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Effects of *Fusarium proliferatum* on Aboveground Physiological Indicators of Superior Apple Rootstock Line 12-2 (*Malus spectabilis*) with Improved Apple-Replant-Disease Resistance

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Abstract: (1) Background: Cultivating resistant rootstocks is an effective way to mitigate apple replant disease (ARD), and we developed superior apple rootstock line 12-2 (self-named), which shows improved ARD resistance. (2) Methods: We used ARD-associated pathogen *Fusarium proliferatum* MR5 (MR5) to test the fungal infection in the 12-2 line. Seedlings of the 12-2, T337, and M26 rootstock lines were planted in a substrate with potato dextrose broth and MR5 spore solution, and aboveground physiological indicators were measured. (3) Results: MR5 had the greatest effect on the leaf growth of T337 and M26. The incidence rates of infectious symptoms in the T337 and M26 lines were 68 and 100%, respectively. MR5 significantly affected the leaf chlorophyll content, ETR, and NPQ of T337 and M26, as well as P_n and T_r of M26. MR5 tended to reduce the leaf photosynthetic parameters of T337, but the decreases were not significant. The leaf reactive-oxygen-species levels of T337 and M26, the leaf antioxidant-enzyme activities of M26, and the superoxide-dismutase activity of T337 were significantly affected by MR5. MR5 also had a significant effect on the leaf *malondialdehyde*, proline, and soluble-sugar contents of T337 and M26. None of these aboveground physiological indicators were affected by MR5 in the 12-2 rootstock. (4) Conclusions: The 12-2 rootstock was more resistant to ARD-associated MR5 and could serve as an important test material for resistant-apple-rootstock breeding in China.

Keywords: cultivation of resistant rootstocks; ARD; ARD-associated *F. proliferatum* MR5; apple rootstock superior line 12-2; aboveground physiological indicators

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1. Introduction

Apple is a popular fruit worldwide, and many countries list it as a major consumer product because of its ecological adaptability, high nutritional value, good storage properties, and long supply cycle [1]. Apple replant disease (ARD) is a compound disease that commonly occurs after replanting an apple orchard. ARD slows the growth of young apple trees, inhibits root development, reduces yield, and can cause death, ultimately shortening the life of a new orchard [2]. ARD is estimated to cost growers USD 70,000–150,000 per acre during the first 4 years of orchard production [3]. ARD is a worldwide problem that seriously hinders the sustainable development of the apple industry [4]. As a result, it is increasingly important to study the etiology, prevention, and control of ARD.

Studies have shown that the main cause of ARD is an imbalance in the microbial community structure [5]. The identified causative agents of ARD include *Pratylenchus penetrans*, oomycetes (*Pythium* and *Phytopthora* spp.), and species from fungal genera *Cylindrocarpon*/*Ilyonectria* and *Rhizoctonia* [6]. However, the specific harmful fungi differ among replanted orchard soils from different regions [4]. *Fusarium* is an important pathogen that has been confirmed to cause ARD in some regions [4,7], including China [8] and South Africa [7]. A

study of 57 perennial trees planted in ARD soil in the Bohai Bay region of China showed that the abundances of *Fusarium* and *Mortierella* species were significantly correlated with ARD severity [9]. *Fusarium proliferatum* and other suspected pathogenic *Fusarium* spp. were also identified in ARD soils in Hebei Province, China [10], and Duan et al. [11] recently identified 89 genera and 219 species, most of which were *Fusarium*. Using pathogenicity assays, *Fusarium* was confirmed to be the most abundant pathogen causing ARD in China. Among *Fusarium spp*., specialized, ARD-associated *F. proliferatum* strain MR5 (MW600437.1) was screened and identified, and it shows strong pathogenicity in apple rootstocks [11,12]. Diseased seedlings, particularly, exhibit leaf chlorosis and the browning of leaf edges, followed by leaf rolling and yellowing, withering, and death [11].

Many methods have been proposed to control ARD, such as appropriate crop rotation, intercropping, soil sterilization, and the use of beneficial bacterial fertilizer [13]. Planting resistant rootstocks is one of the more effective methods to alleviate ARD [14]. This method effectively controls the diseases and pests in the reseeded soil, alleviates the row of the same crop in the same plot of land continuously finding obstacles caused by some pathogenic bacteria [15], strengthens plant stress resistance, and increases fruit yield and quality [16]. Many studies have been carried out on resistant rootstocks in Europe, the United States, and other countries. The G11, G16, and G41 rootstocks developed by the Geneva rootstock-breeding program are tolerant to some ARD-associated pathogens, although this assessment has not been consistently confirmed in all studies [17,18]. However, these rootstocks have not been popularized in China for various reasons. Rootstock breeding for fruit trees has long been neglected in China, as hundreds of apple varieties have been reported since the founding of the People's Republic of China, but few apple rootstock varieties have been developed. In contrast, the T337 and M26 dwarf rootstocks remain the main apple rootstocks used for production in China. T337 is characterized by a yellowish-brown trunk, from red to silvery-white branches, oval dark-green leaves with sharply serrated margins, and a sharp apex [19]. M26 seedlings are characterized by large, smooth, and leathery leaves; wavy serrated leaf margins; thick and upright shoots; and obvious lenticels [20]. T337 has the advantages of early fruiting and high yield [21], and M26 has higher graft compatibility and stronger healing ability [3]. However, these two rootstocks have shallow roots and short lifespans. T337 and M26 are generally considered to be ARD-"susceptible" rootstocks [3,13]. Through the patented technology of in situ breeding [22], our research group selected a new apple rootstock superior line, named 12-2, which is tolerant to ARD. 12-2 is a new line of *Malus spectabilis* that has not been previously identified. This new line has red stems and new purple-red leaves in 30-day- and 3-year-old plants [14]. We initially selected more than 30 high-quality ARD-resistant lines and planted them in replanted soil with 20-year-old Fuji/*Malus* × *robusta* (CarriŠre) Rehder apples in 2010. By November 2014, only 12-2 and other fine strains survived, and the trees continue to thrive to this day [23,24].

