Role of Melatonin in Arbuscular Mycorrhizal Fungi-Induced Resistance to Fusarium Wilt in Cucumber

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ABSTRACT

Melatonin is a multifunctional molecule that confers tolerance to a number of biotic and abiotic stresses in plants. However, the role of melatonin in plant response to *Fusarium oxysporum* and the interaction with arbuscular mycorrhizal fungi (AMF) remain unclear. Here we show that exogenous melatonin application promoted the AMF colonization rate in cucumber roots, which potentially suppressed Fusarium wilt as evidenced by a decreased disease index and an increased control effect. Leaf gas exchange analysis revealed that *Fusarium* inoculation significantly decreased the net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO₂ concentrations (Ci), and transpiration rate (Tr). Intriguingly, either melatonin application or AMF inoculation significantly increased the Pn, Gs, Tr, and dry biomass, and their combined treatment showed a more profound effect under *Fusarium* stress. Further analysis showed that *Fusarium* induced oxidative stress as evidenced by increased lipid peroxidation and electrolyte leakage. Conversely, either

melatonin or AMF drastically attenuated the levels of malondialdehyde, H₂O₂, and electrolyte leakage in *Fusarium*-inoculated plants, and their combined treatment caused a further decrease. *Fusarium* inoculation decreased the activity and transcripts of superoxide dismutase and ascorbate peroxidase, and the content of glutathione and proline. Besides, the activity and transcripts of peroxidase and catalase, the content of phenols and flavonoids increased after *Fusarium* infection. Importantly, melatonin and/or AMF significantly increased those parameters with the greatest effect with their combined treatment under *Fusarium* stress. Our results suggest that a positive collaboration between melatonin and AMF enhances resistance to Fusarium wilt in cucumber plants.

Keywords: antioxidants, arbuscular mycorrhizal fungi, biochemistry and cell biology, biological control, disease control and pest management, disease resistance, Fusarium wilt, melatonin, photosynthesis

Plant pathogenic fungi are one of the major biotic hazards that negatively affect plant growth and productivity around the world. *Fusarium oxysporum* is a filamentous plant pathogenic fungus that causes wilt disease in a number of important crops (Gordon 2017). *Fusarium* colonization clogs the vascular system, leading to severe wilting of aerial plant parts (Zhang et al. 2016). To control Fusarium wilt, a large amount of fungicides are irrationally used all over the world, leading to nontargeted adverse effects on the environment (Gordon 2017). Therefore, the development of efficient and environmentally friendly wilt management strategies has been a key research focus in recent years (Chen et al. 2019). These include enhancement of plant resistance by symbiotic association with beneficial fungi, such as *Trichoderma* spp., as well as

pharmacological treatments with plant growth regulators or signaling molecules that can increase plant defense against fungal pathogens (Chen et al. 2019; Li et al. 2016).

Arbuscular mycorrhizal fungi (AMF) are soilborne beneficial fungi that make symbiotic associations with plant roots and provide a variety of benefits to the host plants (Chen et al. 2017b; Shi et al. 2013, 2017). Moreover, AMF can enhance plant tolerance to many biotic and abiotic stresses and thus they can be used to control fungal diseases (Aseel et al. 2019; Bahadur et al. 2019; Li et al. 2019c; Mathur et al. 2018; Miozzi et al. 2019; Oyewole et al. 2017; Rathod et al. 2011). For instance, inoculation with *Funneliformis mosseae* enhances tolerance to *Phoma medicaginis*, the causal agent of leaf spots in alfalfa (*Medicago sativa*), and combined inoculation with *Glomus intraradices*, *Glomus claroideum*, and *Aphanomyces euteiches* suppresses root rot in pea (*Pisum sativum*) (Gao et al. 2018; Li et al. 2019c). Importantly, AMF-enhanced tolerance to leaf spot disease is attributed to the increased activity of antioxidant enzymes (Gao et al. 2018; Li et al. 2019c). Moreover, it is believed that AMF-enhanced tolerance to plant pathogens is also associated with the stimulation of plant secondary metabolism. For instance, mycorrhizal colonization with *Rhizophagus intraradices* decreases leaf spot disease index by 56.18% in alfalfa, which is associated with differential expression of genes involved in phenylpropanoid biosynthesis, flavonoid biosynthesis, glutathione (GSH) metabolism, and so on (Li et al. 2019c). Moreover, AMF colonization increases resistance to *Tomato mosaic virus* by enhancing the expression of *PHENYLALANINE AMMONIA-LYASE 1 (PAL1)* and the content of flavonoids in tomato (Aseel et al. 2019).

