Effects of Gallic Acid on the Cell Structure of *Xanthomonas oryzae* pv. *oryzicola* (Postprint)

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Date: 2018-10-26T00:00:00+00:00

Abstract

没食子酸（GA）是一种酚类化合物，在植物中具有多种生物活性。我们前期的实验表明，GA 对水稻细菌性条斑病菌（Xoc）具有相对较强的抑制作用。为阐明 GA 对该病原菌细胞结构及膜通透性的影响，采用电子显微镜观察 GA 处理后的细菌形态，并通过测定细胞释放的 260 nm 吸收物质、二乙酸荧光素（FDA）染色细胞荧光变化以及乳酸脱氢酶（LDH）活性来研究 Xoc 细胞膜的完整性与通透性。经 200 g·mL^{-1} GA 处理后，电子显微镜下观察到细胞表面出现大量凹陷和不规则囊泡，表明 GA 可损伤 Xoc 的细胞壁。GA 处理 24 h 后，Xoc 悬液的电导率达 135.48 S·cm^{-1}（对照为 127.85 S·cm^{-1}），处理 2 h 后 Xoc 悬液的荧光强度下降 58.10%，表明细胞发生电解质及细胞质内容物的泄漏。同时，GA 处理的细菌悬液中 LDH 活性亦有所增加，提示 GA 可破坏细菌细胞膜结构。此外，Xoc 悬液在 260 nm 处的吸光度为 1.004（对照为 0.018），表明 GA 对 Xoc 细胞壁完整性具有不利影响。这些结果表明，GA 不仅改变了 Xoc 细胞膜的通透性，还损害了细胞膜的完整性。

Full Text

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Abstract

Gallic acid (GA) is a phenolic compound that presents various biological activities in plants. Our previous experiments demonstrated a relatively strong inhibitory effect of GA on *Xanthomonas oryzae* pv. *oryzicola* (Xoc). To elucidate the effects of GA on the cell structure and membrane permeability of the pathogen, we observed morphologies of GA-treated bacteria by electron microscopy and investigated membrane integrity and permeability by determining

the release of materials absorbing at 260 nm, changes in fluorescence of cells treated with fluorescein diacetate (FDA), and lactate dehydrogenase (LDH) activity. Treatment with 200 g · mL 1 GA resulted in many pits and irregular vesicles on the cell surface under electron microscopy, indicating that GA could damage the cell walls of Xoc. The electrical conductivity of Xoc suspensions 24 h after GA treatment was 135.48 S · cm 1 (control was 127.85 S · cm 1), and the fluorescence intensity of Xoc suspensions decreased by 58.10% after 2 h of GA treatment, indicating that cells leaked electrolytes and cytosolic contents. Meanwhile, the activities of LDH in bacterial suspensions treated with GA also increased, suggesting that GA could damage the structure of the bacterial cell membrane. In addition, the absorbance at 260 nm from Xoc suspensions was 1.004 (control was 0.018), indicating that GA could negatively affect Xoc cell wall integrity. These results indicate that GA not only altered the permeability of the cell membrane of Xoc, but also impacted the integrity of the cell membrane.

Keywords: *Xanthomonas oryzae* pv. *oryzicola*, gallic acid, cell structure, membrane permeability, antibacterial mechanism

Funding: Supported by Natural Science Foundation of Guangxi (2014GXNSFAA118073)

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Bacterial leaf streak of rice, which is caused by *Xanthomonas oryzae* pv. *oryzicola* (Xoc), is one of the important plant diseases in South China. This disease was first reported in Philippines in 1918 and was reported in China in 1953. Bacterial leaf streak of rice can reduce yield loss by 40%-60%, and the disease severely threatens the high and stable yield production of rice. Furthermore, the transportation of rice seeds during production tends to expand and aggravate this disease [**?**, **?**]. Because bacterial leaf streak-resistant rice cultivars are not available, bactericides are mainly used to control the disease. However, few bactericides, such as zinc thiazole and thiodiazole copper, are registered in China [**?**, **?**]. Therefore, the development of new bactericides to manage this disease is desirable.

