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Oxalic acid produced by *Aspergillus niger* Y-1 is effective for suppression of bacterial fruit blotch of watermelon seedlings

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ABSTRACT

Bacterial fruit blotch (BFB) of watermelon caused by *Acidovorax citrulli* (Ac) is a seedborne disease. Seed treatment with bacterial disinfectants is considered as an important measure for suppression of Ac infection. This study was performed to detect the antibacterial activity of the cultural filtrate (CF) of the saprophytic fungus *Aspergillus niger* Y-1 against Ac. The six-day-old CF, citric acid (CA, 4 mmol/L) and oxalic acid (OA, 60 mmol/L) were determined for *in vitro* antibacterial activity against Ac. CF and OA were determined as seed disinfectants for suppression of seedborne infection by Ac. Results showed that production of CA and OA by *A. niger* in potato dextrose broth was consistently detected by HPLC. The CF, CA and OA inhibited growth of Ac and their inhibitory effect disappeared when the pH values of the three solutions was adjusted to 7.0. OA was more effective than CA in suppression of Ac. The potting experiment showed that both CF and OA applied on watermelon seeds effectively suppressed BFB incidence on seedlings. The efficacy was comparable to the seed treatment with HCl (1%, v/v). This study suggests that the CF of *A. niger* and OA can be used as seed disinfectants for elimination of seedborne Ac.

1. Introduction

Bacterial fruit blotch (BFB) caused by *Acidovorax citrulli* is a devastating disease on cucurbit crops, including watermelon (*Citrullus lanatus*) (Schaad et al., 2008; Burdman and Walcott, 2012). The disease was first found in Florida of USA in 1989 (Somodi et al., 1991). Since then, BFB has been spread worldwide and caused great economic losses for cucurbit fruit industries (Burdman and Walcott, 2012). The infected seeds represent the most primary inoculum source of BFB (Rane and Latin, 1992; Hopkins and Thompson, 2002). Seeds even with a low level of *A. citrulli* containment can result in severe BFB epidemics under the favorable environment (Dutta et al., 2012). Moreover, BFB can cause great economic losses for production of seedlings in commercial watermelon nurseries, where the environmental conditions (high temperature, high humidity) are favorable for infection by *A. citrulli* (Burdman and Walcott, 2012).

Nowadays, commercial cultivars with resistance to *A. citrulli* are not available in cucurbit crops (Bahar et al., 2009; Burdman and Walcott, 2012). Therefore, control of BFB mainly depends on use of *A. citrulli*-free seeds and on treatment of *A. citrulli*-contaminated seeds either with

(Fessehaie and Walcott, 2005). However, use of selected filamentous fungi or their metabolites to control BFB has not been reported so far.

Aspergillus niger is a filamentous ascomycetous fungus and a common saprophyte widely living in soil and plant debris. It can produce various organic acids, including citric acid, oxalic acid and gluconic acid (Yang et al., 2017). Previous studies showed that *A. niger* is an effective BCA for control of plant nematodes and oxalic acid was found to be the most toxic compound (Zuckerman et al., 1994; Jang et al., 2016). Whether or not *A. niger* and oxalic acid can suppress *A. citrulli* remains unknown. Therefore, a study was conducted to fulfill the following two objectives: (i) to detect the inhibitory effects of cultural filtrate (CF) of *A. niger* Y-1 and the organic acids (citric acid, oxalic acid) in the CF of *A. niger* against *A. citrulli*; and (ii) to determine the efficacy of the CF of *A. niger* and oxalic acid applied on seeds of watermelon in suppression of bacterial fruit blotch caused by seedborne *A. citrulli*.

2. Materials and methods

2.1. Microbial strains and cultural media

Strain Pslbtw36 of *Acidovorax citrulli* and strain Y-1 of *Aspergillus niger* were used in this study. Strain Pslbtw36 was kindly provided by Dr. T. C. Zhao of the Institute of Plant Protection in Chinese Academy of Agricultural Sciences (Beijing, China). Strain Y-1 of *A. niger* was isolated from a soil sample collected from a field growing with upland cotton (*Gossypium hirsutum*) in Hubei Province of China (Lu, 2010). The cultural media used in this study included King's B medium (KB), King's B agar medium (KBA), potato dextrose agar (PDA) and potato dextrose broth (PDB). KB contained (in 1000 mL water, pH 7.0) peptone 20 g, glycerol 15 mL, $K_2HPO_4{\cdot}3H_2O$ 1.5 g, $MgSO_4{\cdot}7H_2O$ 1.5 g. KBA contained all the ingredients appearing in KB and 1.5% agar (w/v). Both PDA and PDB were prepared with peeled potato tubers using the routine procedures.

