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First Isolation and Identification of *Neopestalotiopsis clavispora* Causing Postharvest Rot of *Rosa sterilis* and Its Control with Methyl Jasmonate and Calcium Chloride

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Abstract: Postharvest rot is a major issue in fruit. However, the cause of postharvest rot on *R. sterilis* fruit has not been clarified, and there are few studies on the disease control. In this study, the fungus causing postharvest rot is isolated from the symptomatic *R. sterilis* fruit, and identified by morphological characteristic, pathogenicity test and molecular identification. Moreover, the effects of methyl jasmonate (MeJA) or calcium chloride ($CaCl_2$) alone and their combination on disease resistance to fruit rot were assessed by the determination of defense-related enzyme activity and other indicators. *N. clavispora* was identified as the main fungus causing the postharvest rot of *R. sterilis* fruit. The infected fruits were treated with MeJA and $CaCl_2$, and these partially controlled the disease, were additive in effectiveness when used together, increased retention of vitamin C content and fruit firmness, and both enhanced and improved the retention of PAL, POD and PPO activities. The treatment of 500 μL/L MeJA and 3% $CaCl_2$ resulted in the high inhibition of the disease. To our knowledge, this is the first report of *N. clavispora* causing *R. sterilis* fruit rot, and the combined treatment is a promising method for controlling postharvest rot on *R. sterilis* fruit.

Keywords: *N. clavispora*; *R. sterilis*; postharvest rot; disease control; induced resistance

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1. Introduction

Rosa sterilis S. D. Shi (*R. sterilis*) is a deciduous shrub with perennial rootstock, which is a wild fruit tree and grows at the altitude of 1000 m above the sea level on valleys, slopes, along roads and with bushes [1]. It has been reported that *R. sterilis* has a very close genetic relationship to *Rosa roxburghii* (*R. roxburghii*) [2,3]. Similar to *R. roxburghii*, *R. sterilis* has also been widely cultivated in Guizhou, China [4]. *R. sterilis* fruit is a nutritional product and a rich source of vitamins, amino acids, minerals, trace elements, functional components, volatile aroma compounds, among others [5–7]. However, postharvest rot on *R. sterilis* fruit predominated in most orchards, and it is considered as the most serious cause of a loss of production and commercial value at postharvest stage. Therefore, it is necessary to clarify the cause of *R. sterilis* fruit rot and develop a reliable method to control and reduce the occurrence of the disease during fruit storage.

Disease investigation, pathogen identification and disease control are commonly used methods in the protection of fruit products worldwide [8–11]. Numerous studies have indicated that the main pathogens causing postharvest fruit rot include *Botrytis cinerea* [8,12], *Alternaria alternata* [13], *Aspergillus tubingensis* [14], *Alternaria tenuissima*, *Fusarium moniliforme*, among others. Many pathogens, mostly fungi, cause the postharvest decay of perishable fruits [15], for example, the families of *Botryosphaeriaceae* (*Diplodia mutila* and *Diplodia seriata* (*D. seriata*)), *Bulgariaceae* (*Phacidiopycnis washingtonensis*) and *Phacidium*

lacerum were considered as the fungal pathogens causing apple rot [16]. *Colletotrichum fructicola* was identified as a pathogen of pear rot [17]. Additionally, *Pestalotiopsis bicilita*, *D. seriata*, *Diaporthe eres* and *Colletotrichum viniferum* were reported as causing fruit rot in grapes [10,18], while *Fusarium solani* and *Phomopsis amygdali* were considered as causing fruit rot in peaches [19,20]. *Botryosphaeria dothidea*, *Lasiodiplodia theobromae*, *Neofusicoccum parvum* and *Fusarium proliferatum* were reported as causing kiwifruit postharvest rot [21,22]. In addition, as an important plant pathogenic species, *Neopestalotiopsis clavispora* (*N. clavispora*) was reported as a causative pathogen on postharvest fruit rot and trunk diseases; it caused canker and dieback on blueberries in Chile [23] and root and crown rot on strawberries in Spain [24]. In the field, initial symptoms of fruit rot disease are mostly observed at the splits and the wounds of the fruits. However, there are no reports on *R. sterilis* fruit rot caused by *N. clavispora*, and the pathogens that cause the postharvest decay of *R. sterilis* in Guizhou, China are not known, and determining them was one objective of our research.

In recent years, many chemical and biological agents have been widely used for controlling fruit diseases. As a naturally occurring plant growth regulator, methyl jasmonate (MeJA) was used as an activator for defense responses against pathogenic infections [11,25–27]. The possible reason is that the exogenous application of MeJA can effectively enhance the activities of antioxidant protective enzymes and defense-related enzymes, increase phenolic content, alter volatile patterns in the fruit and, finally, enhance the innate disease resistance of postharvest fruit against pathogens, extend fruit shelf-life, and improve fruit quality [28]. Additionally, due to its ability to inhibit the spread of postharvest diseases, inorganic salt, such as calcium chloride ($CaCl_2$), has been widely used in the field of disease control. Postharvest fruit handling with $CaCl_2$ have been proposed as a safe and effective way to control fruit rot and prolong storage life [29,30]. However, to the best of our knowledge, the pathogenic role of fungi causing postharvest rot on *R. sterilis* fruit is largely unknown. Moreover, there is still little information about the synergistic inhibitory effect of MeJA and $CaCl_2$ on the control of the disease.

