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Morphological and Lethal Effects of D-Leu on the *Xanthomonas citri* subsp. *citri*

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D-amino acids exist in many bacteria, fungi and insects, but little investigation into their function has been performed. In this study, we determined the antibacterial effect of D-Leu on *Xanthomonas citri* subsp. *citri* (*Xcc*), a plant pathogen that is extremely detrimental to the production of Citrus fruit. Our results showed that 2 mM D-Leu morphologically altered *Xcc* strain 101 from rod to chain form and 7 mM D-Leu caused *Xcc* strain 101 to lose pathogenicity and/or die. Electro-microscopic examination revealed that the bacterial chain was formed by many bacteria own some cell membrane, and the cellular membrane broken and protoplasm prolapse out. This discovery has the potential to provide a new preventative strategy against bacterial diseases of plants and to screen for biocontrol bacteria.

Key words: D-amino acids; plant pathogenic bacteria; morphological change; killing effect;*Xanthomonas citri* subsp. *citri*

For prokaryotic bacteria, the cell wall is the main protective structure against an adverse external environment. The bacterial cell wall contains peptidoglycan, which can contain some D-amino acids (D-aas) including D-glutamic acid (D-Glu) and D-alanine (D-Ala)[25]. Over 60 years ago, several researchers discovered that high concentrations of exogenous D-aas could inhibit the growth of some bacteria, such as *Escherichia coli*,*Caulobacter crescentus*, *Lactobacilli*[3, 30] and *Agrobacterium tumefaciens*[3, 33]. Moreover, high concentrations of exogenous D-cysteine (D-Cys) and D-methionine (D-Met) can be incorporated into the peptidoglycan of *E. coli*[6, 7]; and D-Met and D-leucine (D-Leu) can be substituted for D-Ala and D-Glu in the bacterial peptidoglycan[15, 22, 26]. Many Gram-positive (Gram$^{+)}$ bacteria, such as *Bacillus subtilis*, can also synthesize and release different types of D-aas with concentrations reaching millimolar levels[21]. Such physiological concentrations of D-aas can result in a reshaping of the bacterial cell wall and the degradation of bacterial biofilms[20]. Since *B. subtilis* is able to secrete a variety of antimicrobial substances, it is widely used as a biological control strain for the prevention of an assortment of plant bacterial diseases. *B. subtilis* can also secrete D-aas, including D-Leucine (D-Leu), D-phenylalanine (D-Phe), D-valine (D-Val) and some other D-aas[21]. However, whether or not low concentrations of D-aas affect plant pathogenic bacterial competition is still unknown. Interspecific competition between microorganisms is a common phenomenon in nature. Different

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organisms secrete various substances to compete for nutrients and space; however, it has not been determined whether low concentrations of D-aas (below 3 mM) can affect other plant pathogenic bacteria.

Xanthomonas and *Pseudomonas*, which include more than 250 pathogenic species and pathogenic variants, are the two main types of Gram-negative ($Gram^-$) plant pathogenic bacteria that can cause huge economic losses in agriculture. Of all the agricultural pests and diseases that threaten citrus crops, citrus bacterial canker disease (CBCD) caused by the bacterium *X. citri* subsp. *citri*, is one of the most devastating. More than 30 of the world's citrus-producing countries or regions have suffered from CBCD, including China. Severe CBCD infection can result in a range of negative effects including defoliation, dieback,severely blemished fruit, reduced fruit quality and premature fruit drop. In the current study, the effects of D-aas on the morphology and toxicity of *X. citri* subsp. *citri* strain 101 (*Xcc101*) were investigated. This research provides a basis for the exploration of the antibacterial mechanisms of D-aas and presents a new strategy for the prevention and control of plant diseases.

