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Hydroxycoumarins: New, effective plant-derived compounds reduce *Ralstonia pseudosolanacearum* populations and control tobacco bacterial wilt

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ABSTRACT

Plant wilt disease caused by the soilborne bacterial pathogen *Ralstonia pseudosolanacearum* is one of the most devastating plant diseases; however, no effective protection against this disease has been developed. Coumarins are important natural plant-derived compounds with a wide range of bioactivities and extensive applications in medicine and agriculture. In the present study, three hydroxycoumarins (Hycs), umbelliferone (UM), esculetin (ES) and daphnetin (DA) significantly inhibited the growth of *R. pseudosolanacearum* on solid medium in a concentration-dependent manner, and the minimum inhibitory concentration (MICs) of these compounds was 325 mg L^{-1}, 125 mg L^{-1} and 75 mg L^{-1}, respectively. The percentage of live cells of *R. pseudosolanacearum* when supplemented with UM, ES, and DA was 63.61%, 17.81% and 7.23%, respectively, which were significantly lower than the DMSO treatment with 92%. Furthermore, irrigating roots with hydroxycoumarins (Hycs) 24 h before inoculation with *R. pseudosolanacearum* significantly delayed the occurrence of tobacco bacterial wilt, with the control efficiency of the DA treatment (the most efficient of Hycs treatment) 80.03%, 69.83%, 59.19%, 45.49%, 44.12%, 38.27% at 6, 8, 10, 12, 14, and 16 days after inoculation, respectively. Compared with the DMSO treatment, the pathogen populations of tobacco stems supplemented with 100 mg L^{-1} DA were the lowest, with population significantly reduced by 22.46%, 27.34%, and 18.06% at 4, 7, and 10 days after inoculation, respectively. Based on this study, these Hycs could be applied as potential protective agents in the management of tobacco bacterial wilt.

1. Introduction

Bacterial wilt is a lethal systemic vascular disease caused by *Ralstonia solanacearum*, one of the world's most important bacterial plant pathogens (Genin and Denny, 2012; Mansfield et al., 2012). This pathogen is a Gram-negative soil borne bacterium that can survive and persist in soils and water bodies for long periods and invades host plants through root openings (Álvarez et al., 2010). When the pathogen enters a host, growth is rapid and high cell densities ($> 10^{10}$ CFU/g stem) are reached in the stem, causing wilting and death of the host (Genin, 2010). Recent studies propose to separate *Ralstonia solanacearum* species complex into three species: *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotype I and II), and *R. syzygii* (phylotype IV) (Safni et al., 2014; Prior et al., 2016). The phylotype I strains reported in China comprise 15 sequevars and phylotype II strains are consisting of 2 sequevares (Xu et al., 2009; Liu et al., 2017a,b). Because of the aggressiveness, large host range of more than 450 plant species and broad geographical distribution, bacterial wilt is difficult to control and causes severe yield losses of many economically important crops such as tobacco, tomato, potato, pepper, banana and eggplant (Peeters et al., 2013; Yuliar et al., 2015).

Recently, many strategies have been developed to control bacterial wilt, and include biological, chemical, cultural, and integrated management approaches (Huet, 2014; Liu et al., 2016; Paret et al., 2010; Yuliar et al., 2015). However, the successes have been few because of the high survival capacity of *R. solanacearum* in a complex environment with pathogenicity factors that are complex (Peeters et al., 2013). The use of traditional chemical control methods with streptomycin and thiodiazole-copper for controlling bacterial wilt shows few positive effects in the field (Li et al., 2015a; Xu et al., 2012). Moreover, the frequent and excessive use of these chemicals often results in adverse environmental effects, such as inducing successive development of pesticide-resistant strains, disturbing the ecological balance of soils, and causing safety problems in the environment (Gutiérrez-Barranquero et al., 2013; Xu et al., 2010). The breeding of cultivars resistant to bacterial wilt is considered the most environmentally friendly, economical, and effective method of disease control. However, the resistance to bacterial wilt in many crops is negatively correlated

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with yield and quality (Yuliar et al., 2015). In addition to benefiting the maintenance of soil structure and organic matter, crop rotation can also contribute to pathogen control (Mbuthia et al., 2015), in contrast to continuous cropping with the same susceptible host plant, which can lead to the establishment of pathogenic populations of bacterial wilt, as frequently occurs in many regions cultivated of tobacco in China (Niu et al., 2017). Interest in biological control methods has increased because of concerns over the general use of chemicals (Whipps, 2001). Biological control agents (BCAs) and organic matter (plant residues, animal wastes and simple organic compounds) have potential for effective biological control of bacterial wilt (Yuliar et al., 2015). For biological control, the primary mechanism of action is likely antimicrobial activity, followed by indirect suppression of the pathogen by improving physical, chemical, and biological soil properties (Cardoso et al., 2006). Moreover, simple organic compounds such as amino acids, sugars, and organic acids can improve the soil microbial community structure such that more rapid death of the pathogen occurs (Posas and Toyota, 2010; Posas et al., 2007). However, plant bacterial wilt disease remains a challenging problem in agricultural crop protection. Therefore, new, effective and alternative strategies for the control of soilborne bacterial wilt are required immediately.