Photosynthesis is the most vital physiological process in plants. But infections with disease causal pathogens negatively affect plant photosynthetic capacity, leading to significant yield losses (Sun et al. 2019). RNA-seq analysis revealed that infection with *Phoma medicaginis* down-regulates a number of genes in the photosynthetic pathway, including a gene encoding for the photosystem 1

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reaction center subunit N, suggesting the negative effects of fungal pathogens on photosynthesis (Li et al. 2019c). Notably, enzymes involved in the photosynthetic light reaction, as well as the components of photosystem I and II, are sensitive to reactive oxygen species (ROS) (Ahmed et al. 2018; Sun et al. 2019). It is well established that fungal infections increase ROS accumulation, which can damage biological membranes, such as thylakoid membranes (Chen et al. 2019; Zhang et al. 2016). To ameliorate ROS-caused oxidative stress, plants elevate the activities of antioxidant enzymes (Ahmed et al. 2019). In addition to the enzymatic antioxidants, the nonenzymatic antioxidants, such as flavonoids that are synthesized through the secondary metabolic pathway, are capable of scavenging ROS (Aseel et al. 2019). Thus, enhancement of plant antioxidant defense and secondary metabolism can strengthen plant defense against plant pathogenic fungi.

Melatonin (*N*-acetyl-5-methoxytryptamine), a multifunctional biomolecule in plants, can stimulate photosynthetic processes and growth under biotic and abiotic stresses (Ahmed et al. 2019, 2020; Hasan et al. 2019; Hernández-Ruiz and Arnao 2018; Li et al. 2018, 2019a; Sharma and Zheng 2019). Classically being a strong antioxidant and an inducer of plant stress tolerance, melatonin can enhance plant resistance to a number of fungal diseases, such as gray mold in cherry tomato caused by *Botrytis cinerea* (Li et al. 2019a), anthracnose in banana caused by *Colletotrichum musae* (Li et al. 2019b), tomato fruit decay caused by *Botrytis cinerea* (Liu et al. 2019), powdery mildew in watermelon caused by *Podosphaera xanthii* and crown rot in cucurbits caused by soilborne fungus *Phytophthora capsici* (Mandal et al. 2018), downy mildew in cucumber caused by *Pseudoperonospora cubensis* (Sun et al. 2019), and potato late blight caused by *Phytophthora infestans* (Zhang et al. 2017). In cucumber, melatonin application decreased the levels of lipid peroxidation and relative electrolyte leakage caused by *Pseudoperonospora cubensis*, which was associated with the increased activity of antioxidant enzymes in leaves (Sun et al. 2019). However, melatonin treatment enhances susceptibility to *Penicillium digitatum* in citrus fruits by attenuating the content of H₂O₂, suggesting a negative role for melatonin in defense against fungal pathogens that cause postharvest diseases in citrus (Lin et al. 2019). Despite this fact, both melatonin and AMF are considered effective stimulators of plant defense against fungal pathogens (Hernández-Ruiz and Arnao 2018); however, the role of melatonin in AMF-induced resistance to *Fusarium oxysporum* remains unclear. In this study, we unraveled an additive role of melatonin and AMF that could enhance cucumber resistance to *Fusarium* wilt to a greater extent than their sole treatment. Our study unveiled an eco-friendly wilt management strategy by combining the application of melatonin and AMF in cucumber plants.

MATERIALS AND METHODS

Plant culture, fungal inoculation and treatments. Cucumber (*Cucumis sativus* L. ‘Jinyou 28’) seeds were surface sterilized with a solution of sodium hypochlorite (5%, vol/vol) for 10 min followed by rinsing with distilled water four times. Then the seeds were germinated on layers of wet filter paper at 28°C. When the seeds germinated (after 4 days of germination treatment), they were transplanted in plastic pots (13 × 13 cm) containing sterilized soil substrate (0.88 kg; organic manure/soil/decomposed straw = 1: 2:1) with the composition described previously (Chen et al. 2013). During transplanting, seedlings under the AMF treatments (AMF plants) were inoculated with AMF by adding 10 g of *Glomus claroideum* per pot containing approximately 720 spores. The AMF inocula contained spores, soil, extraradical hyphae, and infected clove (*Trifolium repens* L.) root fragments from a stock culture of *G. claroideum*. The AMF inocula were placed close to the roots of seedlings (Chen et al. 2013). Treatments not receiving the AMF (non-AMF plants) were inoculated with the same amount of sterilized (autoclaved) AMF inocula. From the fifth day after