The roots of 12-2 show good resistance to ARD-associated *F. proliferatum* MR5 [12]. The *Fusarium* infectious process can be divided into root identification, root-surface adhesion and colonization, penetration and colonization of the root cortex, and mycelium proliferation in the xylem vasculum [25]. *Fusarium* that reaches the woody tissue vessels spreads within the plant through the proliferation of hyphae and the production of microspores [26]. Infected plants must adopt a series of stress responses to reduce infection and pathogenic bacteria [27]. Fungi secrete phytotoxins during plant colonization that can reduce plant-cell viability or cause cell death at low concentrations [28]. Fusaric acid (5-*n*-butyl-pyridine-2-carboxylic acid, $C_{10}H_{13}NO_2$) is a nonspecific toxin produced by *Fusarium* bacteria that interferes with the metabolism of infected plants, alters membrane permeability, and inhibits oxygen absorption [25]. Studies have shown that enniatins, equisetin, fusarium acid, and several other mycotoxins are directly involved in the pathological systems of many *Fusarium* host plants [29]. Secondary fungal metabolites have also been widely studied in host-specific pathogenesis mediated by specific toxins. Secondary metabolites released by filamentous fungi into the rhizosphere, such as fusaric acid, enniatins, and equisetin, significantly affect plant growth [30]. Pathogenic fungi of the *Fusarium* complex produce various

hormones, such as auxins, cytokinins, and gibberellins [31]. The accumulation of active cytokinins may lead to organ malformation after *Fusarium* infection [32]. Plants infected with *Fusarium* develop symptoms, such as yellowing, curling, leaf senescence, decreased photosynthesis, cell damage caused by oxidative stress, decreased antioxidant-enzyme activities, and increased malondialdehyde (MDA) and proline contents [33]. Severely infected plants die [34]. Studies have shown that seedlings are more likely to be infected with ARD, and the growth of rootstocks infected with ARD during the seedling period is affected [35]. The higher the resistance of the apple rootstock, the less it is affected by stress [36]. Because 12-2, T337, and M26 are different varieties within the *Malus* genus, it is impossible to determine *Fusarium* resistance by directly comparing the growth of the three varieties. Therefore, we used the intraspecies comparative method to detect *Fusarium* resistance and tested the aboveground physiological indicators. Our study provides useful test materials for the breeding of resistant apple rootstocks in China, which are important for fundamentally solving the ARD problem.

2. Materials and Methods

2.1. Experimental Materials and Treatments

The experiment was carried out from June to September 2021. The test materials were 12-2 (self-named), which is a tolerant rootstock produced by our group using patented breeding technology, and the T337 and M26 tissue-culture rootstocks purchased from Shandong Horticultural Techniques & Services Co., Ltd. (Tai'an, Shandong, China). In mid-June, tissue-culture rootstocks that had been subcultured multiple times were inoculated into a rooting medium (modified 1/2 MS medium with 20 g·L^{-1} sucrose, 7.5 g·L^{-1} agar, 0.2 mg·L^{-1} 6-BA, and 1.0 mg·L^{-1} IBA (pH 5.8)). Five explants were placed in each bottle of induction medium and grown in a tissue-culture room at 25 ± 2 °C with a 16 h light photoperiod and a light intensity of 1,000 lx. In mid-August 2021, rooted seedlings of similar size were selected from each superior line and transplanted into a sterile substrate in early September after acclimating the seedlings. Specialized, ARD-associated *F. proliferatum* strain MR5 (MW600437.1) [11] was characterized by the research group of Professor Mao Zhiquan of Shandong Agricultural University. In early September 2021, a layer of pathogenic fungal strains was inoculated in a potato glucose liquid broth medium (PDB; Qingdao, Shandong, China). After 7 days of culture, a spore suspension was obtained through 8 layers of sterile gauze filtration. A hemocytometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the spore concentration under a microscope (Nikon ni-u, Tokyo, Japan), and the final concentration was adjusted to 10^6 spores ml^{-1} with sterile water. On September 8, the spore suspension (20 mL) was inoculated into plugs containing sterile substrate (substrate: Perlite = 1:1), and a control group received the same volume of PDB medium. One hundred rooted seedlings of each cultivar (12-2, T337, and M26) were randomly divided into two groups and planted in the two media. Inoculated mycelia were grown in a tissue-culture chamber at 24 ± 2 °C under a 16 h photoperiod and 1,000 lx light intensity. All indicators were measured on September 14, 2021 [12]. Five biological replicates were measured in each treatment.

2.2. Assessment of Infection Symptoms

Disease resistance was assessed based on the plant survival rate, disease incidence rate, and the disease index [37]. The disease incidence rate was based on the appearance of brown lesions on the leaves, and the disease index grades were defined as shown below [37].

Grade 0: No symptoms.

Grade 1: Yellowed or wilted true leaves and cotyledons did not exceed 50% of the total area.

Grade 2: Yellowed or wilted true leaves and cotyledons exceeded 50% of the total area;

Grade 3: Leaves wilted or dead, with only the growing points surviving.

Grade 4: The entire plant was severely wilted or dead.

Disease index = ∑ (Plant number in the grade × Grade number)/ (Total plant number × Highest grade number) × 100.

2.3. Measurement of Leaf Chlorophyll Content

The relative chlorophyll contents of fully expanded leaves (leaves 3–5 from the bottom) were measured with an SPAD-502 portable chlorophyll meter (Beijing Harvesting Science and Technology Co., Ltd., Beijing, China). The same leaves were later removed, and the contents of chlorophyll *a*, chlorophyll *b*, and carotenes were measured using the acetone extraction method [38].

2.4. Measurement of Leaf Photosynthetic Parameters

The same fully expanded leaves used for the SPAD-meter measurements were placed in a CIRAS-2 portable photosynthesis measurement system (PP-Systems, Hansha Scientific Instruments, Beijing, China) to measure the leaf net photosynthetic rate (P_n), intercellular CO_2 concentration (C_i), stomatal conductance (G_s), and the transpiration rate (T_r). Five replicates of each treatment were measured.

2.5. Measurement of Leaf Fluorescence Parameters

The chlorophyll fluorescence parameters were measured on fully expanded leaves as described above using a German WALZ Junior-PAM portable fluorometer (Zealquest Scientific and Technology Co., Ltd., Shanghai, China). The measured fluorescence parameters were the quantum efficiency of PSII (F_v/F_m), the actual photochemical efficiency of PSII (Φ_{PSII}), the non-photochemical quenching coefficient (NPQ), the photochemical quenching coefficient (qP), and the electron transfer rate (ETR). Five replicates of each treatment were measured.