MATERIALS AND METHODS

Strain and culture conditions

A bacterial strain was isolated from citrus canker diseased leaves of trees growing at Hepu Citrus Farm, Guangxi Province, China, and its morphological, biochemical and physiological characteristics, together with its 16S RNA sequence were investigated. The strain was identified as *Xanthomonas citri* subsp. *citri*, and was named *X. citri* subsp. *citri* strain 101 (*Xcc101*). The strain was stored at the Genetic Engineering Research Centre, School of Life Science, Chongqing University. Nutrient agar (NA) and Luria bertani(LB) were used as the growth media for *X. citri* subsp. *citri*. The bacterium was initially streaked from –80°C glycerol stock on an NA plate and a fresh single colony was inoculated into LB medium (30 mL) in 150 mL flasks and cultured at 28°C with agitation at 200 rpm. The overnight cultures were further diluted in LB to standardize the cultures to obtain an optical density at 600 nm (OD_{600}) of 1.0 prior to setting up the MIC (minimum inhibitory concentration) assay and recording the cell growth measurements.

Preparation of test D-aas

The D-amino acids (D-phenylalanine, D-leucine, D-valine) and L-amino acids (L-phenylalanine, L-leucine, L-valine) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Stock solutions (500 mM D-amino acids in sterilized distilled water [SDW]) were stored at –20°C and diluted in SDW to create the initial test concentrations [1-30 mMol/l]. A LIVE/DEAD BacLight Bacterial Viability Kit (L7012) was purchased from *Invitrogen* (Carlsbad, CA, *USA*). The other assay chemicals were obtained from Dingguo-Biotechnology. Co. Ltd. (Beijing, China).

Evaluation of D-amino acids and L-amino acids for potential inhibition ability

X. citri subsp. *citri* strain 101 was grown in LB at 28°C with agitation at 200 rpm for 8 h. The cultures were standardized to an OD_{600} of 0.03 (5×10^7 CFU/mL) in LB and then poured into the wells of a 96-well assay plate. Each cell contained 190 µl of liquid culture. The initial test concentrations of the D-aas and L-aas were diluted (1:20) in the culture (10 µl of compound in 190 µl of culture) and incubated at 28°C without agitation. The cultures were monitored at 24 and 48 h at OD_{600}, and the lowest concentration resulting in no growth after 48 h was compared with the control samples and defined as the MIC for *X. citri* subsp. *citri* strain 101. All determinations were conducted in eight replicate wells and repeated three times.

Changes in the cell morphology of *Xcc101*

A change in the cell morphology of *Xcc101* after D-Leu treatment was observed by light microscopy. The cultured *X. citri* subsp. *citri*strain 101 in LB was diluted using LB media to approximately 1×10^7 CFU/mL. The bacteria (1×10^7 CFU/mL) were treated with various concentrations [1-30 mMol/l] of D-leu at 28°C (100 rpm, 24 h). After culturing for a certain time (0.1-48 h), the *Xcc101* was stained using Giemsa and observed by light microscopy.

Bactericidal effect of D-leu

The bactericidal effect of D-leu was determined by confocal laser scanning microscopy. The *Xcc101* cultured in LB was diluted using LB media to approximately 1×10^7 CFU/mL. The bacteria were then treated with various concentrations [1-30 mMol/l] of D-leu at 28°C under

aerobic conditions. After 24 h incubation, the bacteria were washed with PBS(Phosphate buffered saline,Ph 7.2) and stained for 15 min using the LIVE/DEAD BacLight Bacterial Viability Kit (L7012, *Invitrogen, USA*), which was prepared according to manufacturer's instructions. The stained bacteria were observed by confocal laser scanning microscopy (LSM 510, *Zeiss*, Germany). This method is based on two nucleic acid stains: a green fluorescent SYTO 9 stain and a red fluorescent propidium iodide stain, which differ in their ability to penetrate healthy bacterial cells. The SYTO 9 stain labels live bacteria, whereas propidium iodide penetrates only bacteria with damaged membranes.