transplanting, the foliage of seedlings under melatonin treatment was sprayed with 100 µM melatonin (Sigma-Aldrich, supplier Mainland, China) in the dark at 5-day intervals. Melatonin solution was prepared by dissolving the melatonin in a small amount of ethanol followed by dilution at a 1:10,000 ratio with double distilled water (ethanol/ddH₂O, vol/vol). Treatments that did not receive melatonin were sprayed with ddH₂O containing the same ratio of ethanol. Twenty days after transplanting, biotic stress was imposed by inoculating plants with *Fusarium oxysporum* f. sp. *cucumerinum* at 1 × 10⁶ CFU/ml (20 ml per plant) via soil drenching. Thus, the study consisted of eight treatments, such as (i) control (sprayed with distilled water and received only autoclaved inocula), (ii) AMF (sprayed with distilled water and inoculated with active AMF inocula), (iii) MT (sprayed with 100 µM melatonin and inoculated with autoclaved AMF inocula), (iv) AMF+MT (sprayed with 100 µM melatonin and inoculated with active AMF inocula), (v) FO (sprayed with distilled water and inoculated with spores of *Fusarium oxysporum*), (vi) AMF+FO (sprayed with distilled water and inoculated with both AMF and *Fusarium oxysporum*), (vii) MT+FO (sprayed with 100 µM melatonin and inoculated with *Fusarium oxysporum*), and (viii) AMF+MT+FO (sprayed with 100 µM melatonin and inoculated with both AMF and *Fusarium oxysporum*). The methods for the preparation of AMF and *Fusarium oxysporum* inocula were described previously (Chen et al. 2013, 2019). The experimental layout was a completely randomized design, with eight treatments and three replicates for each treatment, whereas each replicate contained six cucumber plants. The present study was carried out in a solar greenhouse and the average temperatures were 28°C/20°C (day/night) during the experimental period with a photosynthetic photon flux density (PPFD) of 600 µmol m⁻² s⁻¹ and relative humidity of 80%. Fifteen days after the inoculation with *Fusarium oxysporum*, six cucumber plants per treatment were randomly harvested and kept in an oven at 80°C for 72 h to measure the dry weights. Eventually, the experiment was terminated 15 days after the inoculation with *Fusarium oxysporum* by measuring the gas exchanging parameters followed by the collection of leaf/root samples (in case of leaf samples, the second fully expanded leaf was collected from each plant) for different analyses. The samples were immediately frozen in liquid nitrogen (unless otherwise stated) and stored at -80°C until further analyses.

Determination of mycorrhizal colonization rate, disease index, and control effect. The gridline intercept method was used to determine the mycorrhizal colonization rate on 35 days after inoculation with AMF as previously described (Chen et al. 2013; Giovannetti and Mosse 1980). Disease index was analyzed based on the indexation of root rot and vascular bundle browning, representing different disease rating scores as follows: healthy roots without any browning were given a value of 0, white roots with scarce browning (less than 5% of total root) were given a value of 1, roots with slight rot and browning (5 to 20% of total root) received a value of 2, mild root rot and browning (above 20 to 50% of total root) received a value of 3, and roots with severe rot and browning (above 50 to 100% of total root) were given a value of 4 (Chen et al. 2017a; Ding et al. 2014). Disease index (DI) was calculated according to the following formula: DI = [Σ(rating score × number of plants rated)/(total number of plants × highest rating score) × 100]. Subsequently, the control effect was estimated based on DI as follows: Control effect (%) = [(control DI – treatment DI)/control DI] × 100 (Chen et al. 2019).

Determination of gas exchange parameters in cucumber leaves. Fifteen days after the inoculation with *Fusarium oxysporum*, leaf gas exchange was measured in each of the second fully expanded leaf of six cucumber plants for each treatment between 8:00 a.m. and 11:00 a.m. Data of gas exchange parameters, such as net CO₂ assimilation rate (P_n), intercellular CO₂ concentration (C_i), stomatal conductance (G_s), and transpiration rate (T_r) were generated by a LI-6400 Portable Photosynthesis System, an open-flow infrared gas analyzer adapted with light and temperature

control systems (LI-6400; LICOR, Lincoln, NE, U.S.A.) for each leaf sample. The following conditions were maintained during gas exchange assay: 25°C of air temperature, 80 to 90% relative humidity, 400 $\mu\text{mol mol}^{-1}$ of CO_2 concentration, and 1,000 $\mu\text{mol m}^{-2}$ PPFD.