2.6. Measurement of Leaf Reactive-Oxygen-Species Levels

Fully expanded leaves were harvested from each treatment as described above to measure H_2O_2 content and the O_2^- production rate. The method by Bai et al. [39] was used to determine H_2O_2 content and the O_2^- production rate in five replicate samples from each treatment.

2.7. Measurement of Leaf Antioxidant-Enzyme Activities and Malondialdehyde Content

Fully expanded leaves were harvested as described above and used to measure enzyme activities. Superoxide dismutase (SOD) activity was measured using the method by Sun et al. [40]. Peroxidase (POD) activity was measured with the guaiacol method as described by Omran [41]. Catalase (CAT) activity was measured according to the method by Singh et al. [42]. MDA content was determined with the thiobarbituric acid method [43].

2.8. Measurement of Leaf Proline and Soluble-Sugar Contents

Fully expanded leaves from each treatment were harvested as described above to measure proline and soluble-sugar contents. Proline content was determined with the ninhydrin color method [3], and soluble-sugar content was determined with the phenol method [44]. Five replicate samples from each treatment were measured.

2.9. Data Analysis

The analysis of variance was performed using SPSS software (version 17; IBM SPSS, Chicago, IL, USA). Differences were detected among treatment means using Student's *t*-test or Duncan's multiple range test. A p-value < 0.05 was considered significant.

3. Results

3.1. Effects on Apple Rootstocks

Seven days after applying the MR5 spore solution, T337, M26, and 12-2 all showed different degrees of disease (Figure 1). On day 3 after MR5 infection, yellowish-brown

spots appeared on the tips of the M26 leaf edges, and the leaves were dry and necrotic, but there were no obvious symptoms on T337 or 12-2. On day 5, yellowish-brown spots were observed on the tips of the T337 leaf edges. The dry and necrotic areas of the M26 leaves had expanded from the outside and inside to cover about one-half of the entire leaf area, whereas the 12-2 leaves had not developed any obvious symptoms. On day 7, the leaf edges of 12-2 also began to exhibit yellowish-brown spots (Figure 1F). At this time, the dry and necrotic areas of T337 leaves had expanded from the inside to the outside, and a circle of yellowish-brown lesions appeared on the leaf edges (Figure 1B). The dry and necrotic areas of M26 had expanded to cover > 50% of the total area (Figure 1D). The plant survival rates of the three rootstocks were all 100% 7 days after MR5 was inoculated (Table 1). The disease incidence rates on T337, M26, and 12-2 were 68, 100, and 24%, respectively (Figure 1G–I). The disease indices for T337, M26, and 12-2 were 68.00, 74.00, and 24.00, respectively (Table 1). These results show that M26 leaves had the most significant disease symptoms 7 days after MR5 infection, followed by T337 and 12-2.

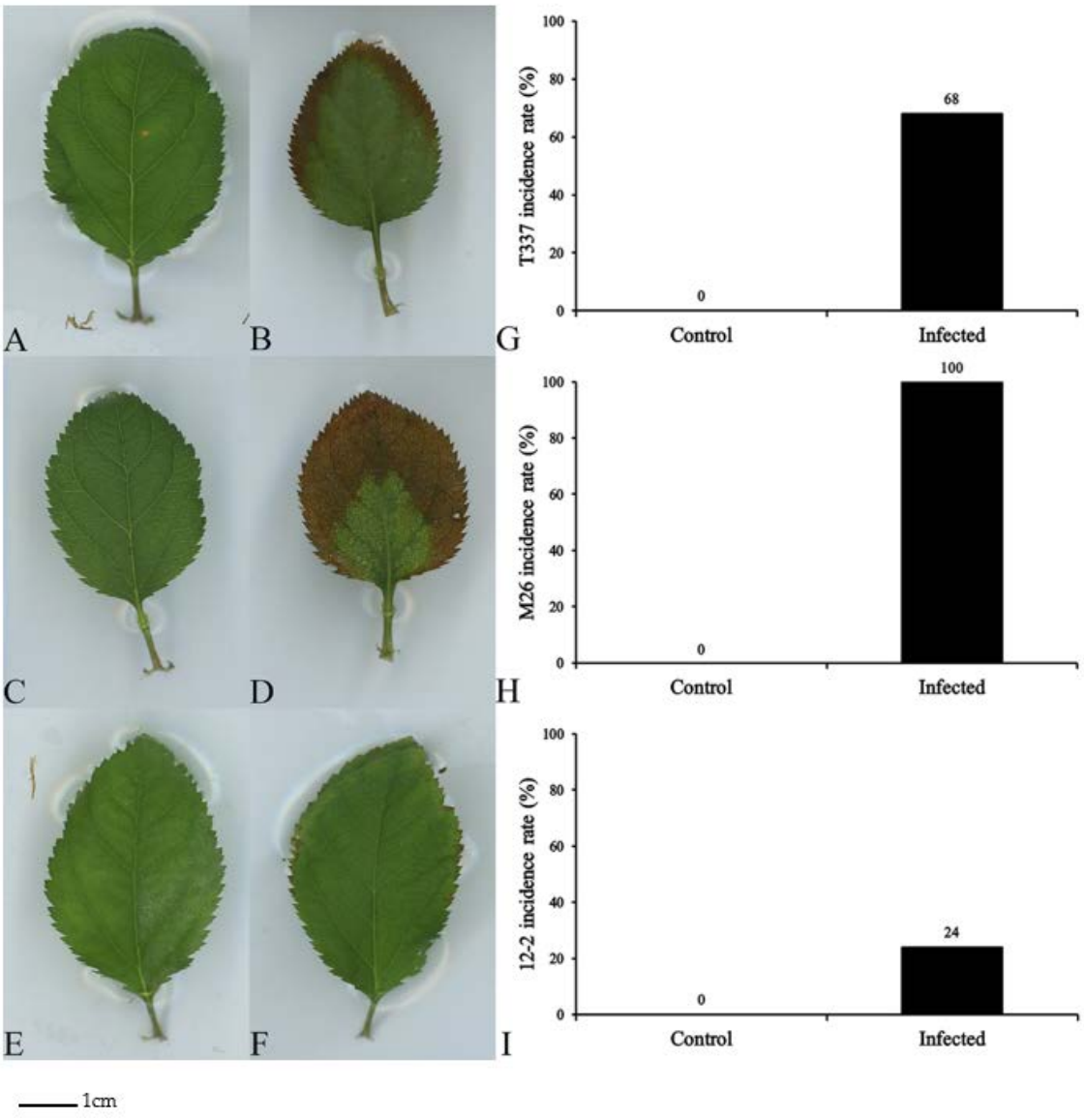

Figure 1. Effects of *F. proliferatum* MR5 on the three rootstocks after 7 days. (**A**) Control T337 leaves. (**B**) Infected T337 leaves. (**C**) Control M26 leaves. (**D**) Infected M26 leaves. (**E**) Control 12-2 leaves. (**F**) Infected 12-2 leaves. (**G**) T337 incidence rate. (**H**) M26 incidence rate. (**I**) 12-2 incidence rate. Note: Scale bars in (**A**–**F**) are 1 cm.