Bacterial virulence test

Immature leaves of young (about 10-week-old) potted grapefruit (summer orange) were prepared in a quarantine greenhouse at the Genetic Engineering Research Centre, School of Life Science, Chongqing University. The LB cultured *Xcc101* was diluted using LB media to approximately 1×10^7 CFU/mL. The bacteria (1×10^7 CFU/mL) were treated with various concentrations of D-leu (1–10mM) at 28°C with 100 rpm agitation for 24 h. The bacteria were then collected (1000 x g, 5 min) and were re-suspended in sterile tap water (1×10^5 CFU/mL). A bacterial suspension (1×10^5 CFU/mL) was injected into the intercellular spaces of leaves with a needleless syringe [27, 32]. Petioles of the treated leaves were immersed in sterile water, placed in Petri dishes and cultured at 28°C for 15 days. Twenty immature leaves at a similar developmental stage were selected from each plant. *Xcc101* medium without D-leu was used as the control. Sterile water was used in the blank control group. Disease symptoms were photographed at 15 days post inoculation. All the tests were independently repeated three times.

Plant testing in the laboratory

In order to validate the effect of D-Leu in the control of CBCD, *in vitro* Citrus leaves (about 10-weeks-old) were divided into two groups with D-Leu treatment applied either prior to, or subsequent to, pathogen infection. Treatment group 1: liquid medium containing the *Xanthomonas* pathogen (1×10^7 CFU/mL) was sprayed onto the back of the leaves. After *Xcc101* infectionat 24 h, 48 h or 72 h, different concentrations of D-Leu (7 mM, 10 mM, 13 mM) were also sprayed onto the back of the leaves. Treatment group 2: The leaves were similarly treated with different concentrations of D-Leu (7–13mM) and 24 h, 48 h and 72 h later were infected with *Xcc101* (1×10^7 CFU/mL). In addition, a control group of citrus leaves was also created which were infected with citrus canker bacteria *Xcc101* (1×10^7 CFU/mL) and 24 h, 48 h and 72 h later were treated with sterile water. All treated leaves were cultured at 28°C under humid conditions. The disease incidence rate of the leaves and the number of lesions on each leaf were recorded 15 days post-treatment.Twenty immature leaves at a similar developmental stage were selected from each plant.The assays were independently repeated three times.