2.2. Incubation of A. niger and preparation of the cultural filtrates

Strain Y-1 was incubated on PDA at 30 °C for five days. The conidia were harvested by washing with sterile distilled water (SDW) amended with Tween 80 (1%, v/v). The concentration of that conidial suspension was adjusted to 1×10^7 conidia/mL with SDW by a hemocytometer under the microscope. Aliquots of the conidial suspension were inoculated in 250-mL-Erlenmeyer flasks each containing 100 mL PDB, 1 mL conidial suspension per flask. The flasks were mounted on a rotary shaker and the cultures were incubated at 150 rpm under 20 °C for 1 to 7 days. Three flasks were randomly removed from the shaker at the 1-day-intervals. The culture of *A. niger* in each flask was filtered through a filter paper (9 cm diameter) (#9, Hangzhou Xinhua Paper-Manufacturing Co. Ltd., Hangzhou, China) to remove the mycelial masses, and the filtrate was further filtered through a 0.22-μm Sterile Filter Unit with Durapore® membrane (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, IRL) to remove the hyphal fragments in the CF. The pH value for the resulting cell-free cultural filtrate was measured using a pH meter (Starter 3C, Ohaus® Instrument, Shanghai, China) and stored at 4 °C. The experiment was repeated three times.

2.3. Incubation of A. citrulli and preparation of the bacterial suspension

Strain Pslbtw36 was cultured on KBA at 30 °C for 48 h. A single bacterial colony was selected and transferred to 100 mL KB medium in a 250-mL-Erlenmeyer flask, which was mounted on the shaker and shake-incubated (150 rpm) at 30 °C for 12 h. The resulting bacterial culture was centrifuged at 8000 rpm for 5 min to collect the bacterial pellet, which was re-suspended with SDW. The bacterial concentration in that suspension was estimated by spectrophotometry at 600 nm. The optical density (OD) value of the final bacterial suspension was adjusted to 0.3 (approximately 1×10^8 CFU/mL).

2.4. In vitro antibacterial assay of the cultural filtrates of A. niger

A time-course trial was done aiming at testing the antibacterial activity of the filtrates of the *A. niger* cultures after shake-incubation for 1–7 days. Aliquots of the bacterial suspension (1×10^8 CFU/mL) of *A. citrulli* were pipetted on Petri dishes (9 cm diameter) each containing 20 mL KBA, 400 μL bacterial suspension per dish. The bacterial suspension drop in each dish was evenly spread using a sterilized glass spatula. Three sterilized stainless-steel Oxford cups (10 × 6 × 8 mm, height × inner diameter × outer diameter) were placed on each *A. citrulli*-KBA dish. The CF of *A. niger* in each flask was pipetted into the three Oxford cups (as three replicates) in a dish, 200 μL CF per cup. In the control treatment, PDB was loaded in the three Oxford cups (200 μL PDB per cup) in a Petri dish. The cultures were incubated at 30 °C for 48 h and diameter of the clear zones around the Oxford cups (indicating the antibacterial activity) was measured. The experiment was repeated three times.

2.5. HPLC analysis of the organic acids in the cultural filtrates of A. niger

To determine the effective compounds in the CF of *A. niger* against *A. citrulli*, high performance liquid chromatography (HPLC) (Model: LC-20AT, Shimadzu, Japan) was applied to detect the organic acids in the CF of *A. niger*. The CF (20 μL per sample) was injected into the HPLC instrument with the procedures recommended by the manufacturer. The organic acids in the CF were separated by NaH_2PO_4 (0.01 mol/L, pH 2.8) plus 2% methyl alcohol (v/v), which was eluted at the rate of 1 mL/min in the high performance column (TC-C18, Agilent, USA) at 25 °C, and detected by the UV-spectrophotometer at 210 nm. Pure citric acid (3–25 mmol/L) and oxalic acid (7–25 mmol/L) from Sigma Chemical Company Limited (St. Louis, MO, USA) were used as standards for quantification of the two organic acids in the CF in the HPLC analysis.

2.6. Evaluation of the organic acids in cultural filtrates of A. niger against A. citrulli

Two trials, a pH-adjustment trial and a pure acid trial, were included in this assay. The pH-adjustment trial aimed at testing the effect of acidity of the *A. niger* CF on its antibacterial activity. The 6-day-old CF of *A. niger* (pH 1.6) were used in this trial. The pH value of CF was adjusted to 7.0 using 0.1 mol/L NaOH solution. Antibacterial activity of both the pH-adjusted CF (pH 7.0) and the original CF (pH 1.6, control) was determined on the *A. citrulli*-KBA dishes. The pure acid trial aimed at testing antibacterial activity of the pure citric acid (CA) and oxalic acid (OA). CA and OA were separately dissolved in SDW to reach the concentrations of 4 and 60 mmol/L, respectively (similar to the concentrations of CA and OA in the 6-day-old CF of *A. niger*). Half of each solution was adjusted to pH 7 using NaOH (0.1 mmol/L) and the other half of each solution was treated as the original solution. Antibacterial activity of the original CA (pH 2.8), neutralized CA (pH 7.0), original OA (pH 1.6) and neutralized OA (pH 7.0) was tested on the *A. citrulli*-KBA dishes using the procedures described above.