Determination of hydrogen peroxide, lipid peroxidation, cell viability, and electrolyte leakage. For the assay of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations, 0.5 g of leaf tissue was used as each sample unit collected from the second fully expanded leaf of each plant and pooled in triplicate from six plants. H_2O_2 content was assayed spectrophotometrically through a peroxidase assay as described by Willekens et al. (1997). In situ H_2O_2 accumulation was visualized through the histochemical staining of leaves with 3,3-diaminobenzidine (DAB) as previously described (Chen et al. 2013). The second fully expanded cucumber leaves of six plants for each treatment were used for DAB staining. MDA content in leaves was assayed as a marker of lipid peroxidation following the thiobarbituric acid (TBA) reaction as described elsewhere (Ahmed et al. 2019). Briefly, leaf tissues (0.5 g) were extracted in potassium phosphate buffer (50 mM). The homogenates were centrifuged for 20 min at 12,000 $\times g$. Then, 4 ml of 0.65% TBA in 20% TCA (trichloroacetic acid) solution was mixed and the mixture was heated in a hot water bath at 95°C for 30 min. Afterward, the tubes were quickly placed in an ice bucket to end the reaction. The samples were then centrifuged for 5 min at 10,000 $\times g$, and the absorbance of the red adduct was measured at 440, 532, and 600 nm with a spectrophotometer (SHIMADZU UV-2410PC, Shimadzu Co., Kyoto, Japan). MDA equivalents were quantified and expressed as MDA content per gram fresh weight (Ahmed et al. 2019). To assess the root cell viability, root tips (six root tips per treatment) were stained with fluorescein diacetate (FDA) and propidium iodide (PI), and the specific fluorescence from the stained roots was observed under a fluorescent microscope (Zeiss LSM510) as described previously (Chen et al. 2017a). For the assay of root activity, 0.5 g of fresh root sample was used as each sample unit in triplicate. Root samples were a mixture of samples from the six plants per treatment, which were cleaned with water and wiped with tissue paper before weighing. The root activity was determined according to the triphenyl tetrazolium chloride (TTC) method as previously described (Chen et al. 2017b). For the assay of electrolyte leakage (%), 0.3 g of fresh leaf sample of equal size was harvested as each sample unit in triplicate from different plants. Electrolyte leakage (%) was analyzed by measuring the electrical conductivity (EC) of leaf suspension. The first reading (EC1) was taken before autoclaving, whereas the second reading (EC2) was taken after autoclaving (Ahmed et al. 2019). The electrolyte leakage percentage was determined according to the following formula: Electrolyte leakage (%) = $100 \times \text{EC1}/\text{EC2}$.

Determination of antioxidant enzyme activity. To extract antioxidant enzymes, frozen leaf tissues (0.3 g sample in triplicate) were crushed with ice-cold HEPES buffer (3 ml, 25 mM, and pH 7.8) containing EDTA (0.2 mM) and insoluble polyvinylpyrrolidone (2%, wt/vol). The buffer was augmented with ascorbic acid (2 mM) for the extraction of ascorbate peroxidase (APX) only. The homogenates were centrifuged at 4°C for 20 min at 12,000 $\times g$. The supernatants were used for the analysis of the activity of antioxidant enzymes. The activity of superoxide dismutase (SOD) was analyzed based on its capacity to inhibit the photoreduction of nitro blue tetrazolium chloride (Giannopolitis and Ries 1977). The activity of catalase (CAT) and peroxidase (POD) was determined as described previously (Cakmak and Marschner 1992). The activity of APX was analyzed based on the H_2O_2 -dependent oxidation of ascorbic acid as described previously (Nakano and Asada 1981). The activity of antioxidant enzymes was presented on the basis of protein content, whereas the protein content was assessed using bovine serum albumin as the standard as described previously (Bradford 1976).

Assay of antioxidant and secondary metabolite content.

For the assay of GSH content, leaf samples (0.2 g sample in triplicate) were homogenized in 6% metaphosphoric acid (pH 2.8), and the homogenates were centrifuged for 15 min at 12,000 $\times g$. Then one half of the supernatant was used for the assay of the total glutathione (GSH + GSSG), while the other half was used for the quantification of the oxidized glutathione (GSSG). Notably, incubation of the supernatants for an hour with 2-vinylpyridine eventually blocked the reduced glutathione (GSH). Then following reagents such as 100 mM phosphate buffered saline (pH 7.5), 5 mM EDTA, 0.2 mM NADPH, 0.6 mM DTNB, and 3 U of glutathione reductase from yeast (Sigma, Santa Clara, CA, U.S.A.) were mixed to initiate the reaction and the absorbance was measured spectrophotometrically at 412 nm for 1 min. The GSH content was calculated by deducting the content of GSSG from total GSH (Rahman et al. 2006).

Proline content was measured based on sulfosalicylic acid colorimetry. In brief, leaf samples (0.5 g sample in triplicate) were homogenized in a 10-ml centrifuge tube, followed by the addition of 3% aqueous sulfosalicylic acid (5 ml). After keeping the tubes in boiling water for 10 min, an aliquot of the supernatant (2 ml) was mixed to initiate the reaction with the freshly prepared acidic ninhydrin (2 ml) and glacial acetic acid (2 ml) in a test tube and kept at 100°C for 30 min. Afterward, the tubes were immediately placed in an ice bath to terminate the reaction, and the mixture was then extracted with toluene (4 ml) and vortexed vigorously for 20 s. Then the chromophore-containing toluene was aspirated from the aqueous phase, and the absorbance was determined photometrically at 520 nm using toluene for a blank (Zhang et al. 2019). For the determination of total phenol content, a method based on Folin-Ciocalteu's reagent reduction was used (Ahmed et al. 2020). Total flavonoid concentration was analyzed spectrophotometrically as previously described. In brief, leaf samples were extracted in 70% ethanol (vol/vol, ethanol/water) at 100°C, and the absorbance of aqueous extract was recorded at 510 nm using AlCl_3 method. Rutin was used as the standard for the calculation of total flavonoid content (Chen et al. 2013).

