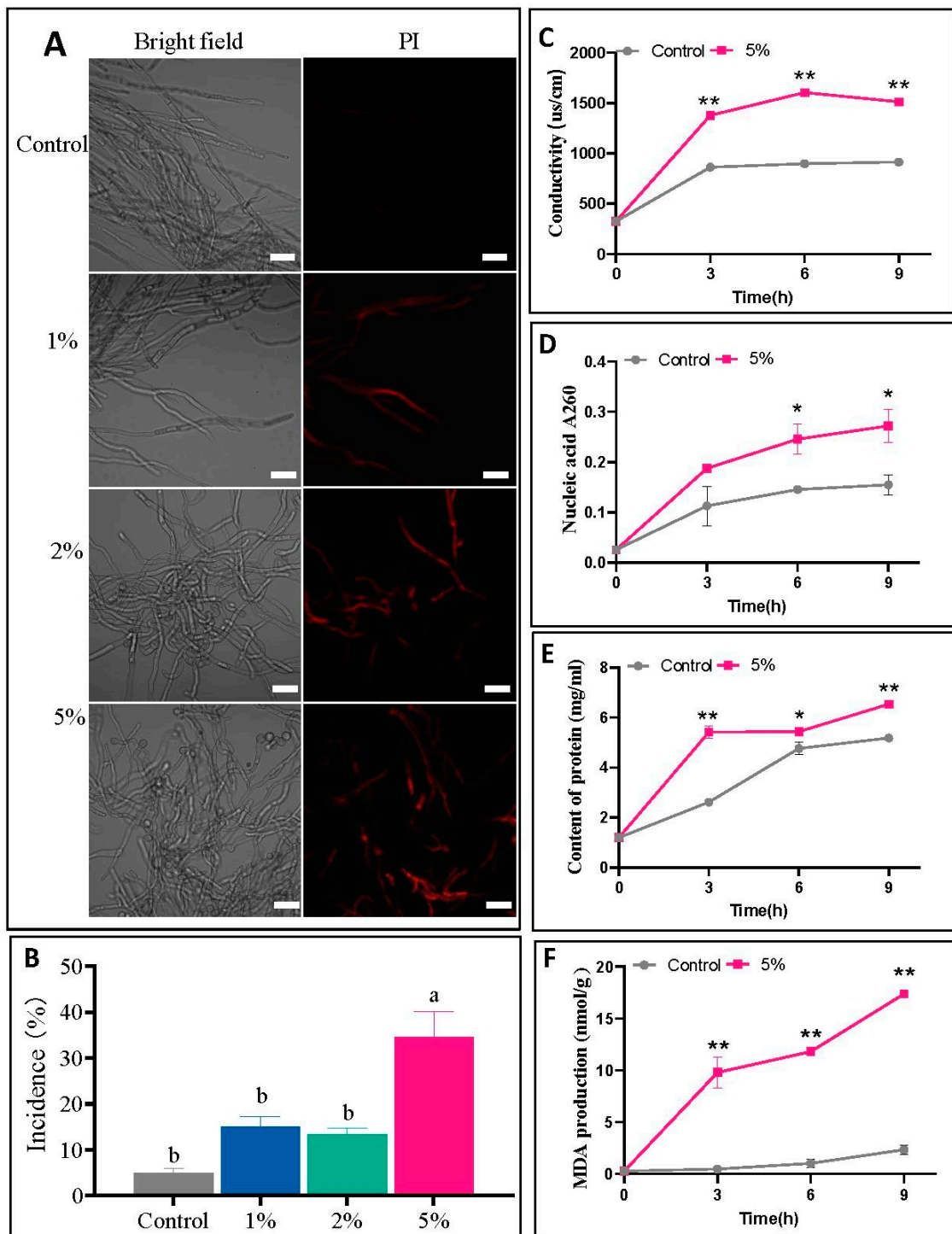


Figure 5. CFS on PI staining (A), the relative intensity of fluorescence (B), and conductivity (C), nucleic acid (D), protein release (E) and MDA accumulation (F) of *P. expansum*. Bar = 20 μ m. Different letters indicated significant differences between groups ($p < 0.05$). * means significant difference between groups ($p < 0.05$), ** means significant difference between groups ($p < 0.01$).