2.7. Watermelon seed germination assay

Seed germination assay was performed as described by Hopkins et al. (2003) with minor modifications. Seeds of watermelon (*Citrullus lanatus* cv. "Zao Jia 8424") (Ming Xin Ke Hong Seeds, Xingjiang, China) were soaked into SDW for 3 h, followed by air-drying overnight. The seeds were divided into five lots, 50 seeds per lot, for the five treatmen (or 7men (pH2%-348. 7.9702 0 0 7.9702 306.594 69.56749 Tm] TJvepe

treatments of control, *A. niger* CF alone and OA alone, three lots of seeds were soaked for 30 min in water alone, *A. niger* CF and OA solution, respectively. Then, the seeds for each treatment were filtered out, immediately wrapped up in four-layers of humid cotton gauze and incubated at 30 °C for 48 h for germination. Finally, the germinated seeds in each treatment were counted. For the treatments of CF plus water washing and OA plus water washing, two lots of seeds were soaked for 30 min in *A. niger* CF and OA solution, respectively. Then, the seeds for each treatment were filtered out and washed under running tap water for 5 min. Finally, the seeds were wrapped up in four-layers of humid cotton gauze and incubated at 30 °C for 48 h for testing seed germination. The experiment was repeated three times.

2.8. Evaluation of the efficacy of the cultural filtrates of A. niger and oxalic acid in suppression of seedborne infection by A. citrulli

Seeds (30 g) of watermelon (cv. "Zaojia 8424") were surface sterilized with 5% sodium hypochlorite (v/v) for 5 min, followed by washing under running tap water and then air-drying overnight under room temperature (20 ± 2 °C). They were soaked into 100 mL bacterial suspension of *A. citrulli* (1×10^8 CFU/mL) for 2 h and the bacterial cells were infiltrated into the seeds by vacuum using the procedures described in previous studies (Chalupowicz et al., 2015; Dutta et al., 2012, 2016). Then, the seeds were air-dried overnight under room temperature and divided into four lots (60 seeds per lot) for the treatments of control, CF, OA and HCl, respectively. The four seeds lots were soaked for 30 min in 100 mL SDW (control), 100 mL CF of *A. niger*, 100 mL OA solution (60 mmol/L) and 100 mL HCl solution (1%, v/v), respectively, followed by washing for 5 min under running tap water. The seeds for these treatments were separately wrapped up in humid cotton gauze and incubated at 30 °C for 48 h for germination. The germinated seeds were sown into Organic Culture Mix with the total content of $N + P_2O_5 + K_2O$ of 3% (Zhengjiang Peilei Organic Manure Manufacturing Co. Ltd., Zheng Jiang City, Jiangsu Province, China) in the plastic pots (9 × 9 cm, height × diameter), 3 seeds per pot, 20 pots for each treatment. The pots were maintained for 10 days in a growth chamber (day/night temperatures: 28 °C/18 °C; air relative humidity: 90%–99%; light regime: 12-h light/12-h dark) and root-watered as required. Diseased seedlings in the pots of each treatment were scored. The assay was conducted for three times.

2.9. Data analysis

The data on pH values, diameter of inhibition zones against *A. citrulli*, concentration of OA in the CF of *A. niger*, seed germination rate and disease incidence in the related experiments were analyzed using PROC ANOVA (analysis of variance) in the SAS software (SAS Institute, Cary, NC, USA, version 9.4). Data for the same treatment, but collected from the different repeats of the same experiment, were pooled when they were not significantly different ($P > 0.05$) in the F-test in ANOVA. Means of each parameter for different treatments in each experiment were separated by Fisher's Protected Least Significant Difference (LSD) test at $\alpha = 0.05$. Before analysis, the values of disease incidence (percentages) were arcsine-transformed into to angular values, which were back-transformed to percentage values after ANOVA.