The aims of this study are to (i) isolate and identify the main fungus causing postharvest decay on *R. sterilis* fruit via morphological observation, molecular identification and pathogenicity test; (ii) evaluate the inhibitory effects of MeJA and $CaCl_2$, used separately or in combination on *R. sterilis* fruit against with postharvest rot. The results of the present work will provide a reference for future research on *R. sterilis* fruit postharvest rot, such as disease prediction, effective disease control and resistance breeding.

2. Materials and Methods

2.1. Sample Collection

In early October 2021, the ripe *R. sterilis* fruits (as shown in Figure 1a) used for the experiment were collected from 3 commercial orchards (no chemical pesticides were used in these orchards during the first 2 months of fruit harvest) in the Puding county, Guizhou province, China, and packed into 6 plastic bags, each containing about 150 pieces. In addition, the symptomatic fruits (as shown in Figure 1b) were obtained from the storehouse in these orchards, which were placed into 3 plastic bags. All the plastic bags were packed into a portable refrigerator (4 °C) and transported to the microbiology lab of College of Biological Sciences and Agriculture of Qiannan Normal University for Nationalities on the same day. The geographical coordinates of Puding county are 105.74285 E longitude and 26.30141 N latitude, and the sea level is about 1205 m. The average temperature and precipitation were about 15.0 °C and 1300 mm in 2021.

2.2. Pathogen Isolation and Morphological Characterization

The pathogens were isolated from the symptomatic *R. sterilis* fruits. The tissue at the junction of disease and health was removed from the rotten fruit and spread on potato dextrose agar (PDA, 200 g potato, 20 g dextrose, 20 g agar, 1 L H_2O, pH 7.0), and then incubated at 28 °C. To obtain a pure culture, after 3 days of initial incubation, the mycelia at the growing edge of each colony was transferred to a new PDA, and then incubated at 28 °C

for 10 days [31]. A total of 30 symptomatic fruits (divide into 3 groups with 10 samples in each group) were selected for the experiment and compared.

The appearance, texture, and color of colony and conidia of the pathogen were observed by naked eye and an optical microscope. Then, preliminary identification was performed based on the morphological features of the colony and conidia.

Figure 1. Postharvest rot caused by *N. clavispora* on *R. sterilis* fruit. (**a**) Fresh fruit (**1**: outside; **2**: inside). (**b**) Symptoms of fruit rot by natural inoculation (**1**: outside; **2**: inside). (**c**) Symptoms on wound inoculated fruit after 14 days (**1**: outside; **2**: inside). (**d**) Control.

2.3. Preparation of Spore Suspension

The spore suspension was prepared by flooding a 10 day old PDA plates with sterile water containing 0.05% (*v*/*v*) Tween 80. The suspension was filtered through sterile cheesecloth to remove the adhering mycelia. The spore concentration of the fungus was measured by hemocytometer and adjusted to 1×10^6 spores/mL with sterile water.

2.4. Pathogenicity Test

A pathogenicity test was conducted on the sterilized surface of matured *R. sterilis* fruits. In detail, a total of 40 fresh fruits were sterilized with 75% ethanol for 2 min, rinsed in sterile water and air dried, and then they were wounded with a 2 mm deep hole with a sterile needle. After that, 20 µL of spore suspension (1×10^6 spores/mL) was injected into the hole of 30 wounded fruits from 3 sources (10 fruits from each orchard), and the remaining 10 fruits (from 3 different orchards) were treated with sterile water as a control. Finally, all the treated fruits were incubated in a constant temperature incubator with a plastic bag at 28 °C, 80% humidity. The symptoms on the treated samples were observed and recorded after 1 day of inoculation. Rotten tissue at the lesion margins of the infected fruit was transferred to PDA plates, and the isolated fungus was re-identified by morphological and microscopic analysis.