Table 1. The number of plants of the three rootstocks in each grade 7 days after MR5 infection.

Grade Number	T337	M26	12-2
0	16	0	38
1	34	0	12
2	0	39	0
3	0	11	0
4	0	0	0

3.2. Effects on Leaf Chlorophyll Contents

The chlorophyll *a*, chlorophyll *b*, carotene, and relative chlorophyll contents of T337 decreased by 82.91%, 77.30%, 83.54%, and 25.46% in treated plants compared with controls 7 days after MR5 infection (Figure 2). The chlorophyll *a*, chlorophyll *b*, carotene, and relative chlorophyll contents of M26 decreased by 78.34%, 60.75%, 42.65%, and 36.43%, respectively. These results show that MR5 significantly reduced the leaf chlorophyll contents of T337 and M26 but had no significant effect on 12-2.

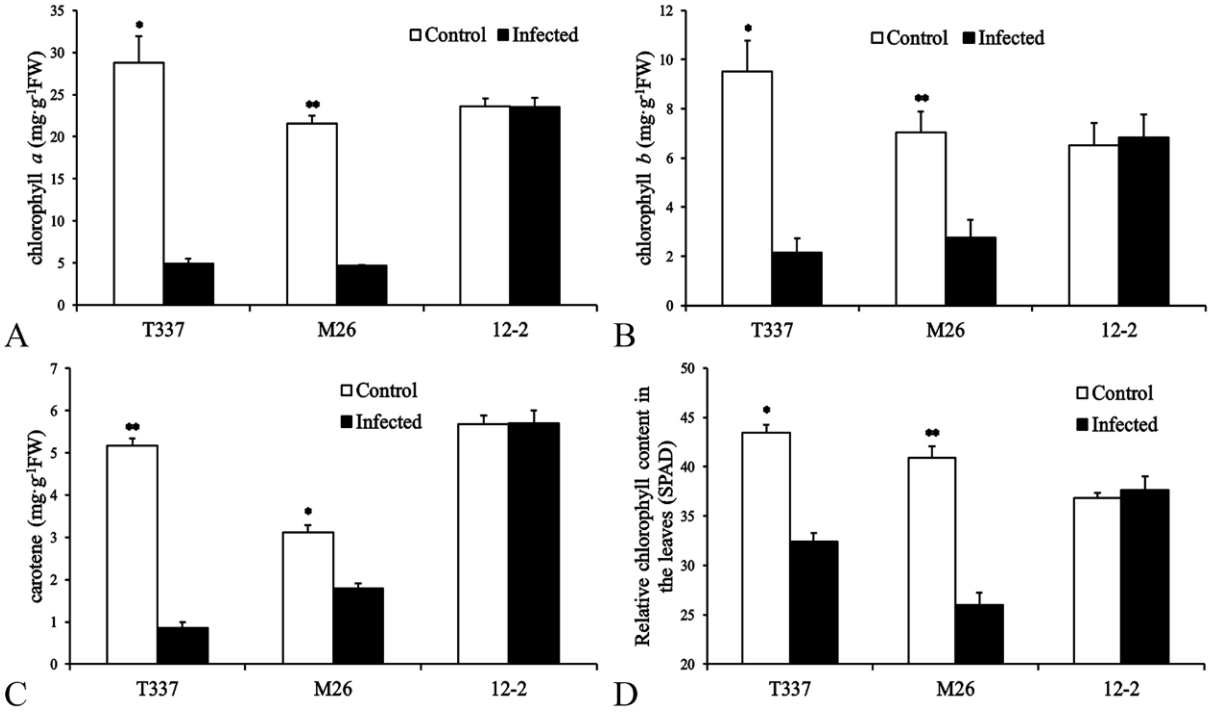

Figure 2. Effects of *F. proliferatum* MR5 on leaf chlorophyll contents: (**A**) chlorophyll *a*; (**B**) chlorophyll *b*; (**C**) carotene; (**D**) SPAD. The Student's *t*-test was used to detect the differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

3.3. Effects on Leaf Photosynthetic Parameters

MR5 significantly reduced P_n and T_r of M26 by 48.26% and 29.50% in treated plants compared with controls 7 days after infection (Figure 3). Although G_s and C_i of M26 decreased, the differences were not significant. MR5 produced nonsignificant reductions in the T337 photosynthetic parameters but did not affect the leaf photosynthetic parameters of 12-2. These results show that MR5 reduced some leaf photosynthetic parameters of T337 and M26 but had no significant effect on those of 12-2.