Previous studies show that many plant-derived compounds (PDCs), including lansiumamide B, methyl gallate, and protocatechualdehyde suppress the survival of the soilborne pathogen *R. solanacearum* through strong antibacterial activities (Li et al., 2014; Li et al., 2016; Yuan et al., 2012). Additionally, some studies reveal that PDCs inhibit the expression of virulence-associated factors, particularly the type III secretion system and biofilm formation (Khokhani et al., 2013; Li et al., 2015b; Lowe-Power et al., 2016; Yang et al., 2017). Because the sources are abundant and the compounds are environmentally friendly and not susceptible to bacteria developing resistance, PDCs are regarded as a potentially useful method for the control of bacterial wilt.

Coumarins and their derivatives are naturally occurring PDCs composed of fused benzene and α-pyrone rings, and the antimicrobial activity of many of these compounds on plant pathogenic bacteria and fungi has been extensively investigated (Céspedes et al., 2006; Gnonlonfin et al., 2012). Our previous studies show that coumarin significantly inhibits the propagation of *R. pseudosolanacearum* and demonstrates a bactericidal effect in vitro and in vivo (Chen et al., 2016). Coumarins demonstrate strong antibacterial activity against *R. pseudosolanacearum*, and hydroxylation at the C-6, C-7 or C-8 position significantly increases the antibacterial activity (Yang et al., 2016). However, little information is available on the effects of Hycs on the control of bacterial wilt in vivo and in vitro. Therefore, to evaluating the control efficiency of these compounds for tobacco bacterial wilt and to identify optimal concentrations and method of application, the effect of Hycs on suppressing the survival of *R. pseudosolanacearum* must be investigated.

The objectives of the current study were to evaluate the effects of Hycs on population of the plant pathogen *R. pseudosolanacearum* and tobacco bacterial wilt. We used culture amendment assays to investigate the antibacterial activity of three Hycs (UM, ES, and DA) against *R. pseudosolanacearum*. We also used epifluorescence microscopy to evaluate the percentage survival of *R. pseudosolanacearum* supplemented with these Hycs. Furthermore, the control efficiency of these compounds on tobacco bacterial wilt and the suppression of *R. pseudosolanacearum* populations in tobacco were investigated.

2. Materials and methods

2.1. Materials and bacterial strain

The plant pathogen *R. pseudosolanacearum* CQPS-1 (phylotype I, race 1, biovar 3) was used in this study (accession number NZ_CP016914.1). The wild-type strain was collected by the Laboratory of Natural Products Pesticides, and was originally isolated from an infected tobacco plant in Chongqing, China (Liu et al., 2017a,b). The pathogen *R. pseudosolanacearum* was grown at 30 °C in rich B medium (10 g/L bactopeptone, 1 g/L yeast extract and 1 g/L casamino acids).

The Hycs (HPLC ≥ 98%) used in the study were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China) and Nanjing Chunqiu Bio-Technology Co., Ltd. (Nanjing, China). The compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare a final concentration of 100 mg mL^{-1}, and this compound solvent was also added to the rich B or B agar medium to prepare different concentrations.

2.2. Antibacterial activity of the Hycs against R. pseudosolanacearum in solid medium

The culture amendment assay used to investigate the antibacterial activity of Hycs against *R. pseudosolanacearum* followed a description in a previous study with minor modifications (Paret et al., 2010). Briefly, an overnight-cultured suspension of *R. pseudosolanacearum* (10^8–10^9 CFU per mL) was used to initiate a 10-fold dilution series (10^{-1}–10^{-6}), and 100 µL of each dilution suspension was spread on agar plates supplemented with a final concentration of 25, 50 and 100 mg L^{-1} Hycs. Then, the populations of *R. pseudosolanacearum* were assessed after incubation at 30 °C for 48 h. Each plate contained ten milliliters of medium, and plates with 0.1% DMSO were used as controls. Each treatment had triplicate plates, and all assays were conducted with two biological replications.

2.3. Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of the Hycs on *R. pseudosolanacearum* was determined using the agar dilution method with a series of final concentrations ranging from 25 to 425 mg L^{-1}, as previously described with minor modifications (Li et al., 2014). Briefly, 100 µL of overnight-cultured bacterial suspension (10^8 to 10^9 CFU per mL) was spread directly on each antibiotic-containing agar dilution plate. The agar plates were incubated at 30 °C for 48 h, and MIC was defined as the lowest concentration at which no visible growth occurred of the pathogen *R. pseudosolanacearum*. All assays were performed at least in triplicate.