Gallic acid [GA: 3,4,5-trihydroxybenzoic acid (C H (OH) COOH)] is a phenolic compound in plants that exists as a free molecule and as a constituent of tannins [**?**, **?**]. Studies have demonstrated the antimicrobial activity of GA against *Salmonella typhimurium* [**?**, **?**], *Escherichia coli* [**?**, **?**], *Bacillus subtilis* [**?**, **?**], *Staphylococcus aureus* and *Candida albicans* [**?**, **?**]. GA may effectively inhibit the invasion ability of gastric cancer MGC-803 cells [**?**, **?**]. Our early work involving the screening of plants for antibacterial activity against Xoc showed

that GA extracted from *Sedum lineare* significantly inhibited the growth of plant pathogens such as Xoc, *X. oryzae* pv. *oryzae*, *X. campestris* pv. *pruni*, *X. axonopodis* pv. *citri*, *Ralstonia solanacearum*, *Pseudomonas syringae* pv. *glycinea*, *P. syringae* pv. *tomato* and *Pectobacterium carotovora* subsp. *carotovora* in vitro.

Reports on the antibacterial mechanisms of GA exist. GA damages the cell membrane of *Pseudomonas fluorescens*, which induced the leakage of intracellular electrolytes and abundant molecular material [**?**, **?**]. GA may potentially capture calcium ions from the calcium-binding proteins on the cell surfaces of *Campylobacter* by chelation, resulting in the loss of vital functions of those proteins and therefore cell death [**?**, **?**]. GA inhibits the proliferation of human hepatocellular carcinoma SMMC-7721 cells by inhibiting the expression of Survivin mRNA [**?**, **?**], inhibits the expression of MMP2 and MMP9 by regulating PI3K/AKT signaling pathway, and also effectively inhibits plankton SH FurnRi activity and biofilm formation by regulating the expression of MDOH gene and OPGH protein [**?**, **?**]. GA plays an antibacterial role by destroying the membrane integrity of *Aeromonas hydrophila* and *Aeromonas sobria* [**?**, **?**]. To our knowledge, no reports on the mechanisms of GA against Xoc have been published. In the present paper, we evaluated the minimum inhibitory concentration (MIC) of GA against Xoc and the effects of GA on the cell structure and membrane permeability of the pathogen. The results will provide a theoretical basis for further GA applications.

1.1 Materials

GA was obtained from the Tianjin Kemiou chemical reagent development center. An assay kit for determining the activity of lactate dehydrogenase (LDH) was obtained from Suzhou Comin Biotechnology Co., Ltd, China. Methanol, ethyl acetate, glutaraldehyde, iodoacetic acid, sodium phosphate, propanedioic acid, phenol, fluorescein diacetate (FDA), 2,4-dinitrophenylhydrazine, trichloroacetic acid, sodium carboxymethyl cellulose, sodium polypectinate, sodium hydroxide and absolute alcohol were of the highest grade commercially available.

1.2 Bacterial Strains and Growth Conditions

Xoc strain Xo-002 was isolated from an infected rice leaf in 2010 in Guangxi and was preserved at the Plant Pathology Research Institute of Guangxi University, Nanning, China. The strain was stored on nutrient agar (NA: 3 g of beef extract, 5 g of peptone, 10 g of dextrose, 17 g of agar, 1,000 mL of distilled water, pH 7.0) at -80 °C and was initially cultured on NA at 30 °C for 2 days, after which the strain was transferred into beef extract broth and shaken at 120 $r \cdot min^{1}$ at 28 °C for 24 h (during the logarithmic phase).

1.3 Antimicrobial Susceptibility Tests

Minimum inhibitory concentration (MIC) was tested using a 2-fold serial broth dilution as described here. Xoc at the logarithmic growth phase were diluted to a 10 colony-forming units (CFU) · mL ^{1} suspension in beef extract. GA dissolved in 10% methanol was added to the bacterial suspension to a final GA concentration ranging from 3.125 to 400 g · mL ^{1}. The bacterial suspension with only 10% methanol served as the control. The suspension was then shaken at 120 r · min ^{1} at 30 °C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent at which cell growth was not visible with the naked eye. Each treatment was replicated three times.

To determine the effects of initial bacterial density on GA inhibition, Xoc at the logarithmic growth phase were diluted to 10 CFU · mL ^{1}, 10 CFU · mL ^{1} or 10 CFU · mL ^{1} in beef extract suspension. GA was added to the bacterial suspension to reach final concentrations of MIC or 2 MIC. The suspension was then shaken at 120 r · min ^{1} at 30 °C for 24 h. Each treatment was replicated three times.