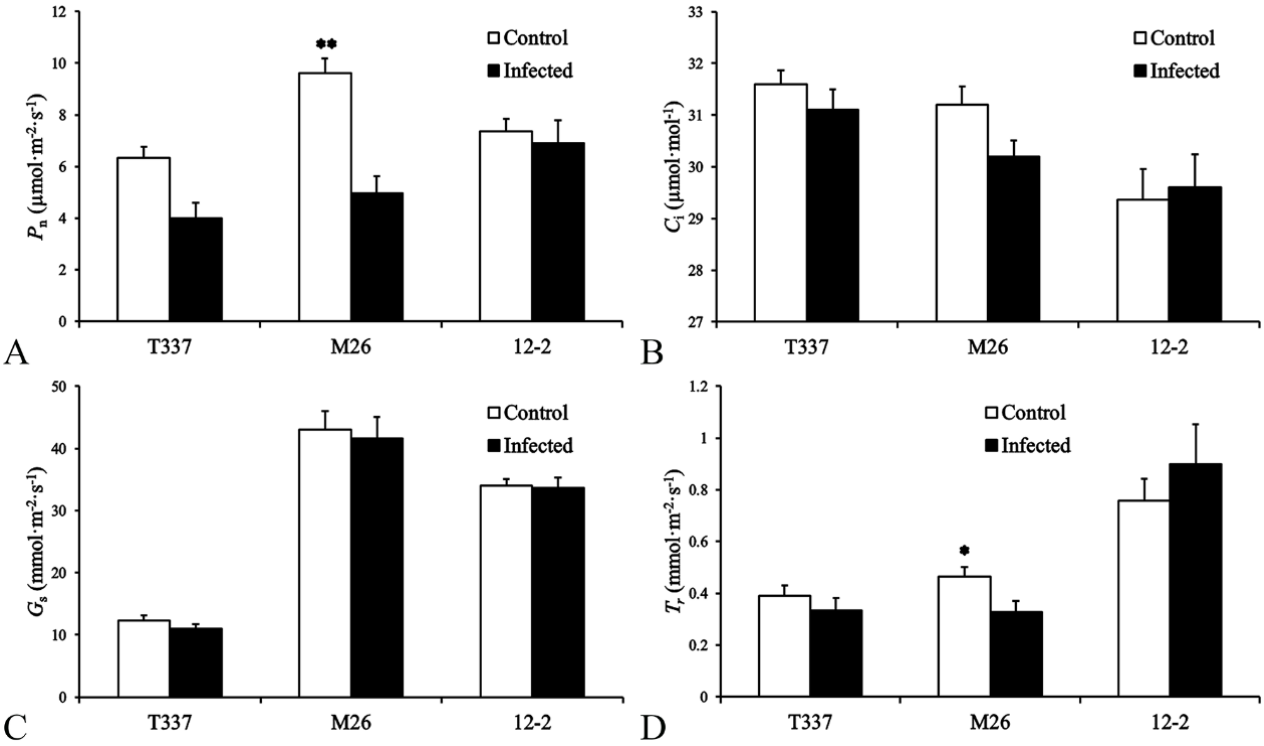

Figure 3. Effects of *F. proliferatum* MR5 on leaf photosynthetic parameters: (**A**) P_n; (**B**) C_i; (**C**) G_s; (**D**) T_r. The Student's *t*-test was used to detect the differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

3.4. Effects on Leaf Fluorescence Parameters

MR5 significantly reduced the ETR of T337 and M26 and significantly increased the NPQ of T337 and M26 but had no significant effect on the other leaf fluorescence parameters of T337 or M26 7 days after infection (Figure 4). MR5 infection had no significant effect on the leaf fluorescence parameters of 12-2. These results show that MR5 had some effect on the leaf fluorescence parameters of T337 and M26 but had no significant effect on those of 12-2.

3.5. Effects on Leaf Reactive-Oxygen-Species Levels

MR5 significantly increased the H_2O_2 content of treated versus control T337 and M26 plants by 14.95% and 75.22%, respectively, and significantly increased the O_2^- production rate of T337 and M26 by 30.23% and 77.96%, respectively, 7 days after infection (Figure 5). These results show that MR5 significantly increased the leaf reactive oxygen species (ROS) levels of T337 and M26 but had no significant effect on those of 12-2.

3.6. Effects on Leaf Antioxidant-Enzyme Activities and MDA Content

MR5 significantly reduced SOD activity of treated vs. control T337 plants by 55.60% and increased MDA content by 28.55% 7 days after the infection (Figure 6). Although no significant differences in POD nor CAT activity were observed in T337, POD and CAT activities decreased by 18.96% and 29.43%, respectively. MR5 significantly increased SOD, POD, and CAT activities in M26 by 77.59%, 43.20%, and 18.15%, respectively, and increased MDA content by 141.20%. MR5 infection increased the antioxidant-enzyme activities and MDA content of 12-2 leaves, but these effects were not significant. These results show that MR5 decreased leaf antioxidant-enzyme activities and significantly increased leaf MDA contents of T337 and M26 but had no significant effect on these parameters in 12-2.