2.4. Effect of the Hycs on surviving percentage of R. pseudosolanacearum using fluorescence microscopy

The percentage of live and dead *R. pseudosolanacearum* cells was determined as described previously (Paret et al., 2010). A live-dead BacLight bacterial viability kit (L13152) was used in this assay, which contains Syto9 green fluorescent stain and propidium iodide red fluorescent stain. Briefly, an overnight-cultured suspension of *R. pseudosolanacearum* (10^8–10^9 CFU per mL) was added to fresh B medium and adjusted to an OD_{600} = 0.4. Ten milliliters of the adjusted bacterial suspension was mixed with 10 µL of the Hycs to give a final concentration of 100 mg L^{-1}, and then the mixture was incubated at 30 °C for 4 h with shaking at 180 rpm/min. After harvest by centrifugation in a microcentrifuge at 8000 rpm for 5 min, the cells were resuspended in 1 mL of 0.85% NaCl. Ten microliters of treated sample was added into 96-well microtiter plates and incubated for 45 min. The cells were stained with 10 µL of the mixed Syto9 stains (6 µM) and propidium iodide (30 µM) for 15 min in the dark. Control cell samples were treated with 0.1% (vol/vol) DMSO. Five microliters of the stained bacterial suspension was placed between a glass slide and a 10-mm coverslip, and then cells were observed with an inverted fluorescence microscope (Axio Observer D1; Zeiss, Jena, Germany). Each treatment was analyzed in triplicate. Syto9 stains cells with intact (live) and damaged (dead) membranes, whereas propidium iodide only strains cells with damaged membranes. The percentage of live cells were calculated according to the following equation:

$$\text{The percentage of live cells} = \frac{Fcell,\ green - Fcell,\ red}{Fcell,\ green} \times 100$$

Where $F_{cell,green}$ = the number of bacterial cells strained by Syto9, $F_{cell,red}$ = the number of bacterial cells strained by propidium iodide.

2.5. Control efficiency of the Hycs on tobacco bacterial wilt under greenhouse conditions

The naturalistic soil soak assay was used to evaluate the control efficiency of the Hycs on tobacco bacterial wilt as described in a previous study with minor modifications (Wu et al., 2015). Briefly, unwounded, six-week-old tobacco plants (Yunyan 87) were irrigated with 15 mL Hycs and thiodiazole copper to make a final concentration of 100 mg g^{-1} soil, same volume with 0.1% DMSO was used as negative control. After irrigation for 24 h, individual plants were inoculated by pouring 15 mL of bacterial suspension into the soil to create a final inoculation density of 1×10^7 CFU g^{-1} soil. In an alternative application method, plants were first inoculated with *R. pseudosolanacearum* (final density of 1×10^8 CFU/g soil), and then 24 h after inoculation, each plant was irrigated with DMSO or 100 mg L^{-1} Hycs treatment. Inoculated plants were placed in the climate room at 28 °C with a 14/10 h light/dark cycle. The symptoms of each plant were scored daily using a disease index scale from 0 to 4 (0, no symptoms appeared; 1, 1–25% of leaves wilted; 2, 26–50% of leaves wilted; 3, 51–75% of leaves wilted; 4 indicated 76–100% of leaves wilted). Individual treatments contained 16 plants for each independent experiment, and the assay was repeated three times. To determine disease index and the control efficiency, we used the following formula:

$$\text{Disease index} = \frac{\sum (ni \times vi)}{N \times 4} \times 100$$

Where ni = the number of plants with respective disease index, vi = disease index (0, 1, 2, 3, 4), and N = the total number of plants used in each treatment.

$$\text{Control efficiency} = \frac{CK - T}{CK} \times 100$$

Where T = the disease index of treatment, CK = the disease index of control group.

To determine *R. pseudosolanacearum* populations in plant stems, 200 mg of tissue was destructively harvested at the base of stems 4, 7, and 10 d after inoculation with *R. pseudosolanacearum*. The tissues were disinfected in 75% alcohol for 1 min, rinsed twice with sterile water and transferred into a 2.5 mL sterile centrifuge tube, grinded with sterile glass beads on MP Biomedicals FastPrep (FastPrep-24™, M. P. Biomedicals, Santa Ana, California, USA). The tissue was diluted in sterile water from 10^{-1} to 10^{-4}, and 100 µL of stem tissue suspension dilution was plated on SMSA medium to quantify CFUs. Each treatment included 16 plants, and the entire experiment was performed three times. The semi-selective SMSA medium used in the assay was previously described (Elphinstone et al., 1996). Water-inoculation by plants was used to quantify bacterial density in root tissue of tobacco supplemented with DMSO or the Hycs (Yang et al., 2017). Briefly, we chose 4-week-old tobacco plants (Yunyan 87), cleared the medium and placed the tobacco root into 0.1% DMSO or 100 mg L^{-1} Hycs for 5 min. The plants were then placed into 50 mL triangular bottles filled with 30 mL of MS medium. After 24 h, each plant was inoculated with 150 µL of *R. pseudosolanacearum* (10^8 CFU per mL) to make the final inoculation density of 5×10^5 CFU mL^{-1}. Four days after inoculation, the root tissue of tobacco was collected and weighed, then the roots were disinfected in 75% alcohol for 1 min, rinsed twice with sterile water and transferred into a 2.5 mL sterile centrifuge tube, grinded with sterile glass beads on MP Biomedicals FastPrep (FastPrep-24™, M. P. Biomedicals, Santa Ana, California, USA). The tissue was diluted in sterile water from 10^{-1} to 10^{-2}, and bacteria quantified by dilution tissue suspension on SMSA medium.