3.7. Alterations in the Expression Levels of Genes Associated with Autophagy Following CFS Treatment

To further investigate the impact of CFS on *P. expansum*, qRT-PCR was employed to analyze the gene expression levels related to autophagy-related genes after treatment with 5% CFS for 24 h and 48 h. Subtilisin-related peptidase S8 (*PePRT*) is a proteolytic enzyme highly expressed in plants and is closely related to the autophagy process and

pathogenicity of *P. expansum* [31]. Compared with the control group, the expression levels of *PePRT* showed no significant change at 24 h post-CFS treatment. After 48 h, *PePRT* was significantly upregulated by 16.75-fold (Figure 7A). Metacaspase is a tightly regulated essential factor that is not only crucial for the removal of damaged or stressed cells but also plays a role in maintaining cellular homeostasis and contributing to the broader ecological balance [34,35]. After 48 h of treatment with CFS, *PeMetacaspase1* was upregulated by 2.06-fold (Figure 7B), and *PeMetacaspase2* was upregulated by 4.96-fold (Figure 7C).

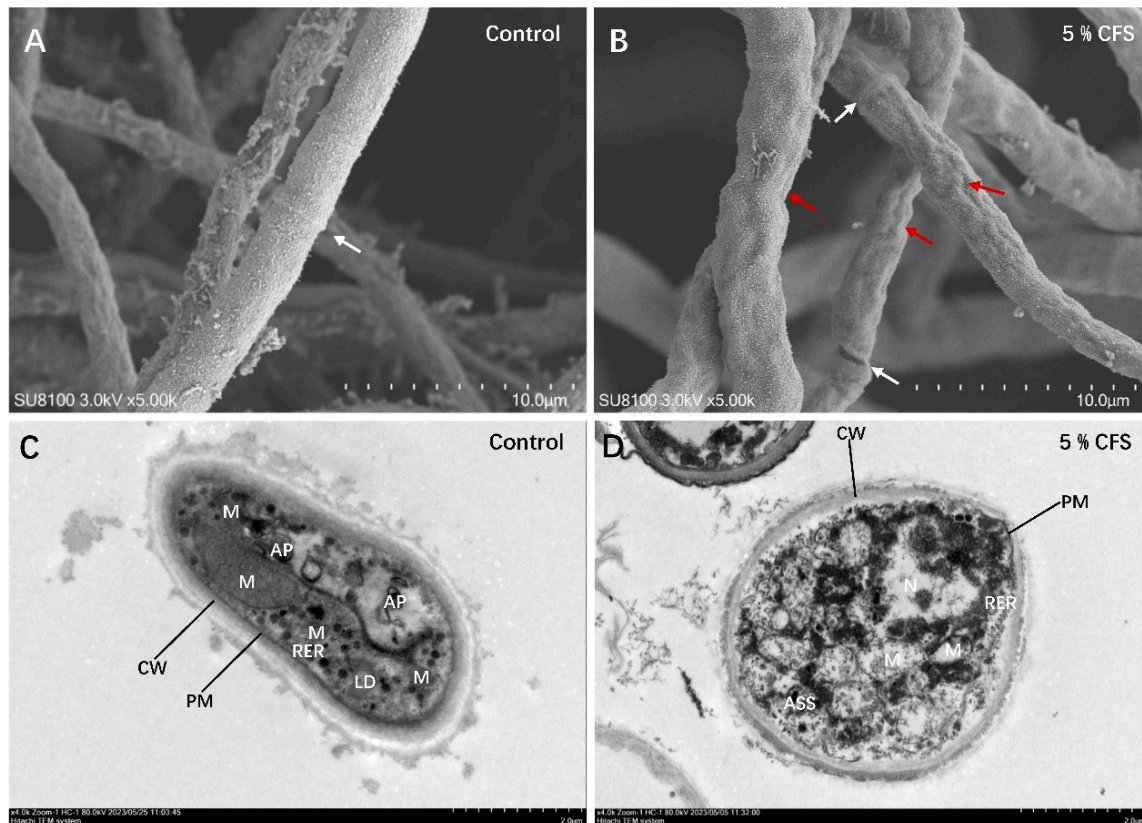


Figure 6. Effect of CFS on the morphology (A,B) and microstructure (C,D) of mycelium. The white arrow points to the septum of the mycelium, while the red arrow indicates the shriveled part of the mycelium. CW: cell wall; PM: cell membrane; LD: lipid droplets; M: mitochondria; RER: endoplasmic reticulum; AP: autophagosome; N: nucleus; ASS: Autophagolysosomes.