1.4 Electron Microscopy

Xoc at the logarithmic growth phase were diluted with beef extract to 10 CFU · mL ^{1}. GA (dissolved in 10% methanol) was added to the bacterial suspension to reach a final GA concentration of MIC. The bacterial suspension with only 10% methanol served as the control. After shaking at 120 r · min ^{1} and 28 °C for 6, 12 or 24 h, the suspension was centrifuged. The cells were washed three times with 0.2 M sodium phosphate buffer (PBS, pH 7.4) and then fixed with 2.5% glutaraldehyde in 0.2 M PBS. The samples were prepared for electron microscopy as previously described [**?**, **?**]. The prepared samples were examined using an electron microscope (H-500, Hitachi, Japan).

1.5 Electrical Conductivity Assay

Pathogens were diluted with beef extract to 10 CFU · mL ^{1}. GA was added to the bacterial suspension to reach final concentrations of 1/2 MIC, MIC or 2 MIC. The bacterial suspension with only 10% methanol served as the control. After shaking at 120 r · min ^{1} at 28 °C for 0, 1, 2, 4, 8 or 24 h, the suspension was centrifuged, after which the supernatant was diluted 20 times with water and measured for electrical conductivity. Each treatment was replicated three times.

1.6 Cell Membrane Integrity Assay

The cell membrane integrity of Xoc was evaluated by determining the release of materials that absorb at 260 nm. Bacteria at the logarithmic growth phase were washed three times with PBS and then diluted with PBS to 10 CFU · mL ^{1}. GA was added to the bacterial suspension to reach final concentrations

of 1/2 MIC, MIC or 2 MIC. The suspension with only 10% methanol served as the control. Each treatment was replicated three times. The suspension was shaken at 130 r · min ^{1} at 28 °C for 0, 2, 4, 8, 16 or 24 h, after which it was centrifuged at 4,000 g for 10 min. The release of materials that absorb at 260 nm was monitored over time using a UV-Visible spectrometer (Shimadzu UV-1800; Shimadzu Corp, Kyoto, Japan).

1.7 Outer Membrane (OM) Permeabilization Assay

The OM permeabilization activity of cells was determined using an FDA assay as described by Zeng et al. [**?**, **?**]. Xoc at the logarithmic growth phase were diluted with beef extract to 10 CFU · mL ^{1}. GA was added to the bacterial suspension to reach a final GA concentration of MIC. The suspension with only 10% methanol served as the control. After shaking at 130 r · min ^{1} at 28 °C for 2 h, the suspension was centrifuged. The cells were washed three times with PBS. FDA was then added to the bacterial suspension to reach a final concentration of 0.25 g · mL ^{1}, and the suspension was incubated at room temperature for 10 min. Fluorescence excited at a wavelength of 460 nm and an emission wavelength of 680 nm was recorded using an RF-5301PC fluorescence spectrophotometer (Shimadzu, Japan). All experiments were replicated three times.

1.8 Determination of LDH Activity in Bacterial Suspension

Xoc at the logarithmic growth phase were diluted with beef extract to 10 CFU · mL ^{1}. GA was added to the bacterial suspension to reach final concentrations of 100, 200 or 400 g · mL ^{1}. The bacterial suspension with only 10% methanol served as the control. After exposure to GA for 0, 2, 4, 8, 16 or 24 h, 2 L of culture was centrifuged at 1,000 × g for 5 min. The LDH activity in the supernatant was determined using an assay kit (Suzhou Comin Biotechnology Co., Ltd, China) according to the manufacturer' s instructions. All experiments were replicated three times.

The formation of 1 nmol of pyruvic acid upon the exposure of 1 mg of bacterial protein to extracellular matrix for 15 min was defined as 1 unit of activity in the reaction systems. Protein concentration was determined in accordance with the Coomassie brilliant blue staining method. A 3 mL Coomassie brilliant blue color reagent was added to 0.05 mL of distilled water, 0.05 mL of standard protein solution and 0.05 mL of sample liquid, representing a control tube, standard tube and testing tube, respectively, for 10 min. The tubes were then measured for optical density at 595 nm (OD) using a UV spectrophotometer, and protein content was calculated as follows:

Protein content (mg · mL ^{1}) = Standard tube concentration × (test tube OD - Standard tube) / (Standard tube OD -Standard tube)

1.9 Statistical Analysis

The data were subjected to analysis of variance using SAS software (version 6.08, SAS Institute, Cary, NC). Mean comparisons were conducted using a least significant difference (Fisher' s LSD) test at P = 0.05. The standard error and LSD results were recorded.