2.6. Assessment of the effect of the Hycs on tobacco growth

A pot experiment was used to investigate the effect of the three Hycs on tobacco growth as described in a previous study with minor modifications (Lakshman, 2010). Fourteen days after the soil irrigated with 15 mL 0.1% DMSO or Hycs, the final concentration of Hycs was 100 mg per gram of soil, we measured the fresh weight and height of tobacco above ground, and the length of roots. Then, the plant dry weight was measured after drying at 100 °C in an incubator for 2 h. Individual treatments contained 16 plants for each independent experiment, and the assay was repeated twice.

2.7. Statistical analyses

The data were analyzed with the SPSS 17.0 statistical software program (SPSS Inc. Chicago, IL) using ANOVA and Student's *t*-test under the significance level of 0.05 (P-value = 0.05).

3. Results

3.1. Antibacterial activity of the Hycs against R. pseudosolanacearum on agar plates

Antibacterial activity of three Hycs (UM, EA, and DA) on tobacco *R. pseudosolanacearum* was tested with culture amendment assay. The results showed that *R. pseudosolanacearum* did not grow on plates supplemented with ES at 100 mg L^{-1} or DA at 50 and 100 mg L^{-1}. Moreover, the population of *R. pseudosolanacearum* was significantly lower on plates supplemented with UM at 100 mg·L^{-1} and DA at 25 mg L^{-1} (ca. 6 log CFU mL^{-1}) than that on controls plates (ca. 9 log CFU mL^{-1}) (Fig. 1). The results demonstrated a concentration-dependent effect of the antibacterial activity of these Hycs against *R. pseudosolanacearum* on plates.

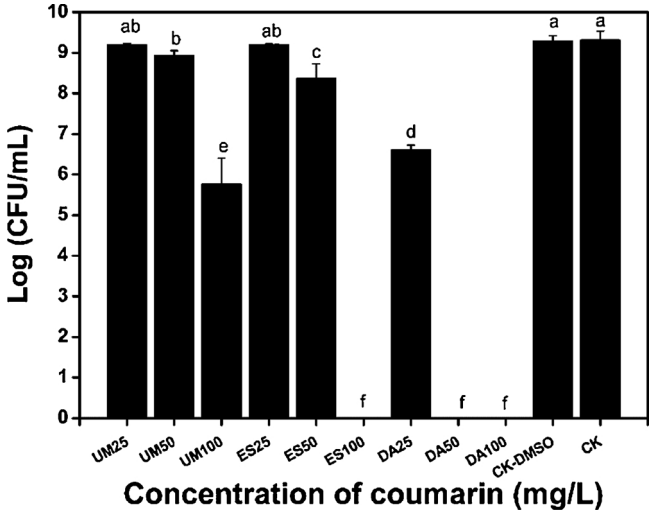

Fig. 1. The antibacterial activity of three Hycs against *R. pseudosolanacearum* in solid medium. An overnight-cultured suspension of *R. pseudosolanacearum* (10^8–10^9 CFU per mL) was used to initiate a 10-fold dilution series (10^{-1}–10^{-6}), and 100 µL of each dilution suspension was spread on agar plates supplemented with a final concentration of 25, 50 and 100 mg L^{-1} Hycs. DMSO used as the positive control, and CK was the untreated treatment. Data shown are the means of independent experiments with a least three replicates. Error bars indicate the standard deviation and different letters indicate significant differences between Hycs treatment and the DMSO treatment ($P < 0.05$, Duncan's test).