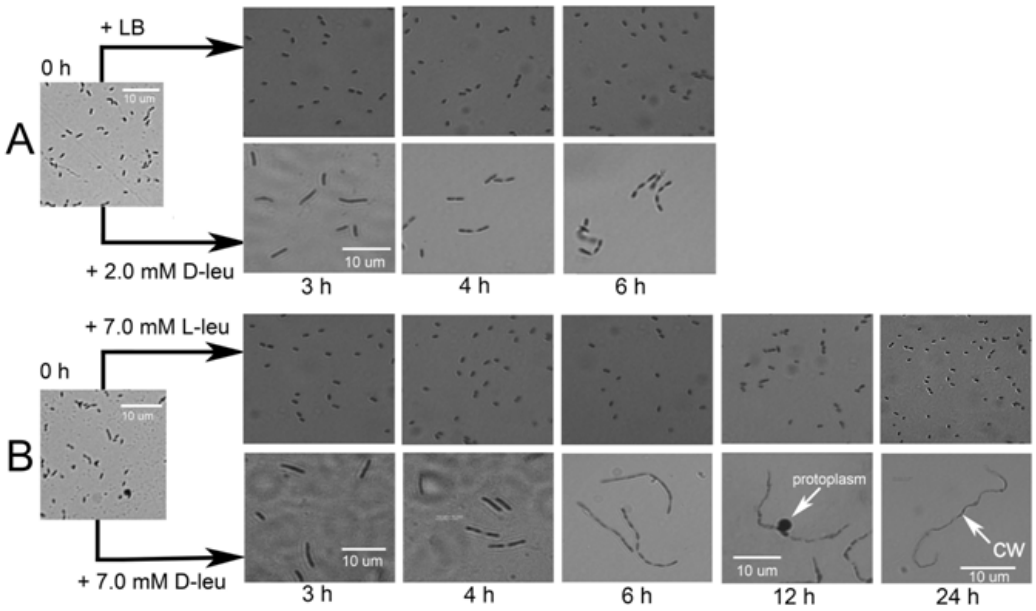
Amino acid assays

Xcc101 was grown in LB at 28°C with agitation at 200 rpm for 12 h. The cultures were standardized to OD_{600} = 0.4 in LB and then D-leu that had been dissolved in ddH_2O and filtered through a 0.22 µm pinhole filter was added to 7 mM . Only LB liquid medium was added to the control group. The cells were cultured at 28°C for 24 h and agitated at 100 rpm. The control and treatment groups were collected after culturing for 3 h and 24 h, respectively. The bacteria were collected by centrifuging the solution at 5000 x g for 5 min at 4°C. The peptidoglycan of the cell wall was extracted and the record method was used for the analysis[14]. In order to preliminarily explain the bactericidal mechanism of D-aas, the abundance of 20 different amino acids in the peptidoglycan from *Xcc 101* were measured and any amino acids that showed significant changes were selected.

RESULTS

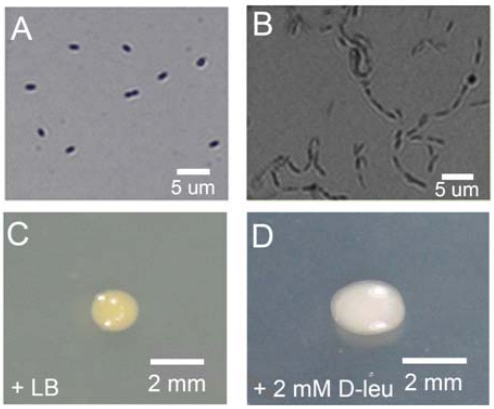
Determination of MICs

The MICs were examined using a 96-well plate assay at 28°C under stationary conditions as described in the Materials and Methods. The MIC was defined as the lowest concentration resulting in no bacterial growth measured at an optical density of 600 nm after 48 h incubation when compared with the control samples. The determinations were repeated three times using eight replicate wells per run. The D-Val MIC for *Xcc101* was highest at 18 mM, followed by D-



(A): Effect of LB and 2mM D-leu on *Xcc 101* cell morphology
+LB: The bacteria of *Xcc101* with the length of 1.5 µm did not change within 6 h. +2mM D-Leu: The length of bacteria increased to 3~7.5 µm within 3 h. The cell length of *Xcc 101* was 1.5 µm, at the 4th hour. The bacteria became chain-like in 6h.
(B): Effect of 7mM L-leu and 7mM D-leu on *Xcc 101* cell morphology
+7mM L-Leu: *Xcc101* with the length of 1.5 µm did not change within 24 h. +7mM D-Leu: The *Xcc101* cell lengths increased to 3~7.5 µm between 0–3 h. *Xcc101* became chain-like in 4h to 11h. Protoplasm of *Xcc101* formed globular structure after protruding from thecell wall (CW), and the protoplasm overflowed in 12 h (As shown by an arrow in Fig.1B.12h). All protoplasm flew out and only filamentous long hollow cell wall left at 24 h.

Fig. 1. Effect of D-Leu on *Xcc101* cell morphology



Colonies in the *Xcc101* + LB control group (Fig.2A and C) did not vary. In the *Xcc 101* + 2.0 mM D-Leu group, chains containing more than 30 single cells (Fig.2B) of a slightly lighter color formed (Fig.2D).

Fig. 2. Effect of D-Leu on *Xcc101* colony morphology

Phe at 15 mM and D-Leu at 7 mM. In contrast, 50 mM L-Leu, L-Phe, and L-Val had no impact on the growth or morphology of *Xcc101*. These results show that D-aas can inhibit *Xcc101* growth. Therefore D-leu was selected for further studies, due to its high toxicity to *Xcc101*.