2.5. DNA Extraction, PCR Amplification and ITS Sequence Analysis

For DNA extraction, the isolated fungal strain was grown on a PDA plate for 7 days at 28 °C. An approximately 20 mg of the mycelia of strain were harvested and ground in a frozen and sterile mortar, and a fine powder was obtained using liquid nitrogen. Genomic DNA was extracted from the fine powder using a plant DNA extraction kit (Tsingke, Biotechnology Co., Ltd., Beijing, China). The internal transcribed spacer (ITS) region of the nuclear rDNA was amplified by the universal primers ITS1 and ITS4. PCR reaction of the ITS gene region was conducted using a thermal cycler, where each reaction was a 50 µL reaction solution containing 45 µL of a PCR master mix (TSE101, Tsingke, Biotechnology Co., Ltd., Beijing, China), 2 µL of each primer set, and 1 µL of genomic DNA. Thermal

cycling parameters were 98 °C for 120 s, 35 cycles of 30 s at 98 °C, 30 s at 56 °C, 60 s at 72 °C, and a final extension for 300 s at 72 °C. The PCR products were verified by 1% AGE (agarose gel electrophoresis). Both strands were custom sequenced (Tsingke, Biotechnology Co., Ltd., Kunming, China) and clustal W was applied to generate consensus sequences. All sequence data were submitted to Genbank. Homologous sequences were analyzed against GenBank sequences by using the nucleotide BLAST search tool on the website of http://www.ncbi.nlm.nih.gov/blast/ (4 January 2022), and phylogenetic analysis was performed by using MEGA 6 software with the neighbor-joining method, and a bootstrap test of 1000 replications.

2.6. Fruits and Treatment

Fruits with uniform size (the diameter was about 20 mm), color and shape, and without physical damaged were selected. All fruits were first sanitized in a 2% (*m*/*v*) sodium hypochlorite solution for 5 min, then rinsed with sterile water, and finally dried prior to wounding.

The fruits were wounded with a sterile needle to make a uniform hole with 3 × 2 mm in diameter and depth at the equatorial zone of fruit. The wounded fruits were randomly divided into four groups, three treated groups and one control group. For treated groups, a portion of 20 µL of 500 µL/L MeJA, 3% $CaCl_2$ and 500 µL/L MeJA-3% $CaCl_2$ solution was pipetted into the wound hole, respectively. The control group was pipetted with distilled water, instead of the chemical solutions. After drying for 30 min, 20 µL of *N. clavispora* spore suspension was pipetted into the hole. All the treated fruits were stored at 28 °C and 80% relative humidity for 7 days.

For physiological and biochemical analysis, approximately 1 cm of healthy fruit tissue surrounding the wound holes was obtained and cut into small pieces at days 0, 1, 2, 3, 5, and 7. The excised samples were frozen in liquid nitrogen, ground into powder, and stored at refrigerator (−80 °C) until determination.

2.7. Inhibitory Effect of MeJA or CaCl2 Alone and Their Combination on Artificially Inoculated Fruit

2.7.1. Determination of Disease Incidence

The number of infected fruit (showing visible mycelium in the inoculated hole) was recorded and the disease incidence was expressed as the percentage of infected fruit. The measurement was performed on the 1st, 3rd, 5th and 7th day after inoculation. Each treatment was replicated 3 times with a total of 20 fruits per replicate.

2.7.2. Assay of Defense-Related Enzyme Activity

Phenylalanine ammonia-lyase (PAL) activity was assayed according to the method of a previous study with slight modifications [12]. In detail, the fruit flesh was weighed (1.0 g) and homogenized in 9.0 mL of 50 mM sodium borate buffer extract (pH 8.8, pre-cooled). The homogenate was centrifuged at 10,000 rpm for 10 min at 5 °C, and the supernatant was used as enzyme source. The enzyme reaction was carried out according to the instruction of test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and then the absorbance at 290 nm was measured by a spectrophotometry (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China). One unit (U) of PAL activity was defined as the quantity of PAL causing increase of 0.1 OD290 per minute. The activity was expressed as U/g (FW).

Peroxidase (POD) activity was measured as described in Xie et al. with some modifications [32]. The fruit flesh (1.0 g) was weighed and crushed with a pestle in an ice-cold mortar with 9.0 mL of 0.1 M phosphate buffer. The homogenate was centrifuged at 3500 rpm for 10 min at 4 °C, and the supernatant was used as enzyme source. The reaction mixture for the POD assay was prepared according to the instruction of test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and the absorbance at 420 nm was recorded by a spectrophotometry (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China).

One unit (U) of enzyme activity was defined as the quantity of POD causing an increase of 0.001 OD420 per minute. The activity of POD was expressed as U/g (FW).

Polyphenoloxidase (PPO) activity was determined following the method of Xie et al. [32]. The fruit flesh (1.0 g) was weighed and ground with a pestle in an ice-cold mortar with 5.0 mL of sodium acetate buffer (100 mM, pH 5.5). The homogenate was centrifuged at 8000 rpm for 10 min at 4 °C, and the supernatant was used as the enzyme source. The reaction mixture for the PPO assay consisted of 4.0 mL of distilled water, 1.0 mL of 0.1 M catechol and 1.0 mL of enzyme source. The absorbance at 420 nm was measured by spectrophotometry (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) for the PPO activity assay. One unit (U) was defined as the quantity of POD, causing an increase of 0.01 OD420 per minute, and the activity of PPO was expressed as U/g (FW).