Total RNA extraction and qRT-PCR assay. Total RNA was isolated from ~100 mg leaf sample in three biological replicates using a total RNA extraction kit according to the manufacturer's instruction (Tiangen, Shanghai, China). An aliquot (1 μg) of total RNA was subjected to reverse-transcription to generate cDNA using specific kits (ReverTra Ace qPCR RT Kit, Toyobo, Japan). For qRT-PCR assay, we used the SYBR Green PCR Master Mix (Takara, Tokyo, Japan). The qRT-PCR assay was performed with Light-Cycler 480 II Real-Time PCR Detection System (Roche, Basel, Swiss) under the default thermal cycling conditions with an added melting curve. The gene-specific primer pairs were designed for each gene and the *actin* was used as an internal control (Supplementary Table S1). The PCR was run at the following conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Relative transcript levels (mRNA abundance) were quantified according to the previously described method (Livak and Schmittgen 2001).

Statistical analysis. At least three replicates were performed for each determination, and the experiment itself was repeated three times independently. All data were subjected to one-way analysis of variance to test various treatment effects and analyzed with SPSS 20.0 statistical software package (SPSS, Chicago, IL, U.S.A.). For multiple mean comparisons, we used Tukey's least significant difference test at 5% level of significance.

RESULTS

Melatonin improves mycorrhizal colonization and enhances resistance to *Fusarium oxysporum*. After 35 days of AMF inoculation, the mycorrhizal colonization rate was 48.9%, which was remarkably increased by 39.45% with exogenous

melatonin application (Fig. 1A). However, the inoculation with *Fusarium oxysporum* (FO) significantly decreased the AMF colonization rate. The AMF colonization rate was decreased by 29.9% following *Fusarium* inoculation; however, melatonin application on AMF+FO-inoculated seedlings significantly increased the colonization rate by 45.3% compared with only AMF+FO (Fig. 1A). Dry weight analysis of cucumber seedlings showed that AMF colonization increased plant biomass by 26.8%, which was further significantly improved by exogenous melatonin application, although only melatonin treatment had no effect on plant biomass (Fig. 1B). However, *Fusarium* inoculation drastically decreased the dry weight by 48.9% compared with the control. Either AMF

colonization or exogenous melatonin treatment, both caused a significant increase in plant biomass in *Fusarium*-inoculated cucumber plants; however, the combined treatment of AMF and melatonin showed the greatest effect, which increased the dry weight by 65.3% compared with the dry weight of only *Fusarium*-inoculated plants. Comparison of disease index revealed that disease incidence was significantly decreased by either AMF or melatonin; however, the combined treatment of melatonin and AMF showed the highest effect. Consistent with the effect of AMF and melatonin on disease index, the control effect (%) significantly increased under combined treatment of melatonin and AMF followed by sole treatment of AMF and melatonin. The control

Fig. 1. Effect of melatonin (MT) on arbuscular mycorrhizal fungi (AMF) colonization as influenced by *Fusarium oxysporum* infection in cucumber seedlings. **A**, Mycorrhizal colonization rate (%), **B**, plant dry weight, **C**, disease index, and **D**, control effect. Cucumber seedlings were inoculated with AMF at 20 days prior to infection with *Fusarium oxysporum* (FO). Meanwhile, foliage was sprayed with melatonin (100 μ M) at 5-day intervals. Fifteen days after infection with FO, colonization rate, dry weight, disease index, and control effect were analyzed. Data are expressed as the mean, and the error bars represent standard deviation ($n = 6$). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

effect (%) was 45.5 and 146.2% higher in the combined treatment of melatonin and AMF than that in the sole treatment of AMF and melatonin, respectively.

Melatonin mitigates *Fusarium*-induced reduction in photosynthesis. Analysis of leaf gas exchange parameters showed that inoculation with *Fusarium* significantly decreased the Pn, Gs, Ci, and Tr by 38.5, 40.9, 24.7, and 36.6%, respectively, suggesting that the reduction in stomatal conductance in response to *Fusarium* inoculation might play a role in the attenuated CO₂ assimilation and transpiration (Fig. 2). Although individual treatment with either melatonin or AMF did not affect the photosynthesis rate, their combined treatment significantly improved the rate of CO₂ assimilation in absence of *Fusarium*. Meanwhile, either AMF inoculation or melatonin application on *Fusarium*-inoculated

plants significantly enhanced the Pn, Gs, Ci, and Tr compared with only *Fusarium* inoculation. Moreover, combined treatment of AMF and melatonin on *Fusarium*-inoculated plants further improved the Pn, Gs, and Tr, suggesting an additive role of AMF and melatonin in promoting leaf gas exchange under biotic stress induced by *Fusarium* inoculation in cucumber plants (Fig. 2).