2.1 MIC of GA on Xoc

Xoc cultured for 24 h grew in NA containing 3.125 g • mL ^{1} to 100 g • mL ^{1} GA . However, the culture solution was transparent in NA supplemented with 200 g • mL ^{1} GA, which indicates that the MIC of GA was 200 g • mL ^{1}.

Table 1. MIC of GA on Xoc

Note: "+++" represents bacterial concentration > 10¹ CFU • mL ¹, "++" represents bacterial concentration ranging from 10 -10¹ CFU • mL ¹, "+" represents bacterial concentration < 10 CFU • mL ¹, "-" represents sterile growth. The same below.

The effects of initial bacterial density on GA inhibition are shown in Table 2 . Initial bacterial densities of 10 CFU • mL ^{1} and 10 CFU • mL ^{1} inhibited the growth of Xoc under conditions of 200 g • mL ^{1} and 400 g • mL ^{1} GA. However, an initial bacterial density of 10 CFU • mL ^{1} resulted in turbid culture media when treated with 200 g • mL ^{1} and 400 g • mL ^{1} GA, indicating Xoc growth.

Table 2. Effects of initial bacterial density on GA inhibition

2.2 Effects of GA on Xoc Cell Morphology

The effect of GA on the cellular structure of Xoc was visualized using transmission electron microscopy (Fig. 1 [Figure 1: see original paper]). Control cells cultured for 12 h had a full shape and smooth surface; the cell membrane adhered to the cell wall, and the cytoplasm was homogeneous (Fig. 1:A). However, GA-treated Xoc showed deformation and roughness of cell walls after 6 h and 12 h (Fig. 1:B,C). After 24 h, the cell outlines appeared blurry, the cell wall was loose, and some cells had an irregular shape and no membrane or cell wall on one side (Fig. 1:D).

Note: A. 12 h control with only 10% methanol (CK); B. 6 h after GA treatment; C. 12 h after GA treatment; D. 24 h after GA treatment (Bars = 1 m). The same below.

Scanning electron microscopy (SEM) showed that Xoc cells treated with GA at the MIC changed remarkably compared with control cells (Fig. 2 [Figure 2: see original paper]). The surface of control cells was smooth and cells were obvious after 12 h of culture (Fig. 2:A). However, granules and holes were observed on the surface of GA-treated cells after 6 h and 12 h (Fig. 2:B,C), and many pits and irregular vesicles were observed on the cell surface after 24 h (Fig. 2:D).

Therefore, GA could damage the cell wall of Xoc, and the effect was greater with longer treatment duration.

2.3 Effects of GA on Electrical Conductivity of Bacterial Culture Media

The effect of GA on the electrical conductivity of the culture solution is shown in Fig. 3 [Figure 3: see original paper]. Electrical conductivity increased with time. GA treatments at concentrations of 100 µg · mL ^{1}, 200 µg · mL ^{1}, and 400 µg · mL ^{1} and the control culture liquid resulted in electrical conductivities of 129.03, 132.80, 135.48 and 127.85 S · cm ^{1}, respectively, after 24 h of incubation. The result indicated that the higher the concentration of GA, the more serious the leakage of electrolytes from the cell.

Note: AA. 100 µg · mL ^{1}; BB. 200 µg · mL ^{1}; CC. 400 µg · mL ^{1}; CK. Only 10% methanol. Columns with different lowercase letters represent significant differences at 0.05. The same below.

2.4 Effects of GA on UV-Absorbing Compounds in Bacterial Culture Media

When antimicrobial agents damage bacterial membranes, intracellular components such as DNA and RNA tend to leak. The release of DNA and RNA from cells can be detected by UV at 260 nm as an indication of membrane damage [**?**, **?**]. The content of UV-absorbing compounds from Xoc suspensions treated with GA is shown in Fig. 4 [Figure 4: see original paper]. GA treatment concentrations altered the absorbance: the higher the GA concentration, the larger the absorbance. Treatment with 100, 200 and 400 g · mL ^{1} GA for 24 h resulted in absorbances of 0.195, 1.004 and 1.720, respectively, and the difference between GA treatment and the control (0.018) was statistically significant. This result indicated that GA could negatively affect Xoc cell wall integrity.