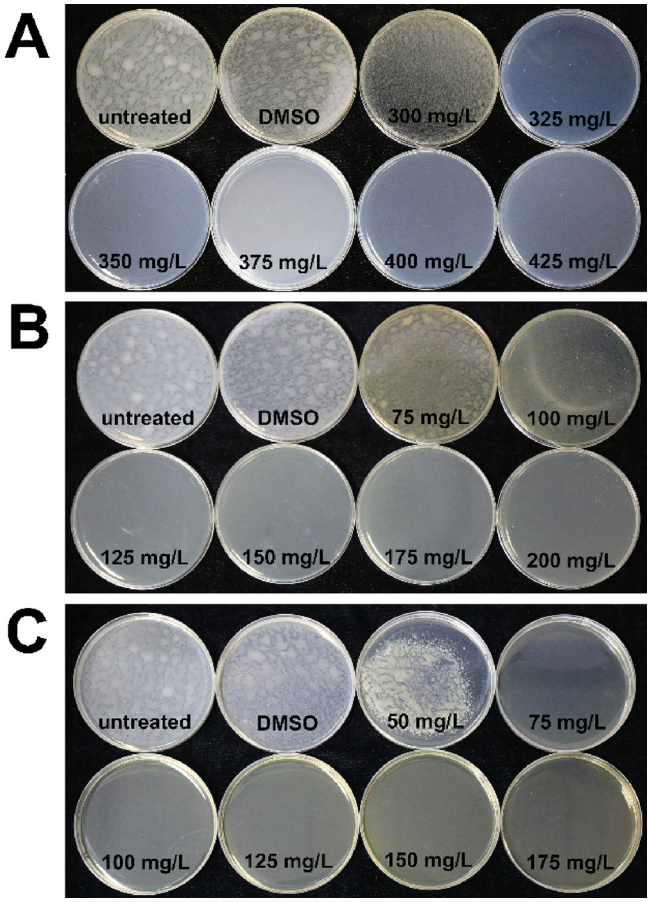

Fig. 2. The minimum inhibitory concentration (MIC) of Hycs on *R. pseudosolanacearum* was determined using the agar dilution method treated with the following: (A) UM at concentrations ranging from 300 to 425 mg L^{-1}; (B) ES at concentrations ranging from 75 to 200 mg L^{-1}; and (C) DA at concentrations ranging from 50 to 175 mg L^{-1}.

3.2. Minimum inhibitory concentration (MIC) of the Hycs on R. pseudosolanacearum

To further determine the antibacterial activity of the three Hycs on *R. pseudosolanacearum*, the minimum inhibitory concentration (MIC) was measured using the serial two-fold agar dilution method. As shown in Fig. 2, the MIC values for UM, ES, and DA were 325 mg L^{-1}, 125 mg L^{-1} and 75 mg L^{-1}, respectively. The Hycs completely inhibited the growth of *R. pseudosolanacearum* at these concentrations.

3.3. Effect of the Hycs on the percentage of live cells of R. pseudosolanacearum

A live-dead BacLight bacterial viability kit (L13152) was used to stain the live and dead cells of *R. pseudosolanacearum*. The results showed that 92% of *R. pseudosolanacearum* cells supplemented with DMSO (control) were stained green, indicating a high percentage of live cells after 4 h of incubation (Fig. 3). However, in the UM, ES and DA treatments, the percentage of live cells was 63.61%, 17.81% and 7.23%, respectively, which were significantly lower than that of the control (Fig. 3**B**). Compared with controls, the larger masses of cellular debris that were observed in ES and DA treatments, suggested that cell membranes were completely damaged and ruptured because of the treatment (Fig. 3**A**).

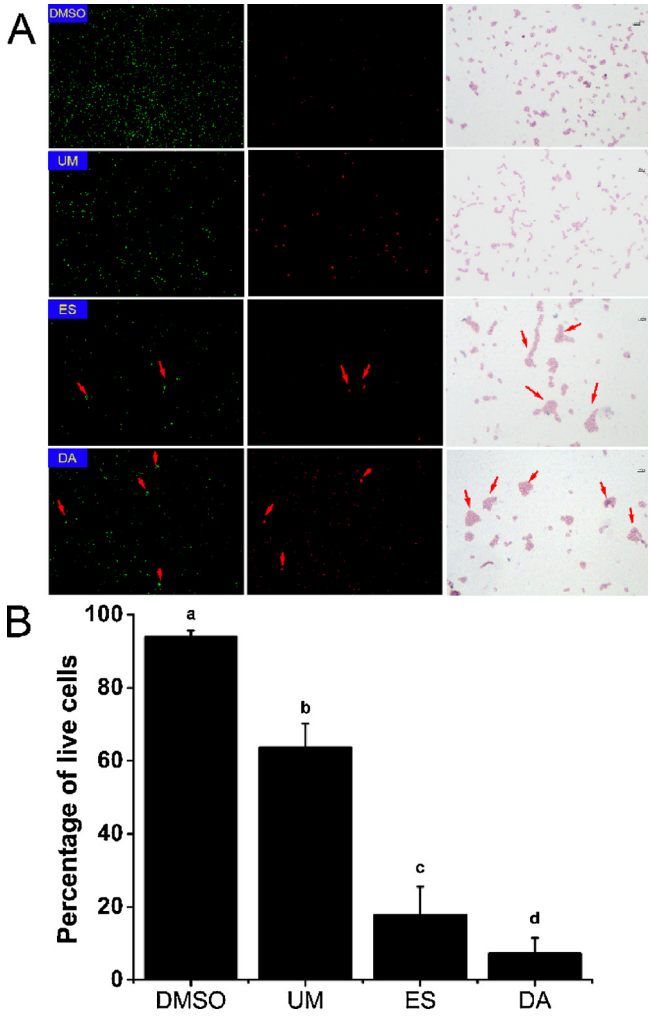

Fig. 3. The antibacterial activity of three Hycs against *R. pseudosolanacearum* determined using epifluorescence microscopy after supplemented with DMSO (control) and Hycs at 100 mg L^{-1} (UM, ES and DA). **(A)** The green and red stained cells indicate live and dead cells, respectively. **(B)** The percentage of live cells supplemented with Hycs after incubation for 4 h. Error bars indicate the standard deviation and different letters indicate significant differences between Hycs treatment and the DMSO treatment ($P < 0.05$, Duncan's test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Control effect of the Hycs on bacterial wilt in tobacco inoculated with R. pseudosolanacearum