Melatonin alleviates *Fusarium*-induced oxidative stress by stimulating the activity of antioxidant enzymes. *Fusarium* inoculation remarkably aggravated cell death as evidenced by intense red fluorescence in the root tip cells (Fig. 3A). However, cell damage greatly decreased when AMF and/or melatonin were applied alongside *Fusarium* inoculation, suggesting that either AMF or melatonin could protect cucumber roots from *Fusarium*-induced damage and their combined treatment showed a

Fig. 2. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on leaf gas exchange as affected by *Fusarium oxysporum* infection in cucumber seedlings. **A**, Net photosynthesis rate (Pn), **B**, stomatal conductance (Gs), **C**, the intercellular CO₂ concentration (Ci), and **D**, the transpiration rate (Tr). Cucumber seedlings were inoculated with AMF at 20 days prior to infection with *Fusarium oxysporum* (FO). Meanwhile, leaves were sprayed with melatonin (100 μM) at 5-day intervals. Fifteen days after infection with *Fusarium oxysporum*, leaf gas exchange parameters were analyzed. Data are expressed as the mean of six replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

better effect. In agreement with the root cell viability, the root activity decreased by 63.2% following *Fusarium* infection; however, AMF inoculation or melatonin application and their combined treatment on *Fusarium*-infected plants significantly increased the root activity by 70.9, 82.1, and 138.4%, respectively compared with only *Fusarium* infection (Fig. 3B). To unveil potential mechanisms

of *Fusarium*-induced damage, we visualized H_2O_2 accumulation by DAB staining and analyzed the levels of H_2O_2 , electrolyte leakage, and lipid peroxidation in cucumber leaves. *Fusarium* infection remarkably increased H_2O_2 accumulation as evidenced by deep brown color deposits in DAB-stained leaves (Fig. 4A). In addition, *Fusarium* inoculation significantly increased the H_2O_2 content,

Fig. 3. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on root cell viability and root activity as affected by *Fusarium oxysporum* (FO) infection in cucumber seedlings. **A**, Root cell viability, and **B**, root activity. Data for root activity are expressed as the mean of three replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

Fig. 4. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on reactive oxygen species accumulation and membrane stability as affected by *Fusarium oxysporum* (FO) infection in cucumber leaves. **A**, Hydrogen peroxide (H_2O_2) in situ visualization, **B**, H_2O_2 content, **C**, malondialdehyde (MDA) content, and **D**, relative electrolyte leakage (%) in cucumber leaves. Data are expressed as the mean of three replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

MDA content, and electrolyte leakage (%) by 160.1, 204.9, and 144.4%, respectively (Fig. 4B, C, and D). However, either melatonin application or AMF inoculation individually decreased the H_2O_2 accumulation, MDA content, and relative electrolyte leakage of *Fusarium*-stressed plants. Importantly, combined treatment with AMF and melatonin caused a further decrease in the levels of H_2O_2 , MDA, and electrolyte leakage (%), which were significantly lower than that of only AMF or melatonin treatment in *Fusarium*-inoculated plants. Although H_2O_2 content was slightly induced by either AMF or melatonin application in absence of *Fusarium*, MDA content was not altered, suggesting that H_2O_2 might function as a signaling molecule rather than an inducer of oxidative stress (Fig. 4C). We then compared the activity and transcripts of some key antioxidant enzymes under different treatments (Figs. 5 and 6). The results showed that *Fusarium* inoculation drastically decreased the activity of SOD and APX in cucumber leaves by 41.1 and 36.9%, respectively, compared with the control. However, the activity of POD and CAT was significantly elevated by 58.8 and 270.9%, respectively, with *Fusarium* inoculation. Both AMF and melatonin

applications individually increased the activity of SOD, CAT, POD, and APX in *Fusarium*-inoculated plants; however, their combined treatment resulted in a further promotion in the activity of antioxidant enzymes. Consistent with the activity of antioxidant enzymes, the expression of *Fe-SOD* and *cAPX* was suppressed by *Fusarium* inoculation, whereas the expression of *POD* and *CAT* was upregulated by *Fusarium* (Fig. 6). Importantly, either AMF colonization or exogenous melatonin application, both upregulated the transcript levels of *Fe-SOD*, *POD*, *CAT*, and *cAPX*, and their combined treatment showed a greater effect on those transcripts in *Fusarium*-infected plants, suggesting that AMF inoculation and melatonin application functioned synergistically to enhance the antioxidant enzyme activity in response to *Fusarium* infection.