2.5 Effects of GA on Bacterial Permeability

FDA, which can enter the cell, is a nonfluorescent compound that is hydrolyzed to fluorescein and acetate by nonspecific esterases. The intracellular retention of fluorescein depends on the integrity of the cell membrane [**?**, **?**]. The change in fluorescence intensity after GA (200 g · mL ^{1}) treatment for 2 h is presented in Fig. 5 [Figure 5: see original paper]. Stained cells displayed the most intense fluorescence at 510 nm. The fluorescence intensities of 200 µg · mL ^{1} GA-treated and control cells were 53.06% and 91.33%, respectively. In addition, the fluorescence intensity of the treated cells decreased by 58.10%, and the cytosolic contents had leaked out from the cells.

2.6 Effects of GA on LDH Activity in Bacteria

The bacterial endoenzyme LDH could leak from bacteria upon cell membrane damage. Therefore, the activity of LDH in the bacterial suspension reflects the change in permeability of the cell membrane [**?**, **?**]. The activities of LDH in the bacterial suspensions are shown in Fig. 6 [Figure 6: see original paper]. The activities of LDH in bacterial suspensions treated with GA (100, 200 and 400 $\mu g \cdot mL^{1}$) and the control were less than 1.5 $U \cdot mg^{1}$ protein after treatment for 2 h; there was no significant difference between the GA treatment and the control. However, the enzymatic activities of GA-treated suspensions were higher than those of the control after 4 h. The enzymatic activities increased with GA dose and duration. The results suggested that GA could damage the structure of the bacterial cell membrane and cause the leakage of LDH enzymes.

3 Discussion

A concentration of 200 $g \cdot mL^{1}$ GA completely inhibited cells at 10 $CFU \cdot mL^{1}$; therefore, the MIC of GA on Xoc was 200 $g \cdot mL^{1}$ under these conditions. The antibacterial function of GA differed against different concentrations of bacteria. However, treatment with 200 and 400 $g \cdot mL^{1}$ GA at an initial bacterial density of 10 $CFU \cdot mL^{1}$ resulted in turbid culture media, indicating Xoc growth.

The cell wall maintains the inherent morphology of cells and facilitates substance exchange. When the cell wall of a bacterium has been disrupted by an antimicrobial substance, cell permeability is altered and materials can leak from the cell [**?**, **?**]. Electron micrographs showed that GA-treated Xoc displayed damaged cell walls with pits and irregular vesicles on the outer surface. A similar finding was reported by Helander et al. [**?**, **?**]. Furthermore, GA treatment resulted in higher culture liquid electrical conductivity than that of the control. Thus, cell wall damage is at least one mechanism by which Xoc was negatively affected by GA.

An essential function of the cell membrane is serving as a selectively permeable barrier. Exposure to high concentrations of antimicrobial agents can alter the permeability of bacterial cell membranes and hinder normal bacterial metabolism [**?**, **?**]. Our experimental results showed that GA enhanced the conductivity of the culture medium, which led to cytoplasm leakage and resulted in the leakage of intracellular DNA and RNA. These phenomena are similar to those reported for *Pseudomonas fluorescens* treated with GA [**?**, **?**], suggesting that GA could negatively affect Xoc cell wall integrity. Furthermore, the fluorescence intensity of cells treated with GA decreased to 58.10%, indicating that cytosolic contents had leaked out from cells. In addition, the activity of intracellular LDH in the bacterial suspension treated with GA was higher than that of the control, suggesting that LDH diffused outside the cell.

Our early work has demonstrated that GA could control rice bacterial leaf streak, with control efficacy of 64.54% in field conditions [**?**, **?**]. GA derivatives, such as methyl gallate, had antibacterial activity against *Ralstonia solanacearum*,

Pseudomonas syringae pv. *lachrymans* and *Pectobacterium carotovora* subsp. *carotovora* [**?**, **?**], and could effectively reduce the incidence of tomato bacterial wilt in the field [**?**, **?**]. Meanwhile, four gallic acid derivatives have also shown antibacterial activity against *Alternaria mali*, *Physalospora piricola*, *Rhizoctonia solani* and *Phytophthora infestans* [**?**, **?**]. Therefore, GA and its derivatives have potential to be developed as a new pesticide to control plant disease.

In conclusion, the results suggest that GA altered Xoc membrane permeability and disrupted membrane integrity. Increasing concentrations of GA resulted in more evident damage. The results showed that the cell membrane was a target for bacterial growth inhibition.

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Note: Figure translations are in progress. See original paper for figures.