Effect of D-leu on *Xcc101* cell morphology

Xcc101 treated with 2 mM D-Leu for 0-3 h stopped dividing, and their length increased to 3 to 7.5 µm, approximately equal to between two and five times the length of the control group (Fig. 1A. 3 h). The larger cells began to divide after 4 h, but many *Xcc 101* cells shared a cell wall. The cell length of single bacteria returned to 1.5 µm (Fig. 1A. 4 h). After 6 h, chain-like colonies containing 5 to 10 or more single bacteria cells of *Xcc101* formed (Fig. 1A. 6 h). In the solid medium, chain-like colonies consisting of more than 30 bacteria were often observed (Fig. 2.B). Under such concentrations of D-Leu, the bacteria did not enter the protoplasmic

expansion and the cell wall phases. The chain-like cell colonies did not change except to become a slightly lighter color (Fig. 2.D).The group, which was cultured in the conventional LB liquid medium at 28°C with agitation at 100 rpm for 24 h, had short rod shaped cells, approximately 1.5 µm long, implying that cellular morphology did not change during each experimental period.

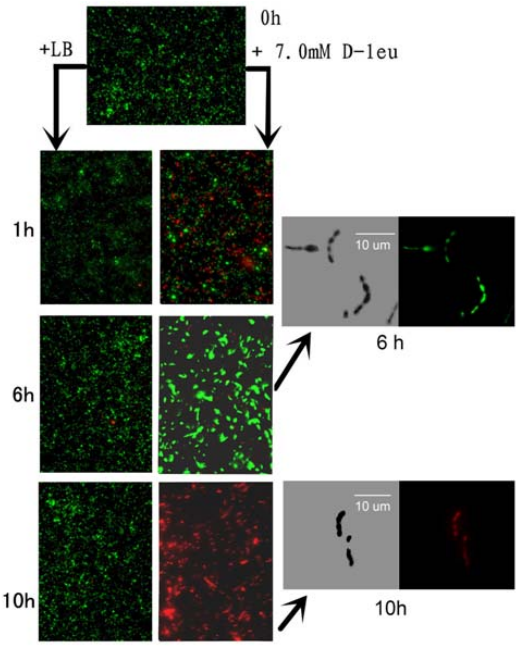
However, when the D-Leu concentration in the LB liquid medium increased to 7 mM, the morphology of *Xcc101* changed. Four distinct morphological periods existed (Fig. 1B):

Bacteria size increase phase

The *Xcc101* bacteria stopped dividing during the first 3 h of incubation in 7 mM D-Leu. The lengths of the cells ranged from 3 to 7.5 µm, which was about two to five times longer than the length of the control group (Fig. 1B. 3 h). *Xcc101*mobility began to decrease or stop;

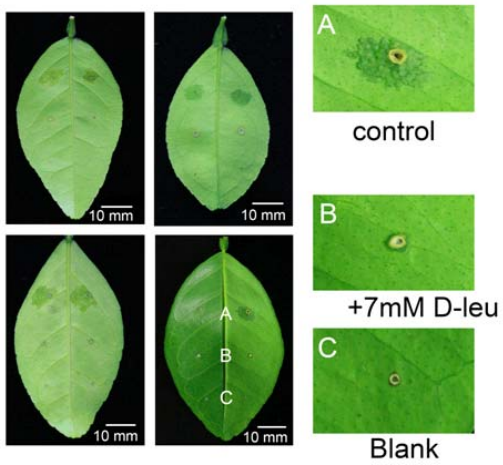
Chain-like bacteria phase

The protoplasm of *Xcc101* bacterial cells began to divide from 3 to 12 h (Fig. 1B. 4 h). However, the cells did not separate from the cell wall, but formed chains that often contained more than 10 to 30 single cells (liquid LB at 100 rpm). At this time, the *Xcc101* cell length returned to 1.5 µm. During this phase, the morphology of the end-to-end cells of *Xcc101*, which shared one cell wall, appeared as chain-like bacilli, with a total length 20 times that of the normal bacteria (Fig. 1B.6 h). However, the cells in the chain lost their mobility; 3) Protoplasmic expansion phase: The protoplasms of the chain-like bacteria cells of*Xcc101* began to expand from 12 h to 24 h. The expanded protoplast began to squeeze the cell wall, leading to the rupture of the cell wall. Then, the protoplasm formed