Based on the strong antibacterial activity of the three Hycs at a concentration of 100 mg L^{-1} against *R. pseudosolanacearum*, the control effect of the Hycs (UM, ES, DA at 100 mg L^{-1}) on the progression of tobacco bacterial wilt disease in pot experiments was evaluated. As shown in Fig. 4, we examined a treatment with irrigated roots with Hycs 24 h before inoculation with *R. pseudosolanacearum*. The Hycs significantly altered the disease index of bacterial wilt. As shown in Fig. 4A, compared with DMSO, the Hycs significantly suppressed the disease index, particularly the DA treatment accelerated the wilting of inoculated plant [$P < 0.05$; repeated-measures analysis of variance (ANOVA)]. Further, DA treatment at 100 mg L^{-1} with control efficiency of 80.03%, 69.83%, 59.19%, 45.49%, 44.12% and 38.27% at 6, 8, 10, 12, 14, and 16 days after inoculation, respectively. For comparison, the control efficiency of the positive control thiodiazole-copper treatment was 38.36%, 4.89%, 15.86%, 17.92%, 17.06%, and 15.44%, which were values significantly lower than those of the DA treatment. The

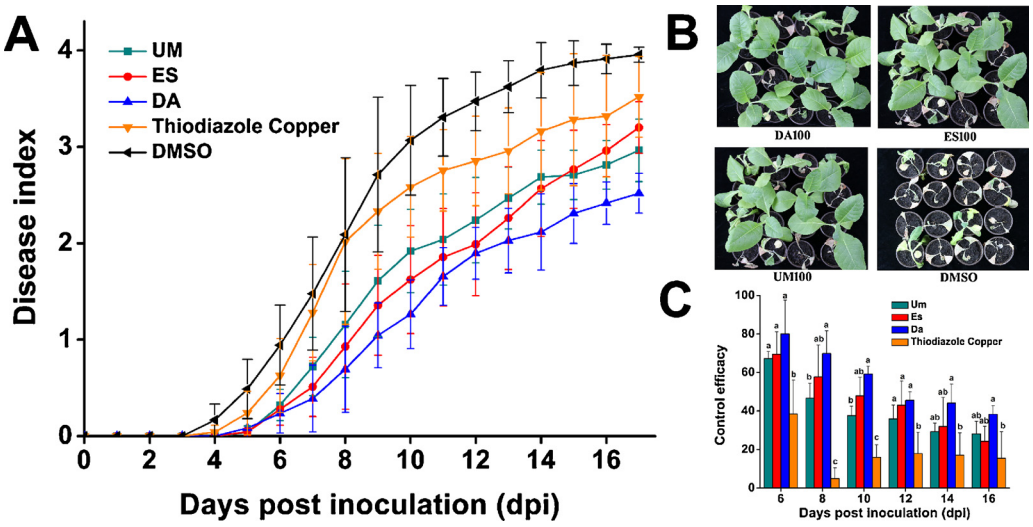

Fig. 4. The effect of Hycs on control of tobacco bacterial wilt applied 24 h before inoculation with *R. pseudosolanacearum*. **(A)** The disease index of tobacco wilt supplemented with UM, ES, DA at 100 mg L^{-1} with DMSO the negative control, and thiodiazole copper treatment with 100 mg L^{-1} was used as positive control. Error bars indicate the standard deviation. **(B)** The occurrence of tobacco bacterial wilt supplemented with DMSO or Hycs 16 d after inoculation. **(C)** The control effect of the Hycs on tobacco bacterial wilt. These results reflect three biological replicates and error bars indicate the standard deviation. Different letters indicate significant differences between Hycs treatment and the thiodiazole copper treatment ($P < 0.05$, Duncan's test).

control efficiency of ES treatment were 69.42%, 57.65%, 47.78%, 42.98%, 31.93%, 24.21% at 6, 8, 10, 12, 14, and 16 days after inoculation, respectively. Another Hycs treatment UM at 100 mg L^{-1} with control efficiency of 67.14%, 46.55%, 37.58%, 35.88%, 29.25% and 28.03% (Fig. 4**C**). Compared with DMSO, the Hycs at 100 mg L^{-1} significantly altered the disease progress of bacterial wilt ($P < 0.05$); in particular, irrigate roots with the DA 24 h after inoculation had a very significant effect on suppression on bacterial wilting disease. The control efficiency of the DA treatment on tobacco bacterial wilt was 63.13%, 47.05%, 33.24%, 31.58%, and 27.71% (Fig. S**1**).