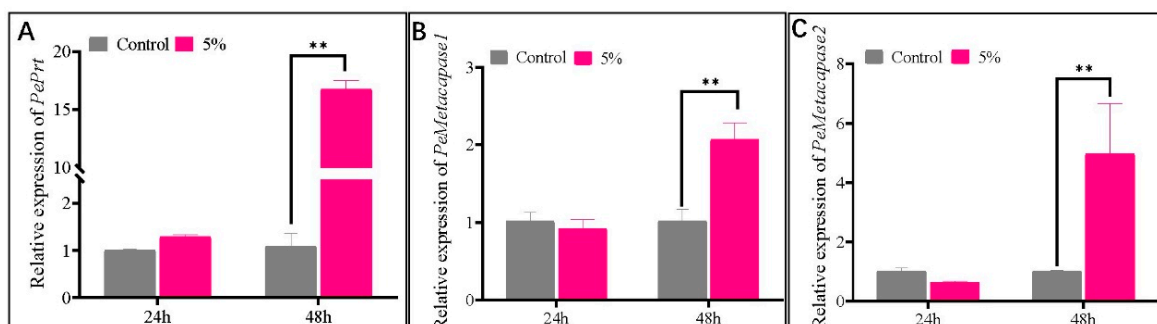


Figure 7. Effect of 5% CFS on relative gene expression of *PePRT* (A), *PeMetacaspase1* (B), and *PeMetacaspase2* (C). ** indicates significant difference between groups ($p < 0.01$). Vertical bars represent the standard deviations of the mean values.

4. Discussion

P. expansum is a fungal pathogen responsible for causing blue mold rot in fruits and is capable of producing patulin, a mycotoxin that raises significant food safety concerns. Utilizing environmentally friendly and safe biological control methods to prevent and control *P. expansum* represents a highly effective and sustainable strategy. *B. subtilis* is widely utilized as a biocontrol agent owing to its capacity to effectively inhibit the growth of various pathogenic microorganisms. The CFS exhibits potent inhibitory activity against plant pathogenic fungi, promotes plant growth, and contributes to environmental remediation, demonstrating various potential applications in agriculture [25]. In this study, the CFS of *B. subtilis* significantly suppressed spore germination and germ tube elongation (Figure 1), while significantly inhibiting the mycelial growth of *P. expansum* in a dose-dependent manner under in vitro conditions (Figure 2). This evidence was similar to that of *B. subtilis*, which can inhibit the *Botrytis cinerea* [36], *Fusarium graminearum* [37], *Verticillium dahlia* [38], and *Botryosphaeria dothidea* [24]. In accordance with previous findings observed in kiwifruit [24] and blueberry [39], CFS significantly reduced the incidence and the lesion expansion on grapes and citrus fruits (Figure 3), demonstrating its potential as a biological control agent for managing blue mold in grapes and citrus fruits.

When exposed to biotic stresses, organisms frequently initiate a range of defensive responses; the production of ROS represents a prevalent and essential mechanism [40]. This study demonstrates that treatment with 5% CFS induced significantly increased ROS accumulation (Figure 4A,B) and upregulation of related synthase genes *PeNoxA*, *PeNoxR*, and *PeRacA* (Figure 4C–E). This aligns with the fact that the antifungal drug from *Bacillus* spp. induced a similar ROS burst and Nox gene upregulation in *Fusarium* sp. [41], *Botrytis cinerea* [23] and *Botryosphaeria dothidea* [24]. In our system, the early and sustained induction of both ROS and Nox genes supports an active role in executing CFS toxicity. However, we cannot exclude the possibility that CFS simultaneously inflicts other primary injuries, with ROS production being one major, albeit not exclusive, component of the ensuing stress response network [42]. Concomitant with oxidative stress, severe membrane damage was observed. CFS destroyed the membrane integrity (Figure 5A,B) resulting in the imbalance of ions and the leakage of intracellular substances (Figure 5C,D). The accumulation of MDA (Figure 5F) further supports the occurrence of lipid peroxidation. This is similar to effects reported for other stressors [23,24]. These findings strongly implicate the plasma membrane as a critical target of CFS. Microscopic observations revealed that the plasma membrane exhibited severe disruption and disintegration (Figure 6D), providing more direct and compelling evidence that CFS compromises the structural integrity of cell membranes. But the observed membrane damage could also be a consequence of more general cellular dysfunction initiated by the treatment. Therefore, the issue of whether the cell membrane is the target of CFS still requires a specific and more in-depth demonstration.