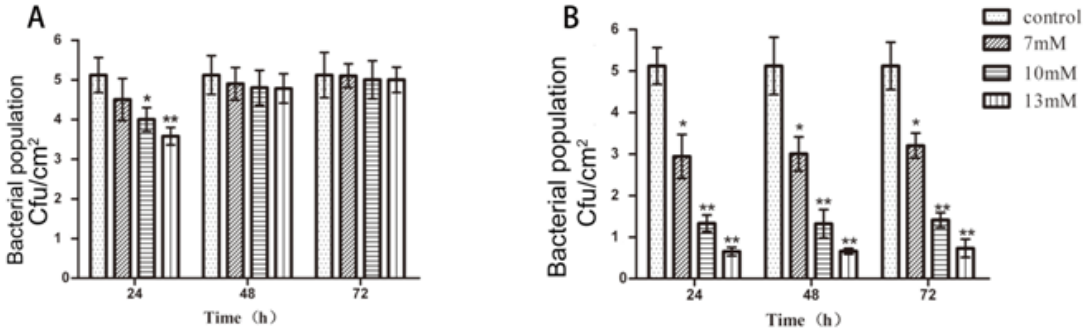
+LB: *Xcc101* was always short rod morph with a length of 1.5 µm after LB was added to. The morphology did not change in each period. The bacteria always showed green-labeled of strong activity in 1h to 10h.The group treated by D-leu 7mM: A lot of red-labeled dead bacteria in 1 h. The bacteria changed into chain-like at the 6th hour, and the bacteria showed green-labeled of strong activity. At the 10th hour, a lot of red-labeled dead bacteria emerged.

Fig. 3. Changes in the activity of *Xcc101* after D-Leu treatment



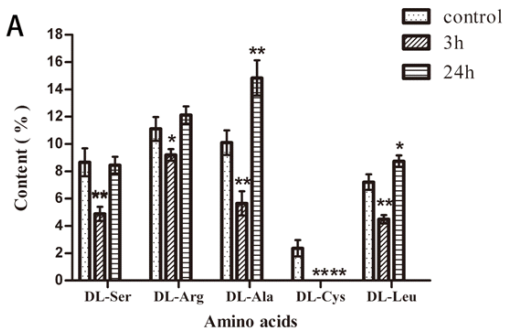
(A) Control: citrus leaves showed characteristics of citrus canker disease. (B) +7mM Leu: did not show the classical characteristics besides slight water soaked spots. (C) Blank: the leaves treated by water did not change. Images are representative of 4 independent replicates at 15 day.

Fig. 4. Changes in the pathogenicity of *Xcc101* after D-Leu treatment



(A) Leaves inoculated with *Xcc101* and then treated with D-leu. The average lesion numbers were 4.5, 4.0 and 3.58 CFU/cm^2 after being inoculated with *Xcc101* for 24 h and sprayed with 7, 10 and 13 mM D-leu, respectively. The average lesion numbers were 4.9, 4.8 and 4.75 CFU/cm^2 after inoculation with *Xcc101* for 48 h and sprayed with 7, 10 and 13 mM D-leu, respectively. The average lesion numbers were 5.1, 5.0 and 5.0 CFU/cm^{2}after inoculation with *Xcc101* for 72 h and sprayed with 7, 10 and 13mM D-leu, respectively,
(B) The average lesion numbers were 2.94, 1.33 and 0.65 CFU/cm^2 after being sprayed with 7, 10 and 13mM D-leu, respectively, for 24 h and inoculated with *Xcc101*, The average lesion numbers were 3.0, 1.33 and 0.66 CFU/cm^2 after being sprayed with 7, 10 and 13mM D-leu, respectively, for 48 h and inoculated with *Xcc101*, The average lesion numbers were 3.2, 1.41 and 0.73 CFU/cm^2 after being sprayed with 7, 10 and 13mM D-leu, respectively, for 48 h and inoculated with *Xcc101*.,P < 0.05(*) or P < 0.01(**).