As shown in **table S1**, tobacco plants in the 100 mg L^{-1} Hycs treatment had similar root length, number of leaves and dry weight to those of the control treatment in *R. pseudosolanacearum*-free soil. However, tobacco plants pre-treated with Hycs at 100 mg L^{-1} were significantly taller and produced more fresh plant weight than those of the control treatment.

3.5. Hycs treatments reduced bacterial density of R. pseudosolanacearum in the roots and base stems of tobacco

As shown in Fig. 5, Hycs treatment significantly reduced *R. pseudosolanacearum* populations in tobacco roots in a concentration-dependent manner after incubation following four dips in the water inoculation assay (Fig. 5**A**), Additionally, the Hycs at 100 mg L^{-1} significantly reduced the populations at the base of tobacco plant stems. In the DMSO treatment, bacterial populations of *R. pseudosolanacearum* tended to increase in tobacco stems following soil-soak inoculation (Fig. 5**B**). Compared with the DMSO treatment, the pathogen populations of tobacco stems supplemented with DA at 100 mg L^{-1} were the lowest and were significantly reduced by 22.46%, 27.34%, and 18.06% at 4, 7, and 10 days after inoculation, respectively. The tobacco stem colonization of ES treatment were 7.6×10^3 CFU per g stem, 9.5×10^5 CFU per g stem, 1.7×10^6 CFU per g stem at 4, 7, and 10 days after inoculation, respectively. The pathogen populations in tobacco stems of UM treatment were 6.8×10^3 CFU per g stem, 1.6×10^5 CFU per g stem, 4.4×10^6 CFU per g stem at 4, 7, and 10 days after inoculation, respectively. Which were significantly lower than the DMSO treatment with 4.6×10^4 CFU per g stem, 3.5×10^6 CFU per g stem, 1.9×10^7 CFU per g stem at 4, 7, and 10 days after inoculation, respectively.

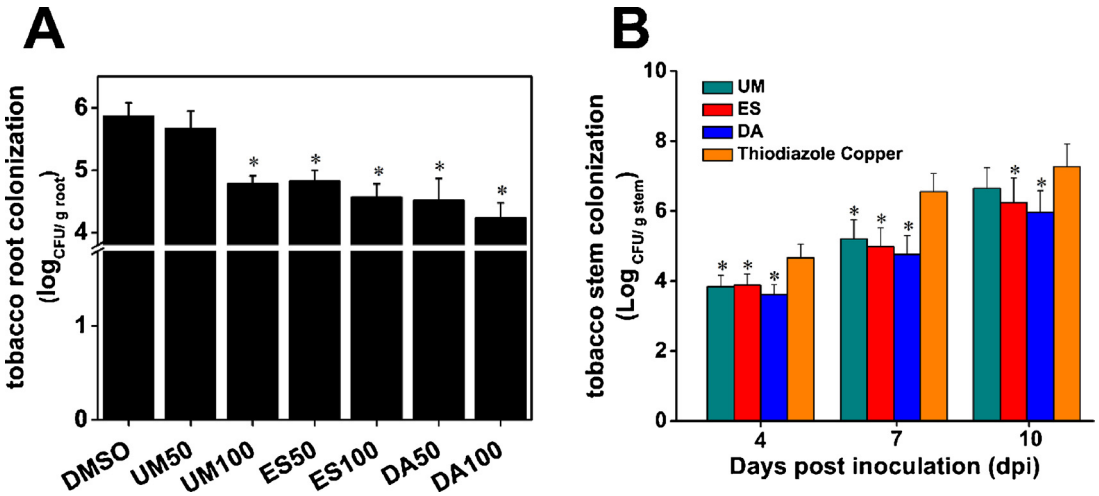

Fig. 5. Hycs treatments reduced bacterial populations of *R. pseudosolanacearum* in the roots and stems of tobacco. **(A)** Bacterial density in root tissue was quantified by dilution plating roots from water-inoculated plants. Error bars indicate the standard deviation and asterisks indicate $P < 0.05$ (Student's *t*-test). **(B)** *R. pseudosolanacearum* population in the stem was quantified by dilution tissue suspensions from 10^{-1} to 10^{-4}, with 100 µL of stem tissue suspension on SMSA medium. Error bars indicate the standard deviation and asterisks indicate significant differences between Hycs treatment and the thiodiazole copper treatment ($P < 0.05$, Student's *t*-test).

4. Discussion

Tobacco bacterial wilt widely occurs in 14 of the 22 primary tobacco-growing regions in China and has caused at least $11.56 million in economic losses in the most recent 3 years (Jiang et al., 2017; Liu et al., 2017a,b). The traditional chemical control methods using streptomycin and thiodiazole-copper for controlling bacterial wilt show few positive effects in the field (Li et al., 2015a). As a landmark discovery in the study, *R. pseudosolanacearum* was impair by certain plant-derived compounds (Guo et al., 2016; Paret et al., 2010). In the present study, it is found that Hycs (UM, ES, and DA) are effective antibacterial compounds against *R. pseudosolanacearum*. Greenhouse experiments demonstrated that irrigating roots with the Hycs 24 h before inoculation with *R. pseudosolanacearum* provided significant control of tobacco bacterial wilt, particularly DA treatment with concentration of 100 mg L^{-1}. It would develop as a new type of plant-type antibacterial agents, and fulfilled its structural modification of DA as a lead compound.