2.7.3. Determination of Hardness and Vitamin C Content

Hardness was measured by a hardness detector (GY-4, Ningbo kecheng insturment co. LTD, Ningbo, China) with a 3.5 mm diameter head.

The 2,6-dichloroindophenol titrimetric method was used to measure the vitamin C content of *R. sterilis* fruit, and the result was expressed as milligrams of vitamin C per 100 g of fresh sample.

2.8. Statistical Analysis

Statistical analysis of experimental data was conducted using SPSS 22.0 software. Analysis of variance was applied followed by Duncan's multiple range tests to separate means. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Typical Symptoms of Rot Fruit and Morphological Identification of Pathogen

Postharvest rot symptoms were observed on both the infected fruits collected from orchards and the stored fruits, and the typical symptoms were as follows: the surface of fruit was covered with an amount of gray-white mycelium, which had small black granular conidia discs, and soft occurred on the outer surface of fruit, then extended to the whole fruit (Figure 1(b1)). Water-soaked flesh tissue could be seen in the sunken area when the collapsed portion was cut (Figure 1(b2)). From the symptomatic fruit, fungal strains with similar features were consistently isolated and cultured on PDA plates for 10 days. In the early stage of growth (within 2 days), the mycelium grew slowly; the colony was white, cottony, and circular growth appearance (Figure 2(a1)). After 3 days of inoculation, the mycelium grew luxuriant; the wheel stripe was observed, and entire colonies formed concentric rings (Figure 2(a2)). On the 7th day of incubation, the Petri dish (d = 5 cm) was filled with mycelium due to the rapid growth (Figure 2(a3)). In the later stage of growth (on the 10th day), small black spots and molds were observed on the surface of the mycelium layer, which developed acervuli that contained scattered conidia (Figure 2(b1)). In addition, there were some granular acervuli buried under the mycelium layer. Conidia were slightly curved, fusiform to clavate, five-celled with four constricted septa; the two dimensions were $17.21 \pm 1.39 \times 6.11 \pm 0.64$ μm ($n = 20$). Three median cells were brown or darker, and the apical and basal cells were hyaline and white, one of which had 2–4 appendages (Figure 2(b2)). It was found that all cultured strains had similar morphological characteristics, thus they were considered to be the same pathogen. The morphological characteristics of the isolated fungus were similar to those described in the literature for the species of *N. clavispora* or *P. clavispora* [24,33]. The isolate could be characterized as *N. clavispora* by colony and conidia morphology.

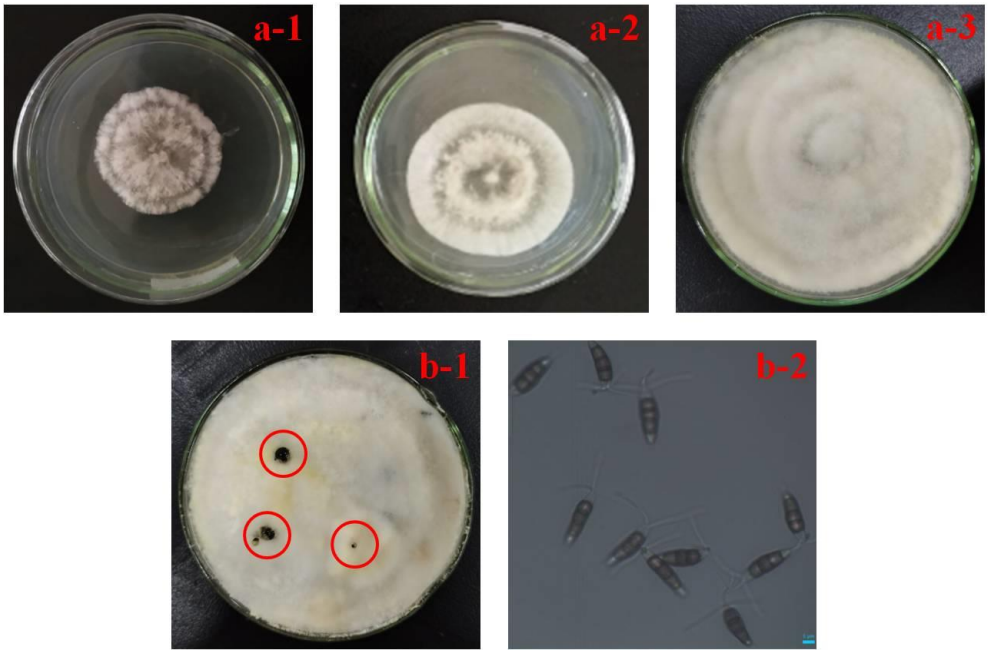

Figure 2. Morphological characteristics of *N. clavispora*. (**a**) Colony on PDA (**1**/**2**/**3**-two/three/seven-day-old); (**b1**) acervuli and (**b2**) conidia.