Fig. 5. Preventive effects of D-Leu on citrus bacterial canker disease (CBCD) under laboratory conditions



Control: Ser: 8.66%, Arg: 11.11%, Ala: 10.10%, Cys: 2.36%, Leu: 7.21%.
Treated by 7mM D-leu after 3 h: Ser: 4.88%, Arg: 9.20%, Ala: 5.65%, Cys: 0%, Leu: 4.49%.Treated by 7mM D-leu after 24 h: Ser: 8.45%, Arg: 12.12%, Ala: 14.84%, Cys: 0%, Leu: 8.74%.P<0.05(*) or P<0.01(**)

Fig. 6. Changes in the *Xcc101* peptidoglycan's DL-amino acid content after D-Leu treatment

globular structures protruding from the cell wall (Fig. 1B. 12 h). Finally, the protoplasm overflowed, and the spheroplasts collapsed;

Cell wall phase

After being treated with D-Leu for 24 h, the protoplasts in the filamentous bacteria were expelled and only the long hollow filamentous cell wall remained (Fig. 1B. 24 h).

Changes in the cell activity of *Xcc101* bacteria treated with D-Leu

The 2 mM D-leu treatment did not cause *Xcc101* death. Therefore, we stained the different culturing stages of *Xcc101* treated with 7 mM D-Leu using the LIVE/DEAD BacLight Bacterial Viability Kit. After being treated for 1 h, a large number of red-labeled dead bacteria were observed illustrating the acute death of *Xcc101* (Fig. 3. 1 h). After being treated for 6 h, the bacteria turned into strong active chains that were green-labeled under fluorescence microscopy (Fig. 3.6h). After 10 h treatment, the bacteria protoplasm did not expand. Large amounts of red-labeled dead bacteria were observed, indicating that the bacterial morphology had not changed, but that the cell membrane was damaged (Fig. 3.10h). Nearly all the bacteria observed in the control group that was not treated by D-leu fluoresced a vibrant green at each culture phase.

Changes in the pathogenic ability of *Xcc101* after D-Leu treatment

Leaves of host summer orange were inoculated in vitro with 10 µl of *Xcc101* (1× 10^{5}CFU/mL) treated with 7 mM D-Leu for 24 h. The infected oranges were stored at 28°C for 15 days. The control group showed volcano and water soaked spots,

which are the classical characteristics of citrus canker disease (Fig. 4A). The treatment group did not show the classical characteristics besides slight water soaked spots (Fig. 4B), implying that *Xcc101* treated by D-Leu had lost its pathogenicity. Leaves of the water control group did not demonstrate any changes besides the pinprick (Fig. 4C).

Preventive effects of D-Leu on CBCD under laboratory conditions

In the control group 15 days after the leaves were infected by the pathogenic bacteria*Xcc101*, the incidence rate in the citrus leaves was 100% and the average lesion on leaf reached 5.12 CFU/cm^2 (Fig.5. Control, A and B). For treatment group 1, treated with D-Leu 24h after infection with citrus canker pathogen, the average lesion on leaf 15 days post-infection reached 3.58 CFU/cm^2, 30% less than that of the control group, indicating that D-Leu has a significant preventive effect on CBCD. Moreover, higher concentrations of D-Leu showed a greater preventive effect. The average lesion 15 days post-infection on leaves treated with D-Leu 48h after *Xcc101* infection, was also less than that on each leaf in the control group, but this difference was not significant. This implies that D-Leu has some therapeutic effect on the primary infection of CBCD, but that the preventive effect declines as the infection of leaves becomes more severe (Fig. 5A). For treatment group 2, treated with D-Leu prior to infection, the average number of lesions on the leaves was significantly lower than that of the control group, and was negatively correlated with D-Leu concentration. The difference between lesion numbers on leaves treated with D-Leu 24 h, 48 h or 72 h prior to *Xcc101* infection, were less than 10%. For the group of leaves treated with 13 mM D-Leu for 24h and then inoculated with *Xcc101*, the lesion number (0.65 CFU/cm^2) was the least, 87.3% less than that of the control group (Fig. 5B). D-leu reduced canker symptom development on Spring Orange grapefruit leaves that had been spray-inoculated with *Xcc101*.