Hydroxycoumarins (Hycs) are a large group of natural plant-derived compounds (PDCs) composed of fused benzene and α-pyrone rings, and because of their wide range of biological activities, including antibacterial, anticancer, antifungal and anti-inflammatory properties, have been the focus of extensive research (Barot et al., 2015; Grover and Jachak, 2015). However, few studies have focused on the antibacterial activity of Hycs and the innovative application of this property in agriculture. Our results proved that the MICs of UM, ES, and DA was 325 mg L^{-1}, 125 mg L^{-1} and 75 mg L^{-1}, respectively. Which were consistent with hydroxylation at the C-6, C-7 or C-8 position significantly increased the antibacterial activity of hydroxycoumarins against the soilborne pathogen *R. pseudosolanacearum* (Yang et al., 2016).

According to some research, the cell membrane is the primary target for the antimicrobial activity of most phytochemicals (Chen et al., 2016; Fan et al., 2014). These compounds destabilize the membrane structure through interaction with the lipid bilayer of the cytoplasmic membrane that increase the space between fatty acid chains (Paret et al., 2012; Setzer et al., 2016). Recently, Hycs, as a naturally plant derived phenolic compounds with antibacterial activity, were considered for use against medical pathogens, such as *Escherichia coli* and *methicillin-resistant Staphylococcus aureus* (Lee et al., 2014; Zuo et al., 2016). Based on the fluorescence microscopy results, these Hycs might destroy the cell membrane of *R. pseudosolanacearum*, causing the death of pathogen bacteria. Consistent with these results, in previous studies, coumarin damaged bacterial cell membranes and prevented swarming motility and biofilm formation of *R. pseudosolanacearum*, with hydroxycoumarins also destroying the cell membranes and inhibiting biofilm formation (Chen et al., 2016; Yang et al., 2016).

The formation and accumulation of coumarins are induced in plant following various stresses or chemical treatment (Prats et al., 2006; Prats et al., 2002). These compounds are identified as phenylpropanoids, which based on modifications of the aromatic amino acid phenylalanine, are derived from the shikimate pathway (Grosskinsky et al., 2012). Recently, studies demonstrated that phytoalexins can be induced by several pathogens in many different plant species, and the hydroxycoumarin scopoletin is considered one of the most important phytoalexins in tobacco to be induced in response to tobacco mosaic virus (Costet et al., 2002; Schmelz et al., 2011). The control effect of irrigated roots with Hycs 24 h before inoculation with *R. pseudosolanacearum* was much better than that of the other application method, which was to irrigate roots with the Hycs 24 h after inoculation with *R. pseudosolanacearum*. Furthermore, we found that these Hycs significantly reduced bacterial populations of *R. pseudosolanacearum* in the roots and stems of tobacco. *R. pseudosolanacearum* invades a host from soil through root openings and then colonizes the root cortex from which the bacteria rapidly reaches the xylem vessels and proliferates to high cell densities ($> 10^9$ CFU/g stem) (González and Allen, 2003). Which indicated the Hycs inhibited the ability of *R. pseudosolanacearum* to adhere to and colonize tobacco roots. In this scenario, Hycs arrive at the tobacco plant and may directly adsorb to the root, and then Hycs alter the colonization, biofilm formation and architecture of *R. pseudosolanacearum* by destroying cell membranes and causing the death of pathogen bacteria. However, when *R. pseudosolanacearum* invades the host through roots and colonizes the root cortex, the pathogen then rapidly reaches the xylem vessels in which growth leads to high cell densities, causing tobacco wilt. Therefore, the most effective application for Hycs against tobacco bacterial wilt is to irrigate or dip roots before tobacco transplant.

In summary, the three Hycs (UM, ES, and DA) have high antibacterial activity and application prospects as plant-derived antibacterial agents in the management of tobacco bacterial wilt. This study was a systematic report on the antibacterial activity of Hycs against the soilborne bacteria *R. pseudosolanacearum*, and a reasonable application method for the control of tobacco bacterial wilt was determined. However, we acknowledge that this study was conclude from laboratory tests, the effect of Hycs application in tobacco field and the control effect of Hycs on tobacco bacterial wilt in different geographic areas, different climates need to be performed in further research. In addition, further studies should be focused the mechanisms of antibacterial activity on *R. pseudosolanacearum* and the structural modification to synthesis of Hycs analogs to analysis the quantitative structure-activity relationship.

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Author contributions

Conceived and designed the experiments: WD, LY. Performed the experiments: LY, LW, XY, JW, SZ. Analyzed the data: LY, SL. Wrote the paper: WD LY.

Competing interests

The authors declare no competing interests.

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