3.2. Pathogenicity

The inoculated fruits were kept at 28 °C in plastic bags. At 7 days after inoculation, the white mycelium developed within the wounds, and discoloration extended outward from the inoculated sites. Slight rot began to develop on the inoculated fruits. At 14 days after inoculation, the area of decay covered more than half of the fruit surface and abundant white mycelia and black spores were observed on the rotten area (Figure 1(c1)). The symptoms on the inoculated fruits (Figure 1(c2)) phenotypically matched those observed on the naturally occurring fruits (Figure 1(b2)) from the appearance of the rotten tissue. The control fruits (inoculated with sterile water) showed discoloration at the edges of the hole, but no disease symptoms developed (Figure 1d). The test results of spore suspensions from different orchards were consistent. The pathogen was re-isolated from the inoculated fruits, and re-identified by morphological features. The resulting colony phenotypically matched the colony obtained from the originally diseased fruit, therefore completing Koch's postulates. Based on the observed pathological symptom, morphology features, and pathogenicity, the pathogen was further identified as *N. clavispora*.

3.3. rDNA-ITS Sequence Analysis of Pathogen

The isolated *N. clavispora* was further confirmed by molecular identification. The gene region of ITS was PCR amplified and sequenced. The rDNA-ITS sequence of the isolated strain was amplified with ITS-1 and ITS-4 as universal primers. The obtained PCR products were detected by 1% AGE. The results showed a single band, no more than specific amplification, and the size of ITS sequence was about 600 bp (as shown in Figure S1).

The sequencing result (Doc. S1) shows that about 600 nucleotide sequences of the rDNA-ITS sequence of the fungal strain were effectively amplified. The sequencing results were submitted to GenBank and subjected to BLAST analysis. The results show that the ITS sequence of the fungal strain was 99–100% homologous to the ITS sequence of *N. clavispora*. The ITS sequence with higher homology was selected and compared with the ITS sequence of the isolated strain to establish a phylogenetic tree, and the result is shown in Figure 3. According to the phylogenetic tree, the isolated strain and *N. clavispora* belong to the same branch of the phylogenetic tree; the relationship was closest, and the homology was over 99%.

The molecular analysis confirmed the morphological identification for the fungal pathogen. Combined with the results of morphological identification, the pathogen was identified as *N. clavispora*.

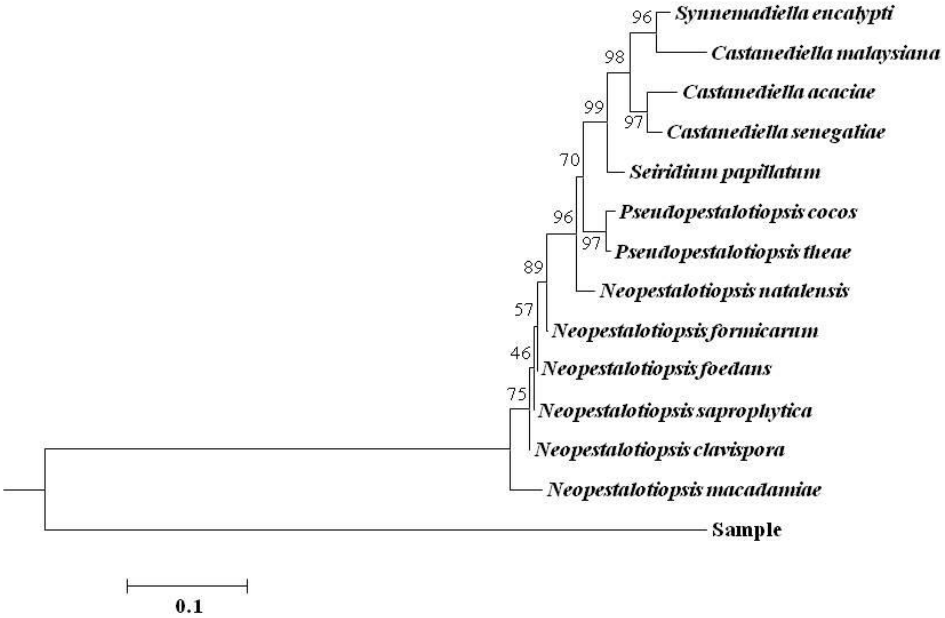

Figure 3. Phylogenetic tree constructed using the neighbor-joining method based on rDNA-ITS sequences.