Melatonin increases the accumulation of antioxidants and secondary metabolites under biotic stress caused by *Fusarium oxysporum*. To assess the role of nonenzymatic antioxidants, we analyzed the content of GSH and proline (Fig. 7). Although AMF and/or melatonin treatments significantly increased the content of GSH in absence of *Fusarium* infection,

Fig. 5. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on the activity of antioxidant enzymes as influenced by *Fusarium oxysporum* (FO) infection in cucumber seedlings. The activity of **A**, superoxide dismutase (SOD), **B**, peroxidase (POD), **C**, catalase (CAT), and **D**, ascorbate peroxidase (APX) in leaves. Data are expressed as the mean of three replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

none of those treatments affected proline content in a condition without *Fusarium*. Compared with the control, *Fusarium* inoculation significantly reduced the concentrations of GSH and proline by 31.9 and 37.4%, respectively (Fig. 7A-B). However, AMF inoculation or foliar application of melatonin on *Fusarium*-inoculated plants increased the GSH content, and their combined treatment showed an additive effect on GSH content, causing a 96.7% increase compared with the GSH content in only *Fusarium*-inoculated plants. Meanwhile, AMF inoculation and/or melatonin application on *Fusarium*-infected plants drastically increased the proline content by 110.4 to 120.7% compared with the *Fusarium*-inoculated cucumber plants, suggesting that combined treatment of AMF and proline did not show any additive effect on proline content under biotic stress with *Fusarium oxysporum* (Fig. 7B). We also

analyzed the phenols and flavonoid concentrations in cucumber leaves and found that *Fusarium* inoculation significantly increased their levels by 87.5 and 96.7%, respectively (Fig. 7C to D). Consistent with the GSH content, AMF or melatonin treatment significantly increased the phenol and flavonoid content, and the combined treatment of AMF and melatonin caused a further increase, suggesting an additive effect of AMF and melatonin on secondary metabolism under the *Fusarium* stress in cucumber plants.

DISCUSSION

The occurrence of both melatonin and AMF is universal across the plant kingdom. While melatonin occurs endogenously in plant

Fig. 6. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on the transcript levels of key genes encoding antioxidative enzymes as influenced by *Fusarium oxysporum* (FO) infection in cucumber seedlings. Relative expression levels of **A**, *Fe-superoxide dismutase* (*Fe-SOD*), **B**, *peroxidase* (*POD*), **C**, *catalase* (*CAT*), and **D**, *ascorbate peroxidase* (*cAPX*) in cucumber leaves. Data are expressed as the mean of three replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

tissues (Arnao and Hernandez-Ruiz 2018), AMF colonize plant roots and provide physiological protections, including enhanced resistance to multiple plant pathogens (Miozzi et al. 2019). However, the effect of melatonin on AMF-enhanced resistance to fungal pathogens remained elusive before this study. Here we found that melatonin positively influences AMF colonization, leading to enhanced resistance to *Fusarium oxysporum*, a devastating plant pathogen that causes significant losses in cucumber production worldwide (Zhang et al. 2016). We also found that melatonin and/or AMF could alleviate *Fusarium*-caused oxidative stress by improving the antioxidative defense and secondary metabolism, which possibly facilitated efficient CO₂ assimilation and biomass accumulation in cucumber plants under *Fusarium oxysporum* infection.

Fusarium oxysporum f. sp. *cucumerinum* typically infects cucumber seedlings via roots and stems; nonetheless, it prefers wounded roots for easy penetration to root tissues (Gordon 2017;

Zhang et al. 2016). Upon entry, the pathogen spreads quickly via vascular system, which eventually interrupts or totally blocks water and nutrient uptake, leading to wilting of aerial plant parts even in the abundance of water in rhizospheric soils (Gordon 2017; Wang et al. 2015). Moreover, plant pathogenic fungi significantly inhibit the photosynthetic process in plants (Li et al. 2019c; Sun et al. 2019). In the current study, *Fusarium* inoculation also drastically reduced the Pn and Tr, along with a significant reduction in Gs, suggesting that stomatal limitations were one of the causes of *Fusarium*-induced reduction in photosynthesis. These results are in agreement with a previous study in cucumber, in which infection with *Fusarium oxysporum* f. sp. *cucumerinum* significantly decreased Gs and Tr, and increased the damage to the leaf cell membranes (Wang et al. 2015). In this study, we also found significantly increased levels of H₂O₂, MDA, and electrolyte leakage in *Fusarium*-infected plants, suggesting potential injury to

Fig. 7. Effect of melatonin (MT) and arbuscular mycorrhizal fungi (AMF) on antioxidant and secondary metabolite content as influenced by *Fusarium oxysporum* (FO) infection in cucumber seedlings. **A**, glutathione (GSH) content, **B**, proline content, **C**, total phenol content, and **D**, flavonoid content in leaves. Data are expressed as the mean of three replicates (error bars represent standard deviation). Different lowercase letters on the histograms indicate the statistically significant differences at $P < 0.05$ according to Tukey's test.