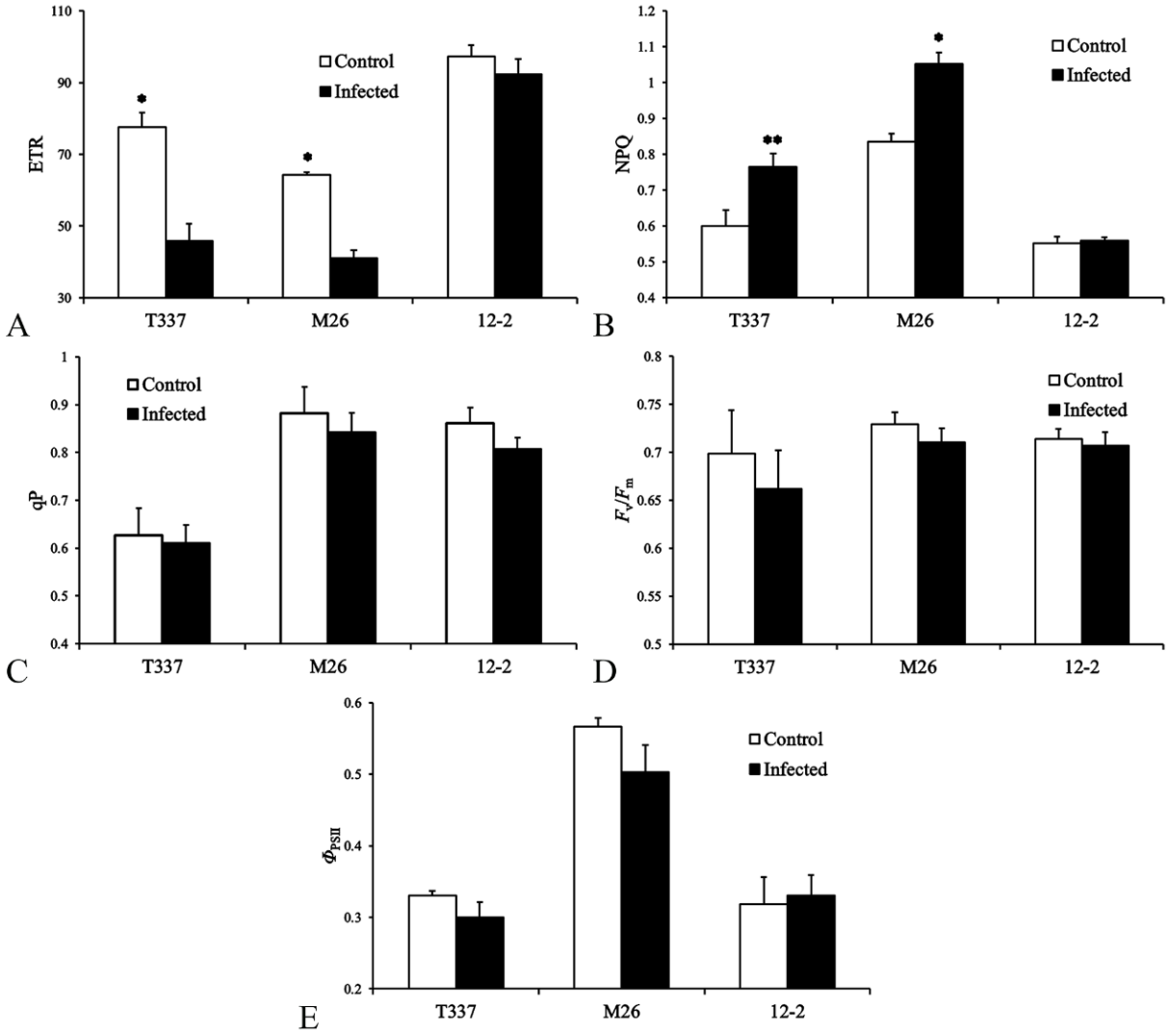

Figure 4. Effects of *F. proliferatum* MR5 on leaf fluorescence parameters: (**A**) ETR; (**B**) NPQ; (**C**) qP; (**D**) F_v/F_m; (**E**) Φ_{PSII}. The Student's *t*-test was used to detect differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

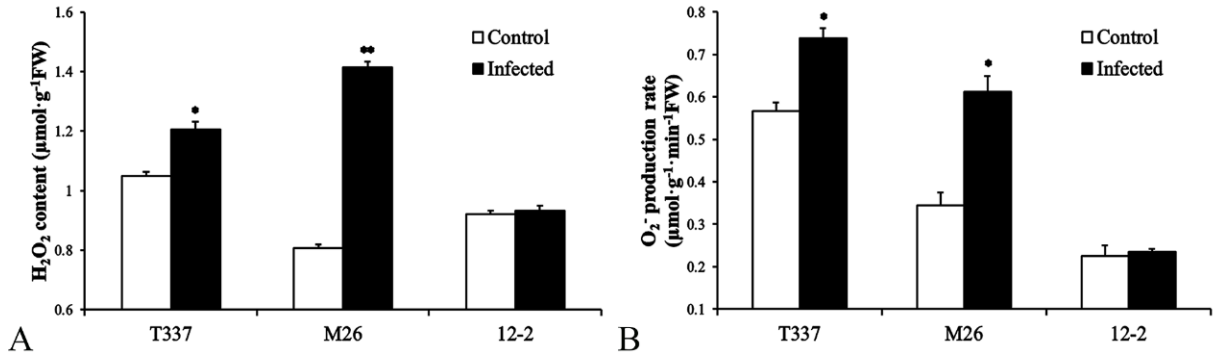

Figure 5. Effects of *F. proliferatum* MR5 on leaf reactive-oxygen-species levels: (**A**) H_2O_2 content; (**B**) O_2^- production rate. The Student's *t*-test was used to detect differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

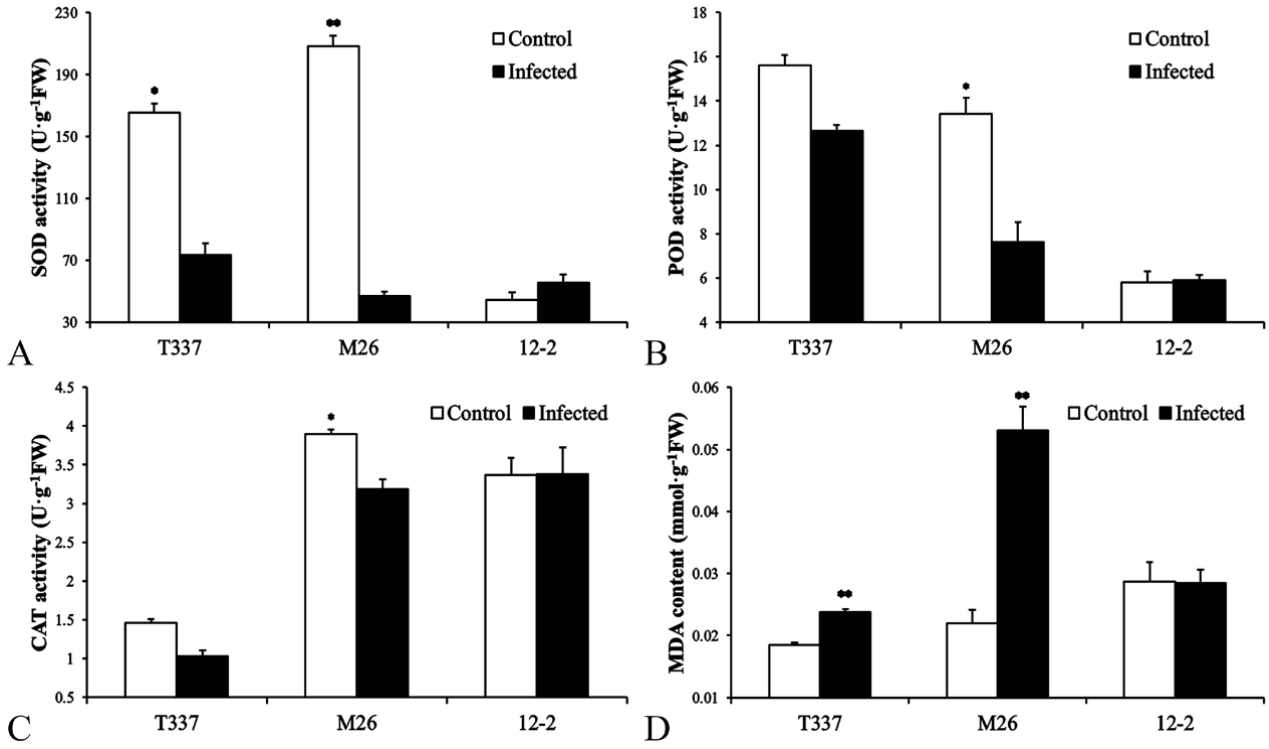

Figure 6. Effects of *F. proliferatum* MR5 on leaf antioxidant-enzyme activities and malondialdehyde (MDA) content: (**A**) SOD activity; (**B**) POD activity; (**C**) CAT activity; (**D**) MDA content. The Student's *t*-test was used to detect differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

3.7. Effects on Leaf Proline and Soluble-Sugar Contents

MR5 significantly increased the proline contents of treated vs. control T337 and M26 plants by 71.82% and 102.35%, respectively, and significantly increased the soluble-sugar contents of T337 and M26 by 57.08% and 117.98%, respectively, 7 days after infection (Figure 7). These results show that MR5 significantly increased proline and soluble-sugar contents in T337 and M26 leaves but had no significant effect on these parameters in 12-2.