Changes in the amino acid constituents of peptidoglycan in *Xcc101*

Within the peptidoglycan extracted from the *Xcc101*, the amount of Arg, Ser, Leu, Ala, and Cys changed significantly (over 17%) following D-Leu treatment, whilst the concentration of other amino acids did not change significantly (less than 5%), in comparison with the control group. The content ratios of Arg, Ser, Ala, Leu and Cys for 3 h declined. Interestingly, the content of Cys was reduced from 2% to 0%. By 24 h, the content of Arg, Ser and Leu had increased to their original level, while Cys remained at 0% (Fig. 6). T-test,$P<0.05$(*) or $P<0.01$(**)

DISCUSSION

The addition of D-Cys could cause the abnormal division of the Gram$^-$ bacteria *E. coli*, resulting in a single huge cell but not in the formation of long chains of single cells. With a high concentration (above 36 mM), D-aas could also lead to the death of *E. coli* [26]. D-aas play many important roles in bacteria [5, 15]. The use of D-amino acids is a recent strategy for combating biofilms. Some studies have indicated that D-amino acids can inhibit biofilm formation and disperse existing biofilms[16, 20, 28]. However, low concentrations of D-aas did not change the morphology of Gram$^+$ bacteria or lead to the death of harmful bacteria, such as *Bacillus* or *Staphylococcus aureus*[11].

This study is the first to report the effects of D-amino acids on the morphological characteristics of *X.* subsp. *citri* and its potential lethal effects. Based on the estimated cost of the application, we only screened D-val, D-phe, D-leu in the experiments. Our study revealed that the plant pathogenic bacteria *Xcc 101* is very sensitive to D-Leu. At a low concentration of D-Leu (2 mM), *Xcc 101* cells became enlarged and formed chain-likestrings of cells. When treated with 7 mM D-Leu for 24 h, the morphology of *Xcc101*changed and they eventually died. Similar results were also obtained using the pathogen *Pseudomonas syringae* pv. *actinidia* (unpublished results).Therefore, screening biocontrol strains (e.g. *B. subtilis*) that can secrete large amounts of D-leu (above 2mM) could lead to useful products that can control plant diseases.

An amino acid analysis showed that several main amino acids in bacterial peptidoglycan first declined, and then rose after the D-aa treatment. However, the total amino acid contents declined, indicating that simple replacement of the

original D-aa by another D-aa may not occur. Disulfide bonds in Cys play a very important role in stabilizing protein structures and increasing the elasticity of the cell wall. The Cys content in the cell wall's peptidoglycan of *Xcc101* after treatment with D-Leu decreased to undetectable levels, implying that the lethal effect of D-aas on *Xcc101* is related to the inhibition of Cys synthesis and the transformation of the peptidoglycan's amino acid contents.

The addition of 7 mM D-Leu could lead to significant morphological changes in the Gram$^-$ plant pathogenic *Xcc101* and eventually death. A variety of other D-aas may also have similar antibacterial effects on other plant pathogenic bacteria. Our results show that, under laboratory conditions, D-Leu has a significant effect on the control of CBCD. Consequently, we suggest that spraying D-Leu directly onto the plant would be an effective method for destroying harmful bacteria. Alternatively or additionally, the addition of D-Leu to botanical fungicides or solvents containing biocontrol strains would enhance their sterilizing effect. Since D-aas are non-toxic and stable, they are good candidates for the inhibition or destruction of plant pathogenic bacteria. However, whether D-Leu can effectively control harmful bacteria in the field requires further study.

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