3.4. Effects of Different Treatments in Controlling Decay on Artificially Inoculated R. sterilis Fruit

The effects of different treatments on the induction of *R. sterilis* fruit resistance based on the disease incidence are shown in Figure 4. Disease incidence at 7 days after inoculation for control, MeJA, $CaCl_2$ and MeJA + $CaCl_2$ treatments was 61.67, 33.33, 38.33 and 26.67%, respectively. The control had significantly more decay than the other treatments, which did not differ significantly from each other. The combined treatment further reduced fruit decay in comparison with the treatment of MeJA or $CaCl_2$ alone, but no significant difference of incidence was observed among different treatment groups. On the 5th day after inoculation, the disease incidence of the control group (31.67%) was significantly higher than the MeJA–$CaCl_2$ treated group (18.33%) and no significant difference of disease incidence was found among different treated groups or the control group, MeJA alone and $CaCl_2$ alone. The differences in disease incidence among different groups were very small during the first three days of inoculation, although the incidence in all treatment groups was significantly lower than that in the control group (3.33%).

3.5. Effect of Different Treatments on the Activity of Defense-Related Enzymes

The activities of all resistance-related enzymes are shown in Figure 5. As shown in Figure 5A, PAL activity exhibited a similar trend in both control and treatment groups; in detail, it can be seen that it first increased and then decreased, but the activity increased to its peak value at different incubation times. PAL activity in MeJA-treated fruits increased on the 1st day of inoculation, and decreased rapidly during the 1st to 3rd days, then became smooth on the 3rd to 5th days, and finally decreased again after 5 days. On the 3rd day of inoculation, the PAL activities in the three groups (control, $CaCl_2$, and MeJA–$CaCl_2$ treatment) peaked simultaneously; the activity in the MeJA–$CaCl_2$ treatment group (56.23 U/g FW) was significantly higher than in the control (47.78 U/g FW), and there was no significant difference between the $CaCl_2$ treatment and control groups, while PAL activity in the MeJA treatment group (40.20 U/g FW) was significantly lower than in the control. On the 5th day, PAL activity in MeJA–$CaCl_2$ treated fruits (50.90 U/g FW) was significantly higher than the control, MeJA and $CaCl_2$ treatment groups. At the end of observation period (on the 7th day of inoculation), PAL activities were still significantly

higher in the treatment groups than in the control (27.34 U/g FW), and the PAL activity in the MeJA–$CaCl_2$ treatment group (38.55 U/g FW) was significantly higher than those of the MeJA and $CaCl_2$ treatment groups.

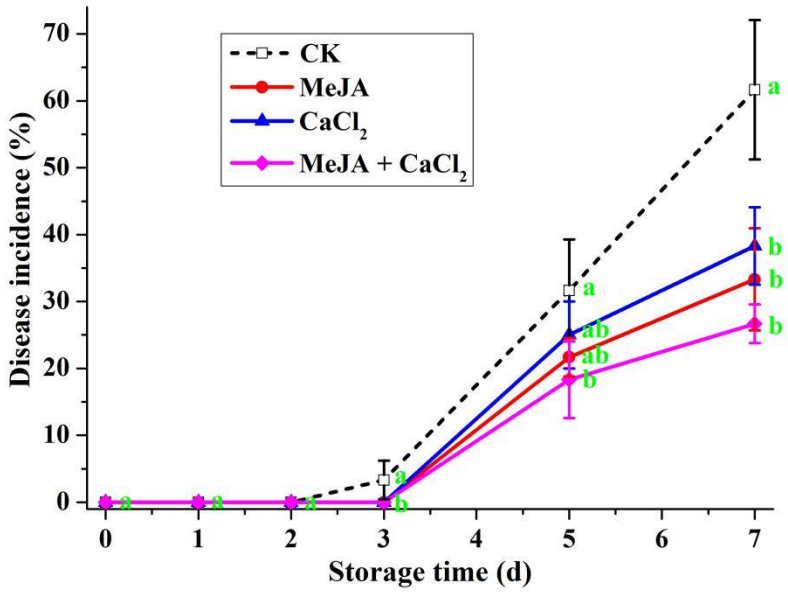

Figure 4. Effect of different treatments on the disease incidence of *R. sterilis* fruit inoculated with *N. clavispora* within 7 days of storage at 28 °C. Data are expressed as mean ± SD of triplicate assays.

As can be seen from Figure 5B, POD activities followed different trends in the control and treatment groups during the monitored time. POD activity in the MeJA–$CaCl_2$ treated fruits showed a slight upward trend on the 1st day and dramatically increased on the 2nd day of inoculation, and then decreased gradually within the remainder of storage. POD activity in the infected fruits treated with $CaCl_2$ slightly increased within the first 2 days, then increased dramatically and reached the highest value on the 3rd day, and finally decreased until the end of the monitored period, while the enzyme activities followed the same trend in both control and MeJA treatment groups, i.e., first increasing and then decreasing. The POD activities of different groups showed significant difference on the 2nd day, and the value was 8.59, 12.22, 5.16, and 15.29 U/g FW for the control, MeJA-treated, $CaCl_2$-treated, and combination-treated group, respectively. There was no difference in the POD activities among different groups on the 3rd day of inoculation, while the differences among these four groups were significant on the 5th day. POD activity in all groups exhibited a downtrend at the later stage of the measurement period (after 3 days of inoculation), and the activities of different groups decreased to almost the same level (corresponding to no significant difference) at the end of the observation period (on the 7th day of inoculation).