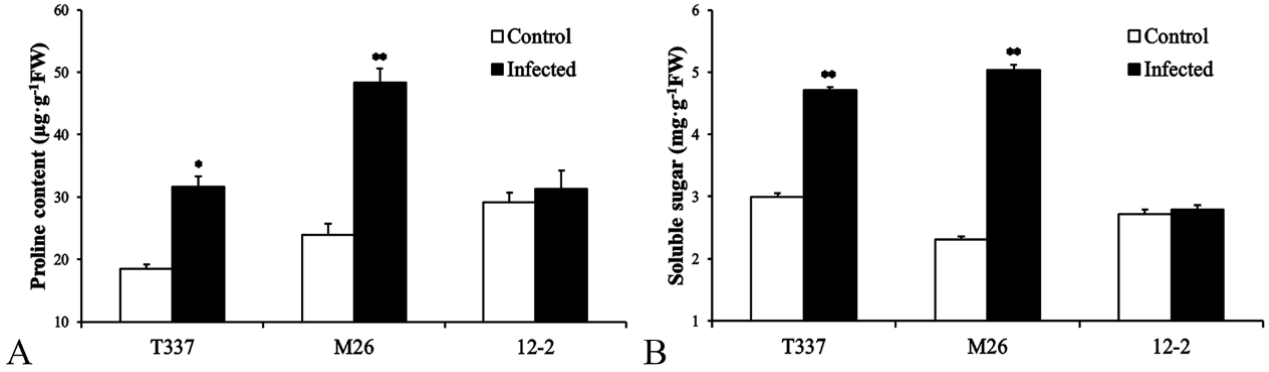

Figure 7. Effects of *F. proliferatum* MR5 on leaf proline and soluble-sugar contents: (**A**) proline content; (**B**) soluble-sugar content. The Student's *t*-test was used to detect differences between the control and infected treatments within a rootstock. * $p < 0.05$; ** $p < 0.01$.

4. Discussion

The morphological structure of and physiological changes in leaves directly reflect the adaptability and stress resistance of plants, as leaves are the most sensitive plant part to disease [45]. Studies have shown that 5–7 days after *Fusarium* infection, the pathogen

invades the vascular cylinder and pith of the host, causing leaves to fall and lodging to occur [46]. The wilting symptoms and physiological responses of plants after *Fusarium* infection are similar to those observed in plants under water stress [47]. In the present experiment, the leaves of the three rootstocks displayed different degrees of dryness and necrosis. The onset of disease symptoms occurred earlier in M26, followed by T337 and 12-2. These findings were similar to the results obtained by Choi et al. [48] in an apple-rootstock tolerance test. The greater the sensitivity of the rootstock was, the earlier the onset of disease was.

Chlorophyll absorbs the optical energy required for photosynthesis. A decrease in chlorophyll content inevitably leads to a decline in the photosynthetic rate and the accumulation of photosynthetic products, which eventually affect the healthy development of plants [49]. Botyanszka et al. [50] reported that chlorophyll content can be used as a technical index to recognize rootstock disease resistance. The worse the resistance of the rootstock is, the more significant the effect of infection is on chlorophyll content. In the present experiment, the chlorophyll contents of infected T337 and M26 were significantly lower than those of the controls. This may have occurred because the pathogen destroyed the structure of the chloroplasts, causing the chlorophyll synthetic rate to be much slower than the degradation rate [51]. In contrast, the chlorophyll content of 12-2 was not significantly affected by MR5. This may be because 12-2 is an ARD-resistant variety with mechanisms that alleviate chloroplast damage, thereby maintaining high chlorophyll levels [52].

Photosynthesis is the principal physiological process of plant growth, and its susceptibility to *Fusarium* infection has been confirmed in bananas, tomatoes, and wheat [53,54]. *Fusarium* disrupts the photosynthetic metabolism [55] and blocks the transport of water through the xylem by clogging the vessels, causing leaf dehydration, stomatal closure, and reduced P_n [56]. Studies have shown that C_i typically increases when G_s decreases, as carboxylation efficiency is reduced [57]. The leaf photosynthetic limits (P_n, G_s, C_i, and T_r) tend to fall under a stressed state [58], and this phenomenon was observed here for T337 and M26. The water loss per unit leaf area of mildly infected plants was significantly lower than that of healthy plants. Reduced G_s and T_r decrease the water loss of infected plants [25]. We speculate that MR5 infection induced photoprotective mechanisms in T337 and M26 and that their reduced P_n was caused by stomatal factors [54,59]. The photosynthetic parameters of 12-2 were less affected by MR5, as 12-2 may have maintained relatively high G_s, which increased T_r and heat dissipation, prevented leaf oxidative damage, and enhanced disease resistance [60].

Stress can also impair normal photochemical reactions. PSII is more susceptible to environmental stress damage than PSI, so chlorophyll fluorescence parameters can be used to assess plant stress tolerance [61]. Previous studies have shown that the carboxylation efficiency and photochemical ability of PSII decrease with *Fusarium* infection, indicating that the infection negatively affected Rubisco and the PSII reaction center [53]. *Fusarium* infection depresses PSII performance and impairs the coordination between PSI and PSII by inducing pathological wilting in apple seedlings [62]. F_v/F_m is commonly used as an indicator of photoinhibition or PSII damage [63]. After MR5 infection, F_v/F_m declined in all three varieties, but this decrease was less severe in 12-2 than in T337 and M26. PSII of 12-2 may have experienced less damage under MR5 stress than T337 and M26, resulting in a lesser slowing of photosynthetic electron transport and preserving the redox ability of initial electron acceptor Q_A, thereby maintaining photosynthetic carbon assimilation at a higher level [64]. The ETR values of the three varieties were consistent with this scenario. At the same time, qP decreased in T337 and M26, indicating that the actual efficiency of light capture, carbon assimilation capacity, and the ratio of the open PSII reaction center decreased, limiting electron transfer from the PSII reaction center, and inhibiting photosynthesis [65]. NPQ significantly increased in T337 and M26 in response to infection, showing that the leaf heat dissipation mechanism was destroyed and light energy dissipation increased in the form of heat [66]. Therefore, a large amount of excitation energy likely accumulated, damaging the thylakoid membranes and photosynthetic complexes,

leading to a loss of photoprotective ability [67] and the appearance of lesions on diseased leaves (Figure 1). No significant changes in the NPQ of 12-2 were observed, indicating that the light energy conversion rate and photosynthesis remained unchanged under stress [68].