leaf cell membranes and nonstomatal water loss (Wang et al. 2015). However, AMF and melatonin treatment improved the CO₂ assimilation rate possibly by improving Gs. Notably, melatonin can improve the activity of Calvin cycle enzymes, which may lead to an increase in photosynthetic carbon assimilation (Ding et al. 2017). In this study, consistent with the role of melatonin in ROS scavenging, the increased activity and transcript levels of antioxidant enzymes upon combined treatment of melatonin and AMF remarkably minimized the levels of H₂O₂, MDA, and electrolyte leakage, suggesting that melatonin and AMF mitigated the oxidative stress caused by *Fusarium oxysporum* (Sharma and Zheng 2019). These results are in accord with a recent report in cucumber, in which exogenous melatonin application increased antioxidant enzyme activity and decreased MDA content under biotic stress caused by *Pseudoperonospora cubensis* (Sun et al. 2019).

Melatonin functions as an antioxidant and it can reduce oxidative stress by enhancing ROS scavenging (Kanwar et al. 2018). In vitro assays show that melatonin can inhibit the growth of different fungal pathogens, such as *Fusarium* spp., *Phytophthora* spp., *Alternaria* spp., and *Botrytis* spp., suggesting its antifungal activity (Arnao and Hernandez-Ruiz 2018; Hernández-Ruiz and Arnao 2018; Mandal et al. 2018). Melatonin enhances plant resistance to fungal pathogens by inhibiting pathogen expansion, enhancing antioxidant defense and thus minimizing damage (Hernández-Ruiz and Arnao 2018). Moreover, melatonin improves resistance to *Fusarium* wilt in banana plants by increasing the expression of heat-shock protein 90s (Wei et al. 2017). Notably, AMF inoculation can also suppress fungal diseases in many plant species (Aseel et al. 2019; Oyewole et al. 2017). Consistent with this, in the present study, exogenous melatonin application or AMF inoculation remarkably increased the resistance of cucumber plants to *Fusarium* wilt as evidenced by decreased disease index and increased control effect. Notably, melatonin biosynthesis also occurs in endophytic

bacteria and beneficial soil fungi *Trichoderma* spp., which may function in host–microbe interaction as well as in resistance to plant pathogens (Arnao and Hernandez-Ruiz 2018). Therefore it is logical to speculate that exogenous melatonin might act as an inducer of plant resistance and function additively with AMF to increase resistance to *Fusarium* wilt in cucumber plants.

Plants produce a number of metabolites through secondary metabolic pathways, which function mostly in plant defense responses to biotic and abiotic stresses. Flavonoids and phenols are the major groups of secondary metabolites that accumulate in response to pathogen attacks. Here we found that the application of melatonin and/or inoculation with AMF increased the content of phenols and flavonoids, which might enhance defense against *Fusarium* wilt in cucumber. Previous studies revealed that AMF colonization could increase the accumulation of phenols and flavonoids in leaves (Chen et al. 2013). Moreover, proteomic analysis revealed that melatonin could also influence flavonoid biosynthesis (Arnao and Hernandez-Ruiz 2018). Since combined treatment of melatonin and AMF resulted in a greater accumulation of flavonoids than their sole treatment, it is highly possible that AMF and melatonin additively stimulated secondary metabolism in cucumber plants, leading to enhanced resistance to *Fusarium* wilt.

To sum up, we found that melatonin application increased the AMF colonization rate in cucumber roots and enhanced the control effect of AMF against *Fusarium oxysporum*. In addition, both melatonin and AMF promoted the photosynthetic capacity, antioxidant defense and secondary metabolite content in cucumber plants, which potentially functioned to enhance resistance against *Fusarium* wilt (Fig. 8). Importantly, combined treatment of melatonin and AMF showed an increased disease suppression compared with their sole treatment, suggesting that melatonin improves the AMF-enhanced plant defense against *Fusarium oxysporum* in cucumber. Since melatonin is considered a naturally

Fig. 8. An illustration showing the role of melatonin and arbuscular mycorrhizal fungi (AMF) in plant resistance to *Fusarium oxysporum* infection in cucumber plants. *Fusarium* infections induced oxidative stress by enhancing reactive oxygen species (ROS) accumulation, thereby reducing the cell viability and photosynthesis. Exogenous melatonin application and/or AMF inoculation suppressed *Fusarium*-induced deleterious effects on cucumber plants by strengthening the antioxidant potential and secondary metabolism, and combined treatment of melatonin and AMF showed a better effect than their sole treatment on controlling *Fusarium* wilt in cucumber plants. (The figure was created based on the data from the current study with Biorender.com and Adobe Photoshop 7.0 program, Adobe Systems, U.S.A.)

occurring safe compound, the combined application of melatonin and AMF could be an eco-friendly strategy for the effective management of wilt disease in cucurbits.

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