The PPO activities of different fruits are shown in Figure 5C. The PPO activities followed the same trend in the four groups (increasing and then decreasing), but they did not take the same amount of time to reach their highest value. After inoculation, the PPO activity in the control fruits started to rise and peaked on the 3rd day (9.54 U/g FW);–similarly, the PPO activity in fruits treated with MeJA-$CaCl_2$ was also at a maximum during this time (17.50 U/g FW), while PPO activities in MeJA-treated and $CaCl_2$-treated fruits reached their maximum value on the 5th day. The activities in the treatment groups were always higher than that in the control group. On the 3rd day of inoculation, the PPO activity of MeJA–$CaCl_2$ treatment group was significantly higher than those of the other three groups. On the and 5th day, the PPO activities for all treatments were significantly higher than that of the control (3.72 U/g FW). On the 7th day of inoculation (the end time of the experiment), the activity in control fruits (1.59 U/g FW) was significantly lower than those in the MeJA and MeJA–$CaCl_2$ treatment fruits, while no significant difference

between the control and CaCl2 treatment groups was observed. The PPO activity in the MeJA–$CaCl_2$ treatment fruits (7.53 U/g FW) was significantly higher than those in the control and $CaCl_2$ treatment fruits, while there was no significant difference between the fruits treated with MeJA alone and in combination with $CaCl_2$.

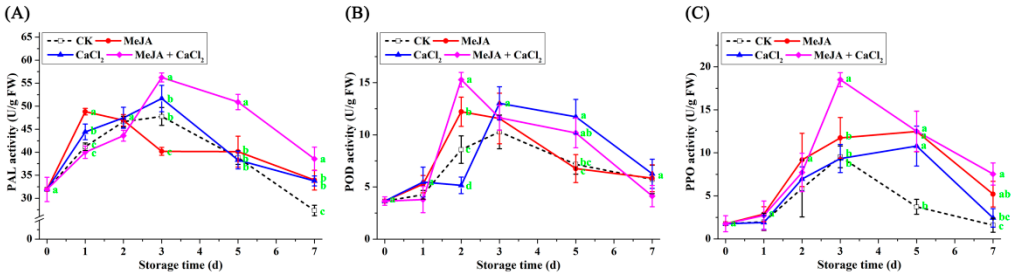

Figure 5. Activities of resistance-related enzymes in the control and treated *R. sterilis* fruits inoculated with *N. clavispora*. (**A**) PAL; (**B**) POD; and (**C**) PPO.

3.6. Effect of Different Treatments on Hardness and Vitamin C Content

As shown in Figure 6A, fruit hardness in both control and treatment groups decreased gradually with the prolongation of incubating time; the decrease in hardness may be due to the hydrolysis of pectin and carbohydrates and the occurrence of fruit rot. The degree of decrease in hardness was less in the MeJA–$CaCl_2$ and $CaCl_2$ treated fruits. On the 7th day of inoculation, the fruits treated with $CaCl_2$ alone or in combination with MeJA maintained a significantly high hardness (26.51 kg/cm^2 or 26.24 kg/cm^2) compared with the control (18.24 kg/cm^2). MeJA showed no significant effect on the hardness of *R. sterilis* fruits throughout the whole storage period.

The vitamin C contents of different fruits are given in Figure 6B. The vitamin C content during storage was retained significantly better after the MeJA, $CaCl_2$ or MeJA+$CaCl_2$ treatments than the control. On the 7th day of inoculation, vitamin C contents in *R. sterilis* fruits were well maintained by different treatments.

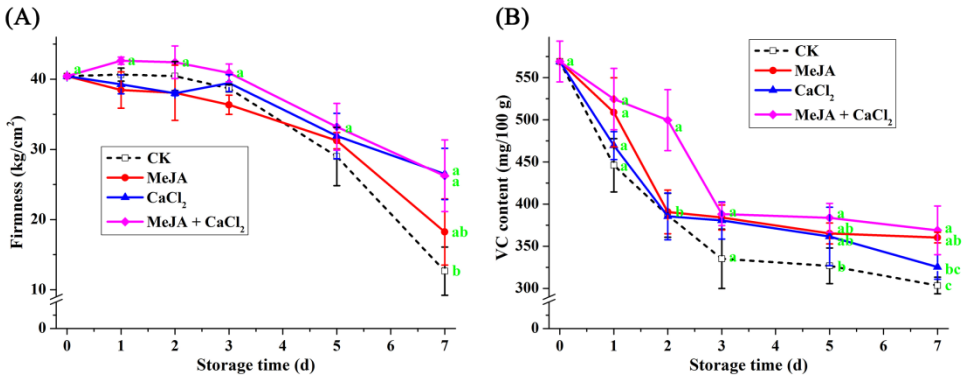

Figure 6. Hardness and vitamin C content of the control and treated *R. sterilis* fruits inoculated with *N. clavispora*. (**A**) Hardness; and (**B**) Vitamin C content.