Active disease resistance is a host defense response induced by pathogen infection, and ROS are the earliest defense response of host plants to invasion by a causative agent [69]. Bacterial infection affects the balance of ROS metabolism in plants, destroying or reducing ROS scavengers, increasing ROS production, and damaging membrane structures [70]. Here, MR5 infection caused a significant increase in H_2O_2 content and O_2^- production rate in T337 and M26, suggesting that the ROS produced by T337 and M26 actively participated in the plant disease-resistance response, as ROS are toxic to pathogenic bacteria and may directly kill them [70]. The ROS content did not significantly increase in 12-2, because disease-resistant varieties are more capable of eliminating ROS than susceptible varieties [71], and ROS induce the immune-mediated necrosis of host cells, thereby inhibiting the spread of a pathogen [72].

However, overproduced ROS react with proteins, lipids, and nucleic acids in the host cell, resulting in cell-membrane peroxidation and damage, enzyme inactivation, cell metabolic disorders, and the eventual death of the host cell [73]. The host plant's ROS scavenging system regulates the ROS balance, thereby maintaining a normal cellular redox state and reducing ROS damage to the host cell [74]. Excessive ROS in plant leaves are removed by SOD, POD, CAT, and other defense-related enzymes [47]. Here, the activities of SOD, POD, and CAT decreased to varying degrees in T337 and M26 7 days after MR5 infection. It may be that when the stress exceeded a certain threshold, T337 and M26 could not remove the oxygen free radicals rapidly enough, eventually leading to reduced enzyme activities and oxidative damage [75]. The POD activity of 12-2 increased 7 days after MR5 infection. One possible reason is that the O_2^- generation rate of 12-2 increased, resulting in increased SOD activity and rapid disproportionation of O_2^- to H_2O_2 and O_2 [76]. At this time, POD plays a protective role in decomposing O_2^- into H_2O_2 and H_2O [68]. CAT activity acts as a reserve force for POD, removing H_2O_2 and converting it to O_2 and H_2O [77].

MDA is the final product of membrane lipid peroxidation. MDA content has been used as a representative of the degree of membrane damage [78]. The more serious the lipid peroxidation is, the higher the MDA content is, indicating a greater plant-damaging effect [79]. Here, the MDA content of T337 and M26 significantly increased 7 days after MR5 infection, whereas that of 12-2 did not significantly change. Therefore, we speculated that the cell membrane functions of T337 and M26 were severely damaged, whereas 12-2 responded to stress in time, and its cell membranes experienced less injury.

Soil-borne pathogens infect the xylem of plant roots and hinder water transport in the xylem [62]; this leads to severe water stress and the pathological wilting of apple seedlings [47]. To restrict water coerced loss, plants synthesize specific consistent solutes to abate the cell osmotic potential and promote water absorption [80]. Soluble-sugars and proline act as plant protective substances under stress. Their production is one of the basic mechanisms for plants to react to stress, and it is probably a manifestation of cell structural damage [81]. Here, the proline and soluble-sugar contents of T337 and M26 increased significantly 7 days after MR5 infection. MR5 infection may have led to a rapid burst of ROS production and an excessive oxidation of membrane lipids, causing cellular damage and inactivating antioxidant enzymes, leading to an increase in proline and soluble-sugars [70]. No significant changes in proline or soluble-sugar contents were detected in 12-2. We speculated that 12-2 was more tolerant to MR5, experienced less stress, and thus maintained relatively normal cellular function [82].

Although we provided some evidence that 12-2 is resistant to ARD-associated *F. proliferatum* strain MR5, the molecular mechanisms by which 12-2 inhibits MR5 are unknown. We only tested MR5. Whether 12-2 is resistant to other pathogens is an important question for future research. We do not have the Geneva apple rootstock, which would also be a good test material. Although the performance of the three varieties was

significantly different within 7 days of infection, 12-2 exhibited mild symptoms. Because there were no other disease-resistant varieties with which to compare 12-2 and the experimental soil contained much higher concentrations of pathogenic fungi than actual soil, it was not necessary to continue this particular experiment. However, the behavior of 12-2 under continued inoculation with a fungal solution would be worth studying. The risk of the damaging effect of low-molecular-weight compounds liberated by *Fusarium* into the plant rhizosphere is another research topic. The interaction between 12-2 and secondary metabolites released by fungi should also be the focus of future research. Many experiments should be performed on new rootstocks, including evaluations of their graft consistency, gain outputs, and survival and growth in other areas.

5. Conclusions

The 12-2 rootstock had good resistance to ARD-associated *F. proliferatum* MR5. Leaf growth, the disease incidence rate, chlorophyll content, photosynthetic and fluorescence parameters, ROS levels, antioxidant-enzyme activity, MDA content, and proline and soluble-sugar contents did not significantly differ between 12-2 plants treated with PDB and those treated with the MR5 spore solution. The MR5 spore solution had the greatest effect on most of the aboveground physiological indicators of T337 and M26. An infection test showed that 12-2 was more resistant to ARD-associated *F. proliferatum* MR5 and may serve as an important test material for apple-rootstock resistance breeding in China.

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Abbreviations

ARD, apple replant disease; 12-2, apple rootstock superior line named 12-2; MR5, ARD-associated *F. proliferatum* MR5; P_n, net photosynthetic rate; C_i, intercellular CO_2 concentration; G_s, stomatal conductance; T_r, transpiration rate; F_v/F_m, PSII original light energy conversion efficiency; Φ_{PSII}, PSII actual photochemical efficiency; NPQ, non-photochemical quenching coefficient; qP, photochemical quenching coefficient; ETR, electron transfer rate; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; MDA, malondialdehyde.

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