Microscopic observations provide more direct and compelling morphological evidence to support the earlier assertion that CFS compromises the structural integrity of cell membranes. CFS induced mycelial damage, shrinkage, and deformation, resulting in an irregular surface morphology (Figure 6B). In mammalian cells, such cell shrinkage is an early marker of apoptosis [43]; however, in fungi, this feature is not pathognomonic and can occur in various forms of regulated cell death (RCD) [44]. TEM analysis showed severe plasma membrane disruption (Figure 6D), organelle swelling, vacuolization, and decreased cytoplasmic density, culminating in organelle rupture. The presence of autophagosome-like structures (Figure 6D), coupled with the upregulation of autophagy-related gene *PePRT*, *PeMetacaspase1*, and *PeMetacaspase2* (Figure 7), suggests the concurrent activation of autophagy and apoptosis-like pathways. These morphological and molecular changes are consistent with a complex, regulated cell death process being initiated.

The observed potent antifungal activity of CFS prompts a critical question: what are the key effector molecules responsible? Based on the well-documented biocontrol arsenal of *Bacillus subtilis*, the CFS likely contains a mixture of secondary metabolites, such as surfactin, iturin, and fengycin, as well as hydrolytic enzymes, with chitinases and glucanases being the primary candidates [45]. Our mechanistic findings align closely with the known modes of action of these compounds. For example, lipopeptides directly disrupt fungal membranes, forming ion-conducting pores that cause rapid depolarization, leakage of cellular contents, and metabolic collapse [46]. This directly parallels our observations of immediate membrane integrity loss and ion leakage. Therefore, our study provides a strong phenotypic and mechanistic foundation for subsequent biochemical characterization. Future work should first focus on the fractionation of the CFS coupled with bioassay-guided purification to identify the specific compound responsible for the ROS induction and membrane damage phenotypes.

Based on these integrated results, we hypothesize that CFS imposes an initial stress, potentially at the membrane level, triggering a rapid defense response. This includes the activation of the *PeRacA-PeNoxR-PeNoxA* pathway, leading to a significant ROS burst. However, as stress continues, the accumulated ROS then initiate a self-amplifying cycle: they gradually disrupt intracellular membrane integrity, directly cause oxidative damage to lipids (MDA accumulation) and impairment of critical organelles such as mitochondria structures essential for sustained energy production. Concurrently, most organelles exhibit pronounced swelling and vacuolization, the cytoplasm becomes less dense, and electron density markedly decreases, culminating in organelle rupture and disruption of the regulated apoptotic cascade. Moreover, numerous organelles involved in biosynthetic processes undergo vacuolization and functional decline, resulting in widespread protein degradation, leakage of intracellular contents, and loss of membrane integrity. This widespread damage, in turn, may further stimulate ROS production, creating a vicious feedback loop akin to that described in other oxidative stress models [47]. Within this deteriorating cellular environment, metacaspase activation and autophagic activity are likely engaged as part of a coordinated, yet ultimately unsuccessful, cellular response. We speculate that the initial attempt to mount a protective apoptotic-like response is overwhelmed by the scale of oxidative and structural damage, steering the cell toward an irreversible, disintegrative form of death. This model remains a hypothesis, and future studies employing temporal-resolution analyses and genetic interventions are required to delineate the precise sequence of events and causal relationships between ROS accumulation, membrane damage, and the activation of specific cell death pathways.

5. Conclusions

In summary, the CFS of *B. subtilis* exhibited significant antifungal activity against *P. expansum*, effectively suppressing spore germination, germ tube elongation, and mycelial growth. Furthermore, in vivo experiments confirmed that CFS treatment significantly reduced the incidence and lesion diameter caused by *P. expansum* in postharvest grape and citrus fruits. Further evidence demonstrated that CFS triggers a reactive oxygen species burst, leading to lipid peroxidation and severe disruption of cell membrane integrity, consequently resulting in the leakage of intracellular components. Additionally, the apoptotic-like response induced by treatment with 5% CFS is overwhelmed by the scale of oxidative and structural damage, steering the cell toward an irreversible, disintegrative form of death. Collectively, these findings provide a theoretical basis for the mechanism of action of *B. subtilis* and highlight the potential of its CFS as an effective, eco-friendly bio-logical control agent for managing postharvest diseases in fruits.

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