4. Discussion

In this paper, a systematic study of postharvest rot occurring on *R. sterilis* fruit during the postharvest storage period was carried out. *N. clavispora* was firstly identified as the main pathogen causing postharvest rot on *R. sterilis* fruit based on the results of morphological identification, molecular analysis and pathogenicity test. *N. clavispora* is widely distributed as opportunistic plant pathogen [24,34], and our experiments showed that the wounded *R. sterilis* fruit can quickly become ruined by the action of the fungus.

It is well known that MeJA, as a naturally growth regulator, plays important roles in regulating a great diversity of biochemical and physiological processes [26]. Previous studies have showed that MeJA has been widely used to control the postharvest diseases

of various fruits, including Chinese bayberry [25,26], kiwifruit [11] and cherry tomato [27], possibly by inhibiting pathogen growth or/and inducing disease resistance. In this study, we found that the treatment with 500 μL/L MeJA in combination with 3% $CaCl_2$ resulted in the better control of *N. clavispora* on *R. sterilis* fruit than the use of MeJA or $CaCl_2$ alone due to the fact that it can significantly reduce disease incidence and increase the induction effect of *R. sterilis* fruit infected with *N. clavispora*. PAL acts as a marker of plant-induced disease resistance due to being the first key enzyme in the phenylpropanoid pathway, which is highly associated with the biosynthesis of lignins, phenolic compounds and phytoalexins to prevent pathogen invasion and slow disease development during pathogenic infection [12,35]. In this study, PAL activities were generally higher in treatment groups than in the control during the whole infection period (especially in the initial period), indicating that the treated fruits showed higher resistance. Many researches suggest that there is a direct relationship between the increase in POD activity and the natural resistance of fruit [36]. This is why a rapid increase in POD activity was observed after 2 days of inoculation. POD plays a key role in the accumulation of lignin since it can catalyze the biosynthesis of lignin [35,37]. The higher POD activities in fruits treated with MeJA alone and in combination with $CaCl_2$ than in the control may be related to MeJA-induced resistance. The activation of PPO is associated with triggering and accelerating the infection process in the treated fruits [35]. In general, the activities of PPO were higher in the inoculated fruits (especially in the MeJA–$CaCl_2$ and MeJA treatment groups) compared to the corresponding healthy fruits. These results suggest that the effect of MeJA in reducing the decay of *R. sterilis* fruit is mainly due to inducing disease resistance in the fruit. In addition, our data have shown that *R. sterilis* fruit treated with $CaCl_2$ alone or $CaCl_2$ with MeJA exhibited significantly higher level of hardness compared with those treated with MeJA alone and control, which may be attributed to the key role of calcium ions in reducing fruit softening by strengthening the cell walls [36]. These results indicate that MeJA in combination with $CaCl_2$ also had an additive effect in inhibiting the decrease in the hardness of the fruit. Vitamin C, an important organic acid in fruits and plants, has great potential in suppressing fungal disease [38,39]. The vitamin C contents of the treated fruits always remained at high levels during the infection period, indicating that the fruits in the treatment groups showed higher resistance to fruit rot disease. Therefore, the combination of MeJA and $CaCl_2$ has a potential application in postharvest treatment for reducing rot in *R. sterilis* fruit during postharvest storage and distribution.

5. Conclusions

To the best of our knowledge, *N. clavispora* was identified as the main pathogen causing postharvest rot on *R. sterilis* fruit in China for the first time. In addition, MeJA in combination with $CaCl_2$ treatment can reinforce the resistance of postharvest rot on *R. sterilis* fruit during *N. clavispora* infection. The possible mechanism is that the MeJA–$CaCl_2$ treatment induced disease resistance against *N. clavispora* in the infected fruits by priming of defense responses, such as increasing the activities of PAL, PPO and POD in the phenylpropanoid pathway and maintaining a high level of vitamin C content. Further research is needed to understand the pathogenicity and infection process of the *N. clavispora* related to fruit rot, which can be carried out as follows: first, to further confirm that the postharvest rot is also caused by other pathogens by the isolation and identification of strains from the symptomatic fruits collected from more different regions. Second, to elucidate the mode of action of different treatments on the disease of *R. sterilis* fruit. Third, to explore the possible mechanisms involved in postharvest disease control by the determination of other indicators, such as the activities of cinnamate-4-hydroxylase and cinnamyl-alcohol dehydrogenase, total phenol and flavonoid contents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8030190/s1, Figure S1: Gel electrophoresis of pathogen PCR-amplification results; Doc. S1: The full-length rDNA-ITS sequence of the strain to be tested as follows.

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