3. Results

3.1. Antibacterial activity of the cultural filtrates of A. niger against A. citrulli

On *A. citrulli*-KBA cultures (30 °C, 48 h), no clear zones formed around the Oxford cups loaded either with fresh PDB or with the 1-day-old CF of *A. niger* (Fig. 1). In contrast, formation of clear zones (indication of inhibition against *A. citrulli*) was consistently observed around the Oxford cups loaded with the 2- to 7-day-old CF of *A. niger*

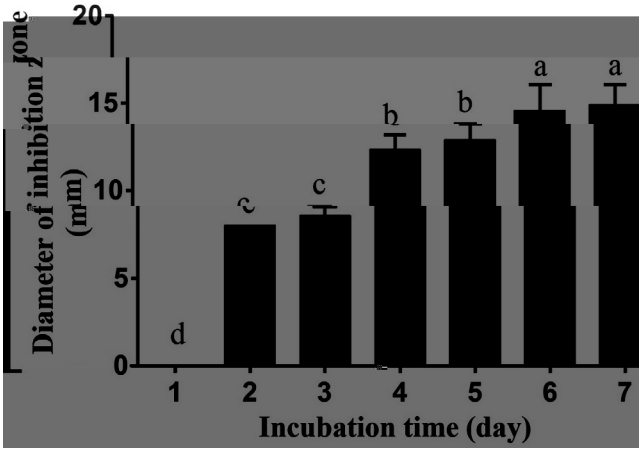

Fig. 1. Antibacterial activity of the cultural filtrates of *Aspergillus niger* Y-1 against *Acidovorax citrulli* (*in vitro* assay). Means with different letters indicated the significant difference between treatments by Fisher's LSD test. Bars indicate the standard deviations of means ($n = 9$) with three replicates in three independent assays.

with the average diameters ranging from 8 to 15 mm. Diameter of the clear zones (CZ) caused by the CF of *A. niger* was positively correlated with the incubation days (D): $CZ = 7.4527\text{Ln}(D) + 1.0664$ ($R^2 = 0.9623$, $P < 0.01$).

3.2. Antibacterial activity of oxalic acid produced by A. niger against A. citrulli

The initial pH value for the PDB medium used for incubation of *A. niger* was 5.9 on average. When *A. niger* was shake-incubated at 20 °C for 1–7 days, the resulting CF became more and more acidic. The average pH value for the CF of *A. niger* was consistently decreased to 5.4, 3.1, 2.6, 1.8, 1.7, 1.6 and 1.5 at 1, 2, 3, 4, 5, 6 and 7 days post-incubation (dpi), respectively (Fig. 2A). HPLC analysis identified two organic acids in the CF, namely oxalic acid (OA) and citric acid (CA) (Fig. S1). The concentration of OA in the CF of *A. niger* was consistently increased from 1.8, 4.0 and 10.2 mmol/L at 1, 2 and 3 dpi, respectively, followed by further increase to 43.1–62.5 mmol/L at 4–7 dpi (Fig. 2B). The concentration of CA was lower than 4.0 mmol/L in the 1- to 7-day-old CF of *A. niger* (Fig. 2B). The pH values (pH) for the CF of *A. niger* were negatively correlated with the OA concentration: $pH = 5.6331[OA]^{-0.3154}$ ($R^2 = 0.9572$, $P < 0.01$). However, the pH values for the CF of *A. niger* were not correlated at all with the CA concentration ($P > 0.05$).

On *A. citrulli*-KBA cultures (30 °C, 48 h), both CA (4 mmol/L, pH 2.8) and OA (60 mmol/L, pH 1.6) could inhibit growth of *A. citrulli* with formation of the clear zones at 8 and 14 mm in diameter, respectively (Fig. 3A, B). When the pH value of the two solutions was adjusted to 7.0, the resulting solutions failed to produce clear zones on the *A. citrulli*-KBA cultures (Fig. 3A, B). Meanwhile, when the pH value of the 6-day-old CF of *A. niger* was adjusted from 1.6 to 7.0, the resulting CF also did not produce clear zones (Fig. 3A, B).

3.3. Suppression of seedborne infection of A. citrulli by the cultural filtrates and oxalic acid of A. niger

In the seed germination assay (30 °C, 48 h), while the seeds in the control treatment (water) germinated by 93.8% (Fig. 4), the seeds in the treatments of the *A. niger* CF and OA (60 mmol/L) without water washing germinated by 26.7% and 28.2%, respectively (Fig. 4). When the seeds in the treatments of the *A. niger* CF and OA were water-washed, the germination rates were largely increased to 84.7% and 83.6%, respectively.

Results of the potting experiment showed that after incubation at

28 ˚C/18 ˚C (day/night temperatures) for 10 days, most seedlings in the treatment of *A. citrulli* alone became severely diseased with formation of water-soaked lesions on cotyledons and hypocotyls, and collapse of the whole seedlings (Fig. 5A). The disease incidence reached as high as 91.7% in this treatment (Fig. 5B). In the treatments of *A. citrulli* plus *A. niger* CF, *A. citrulli* plus OA, and *A. citrulli* plus HCl, however, most seedlings appeared healthy (Fig. 5A). The disease incidence values were

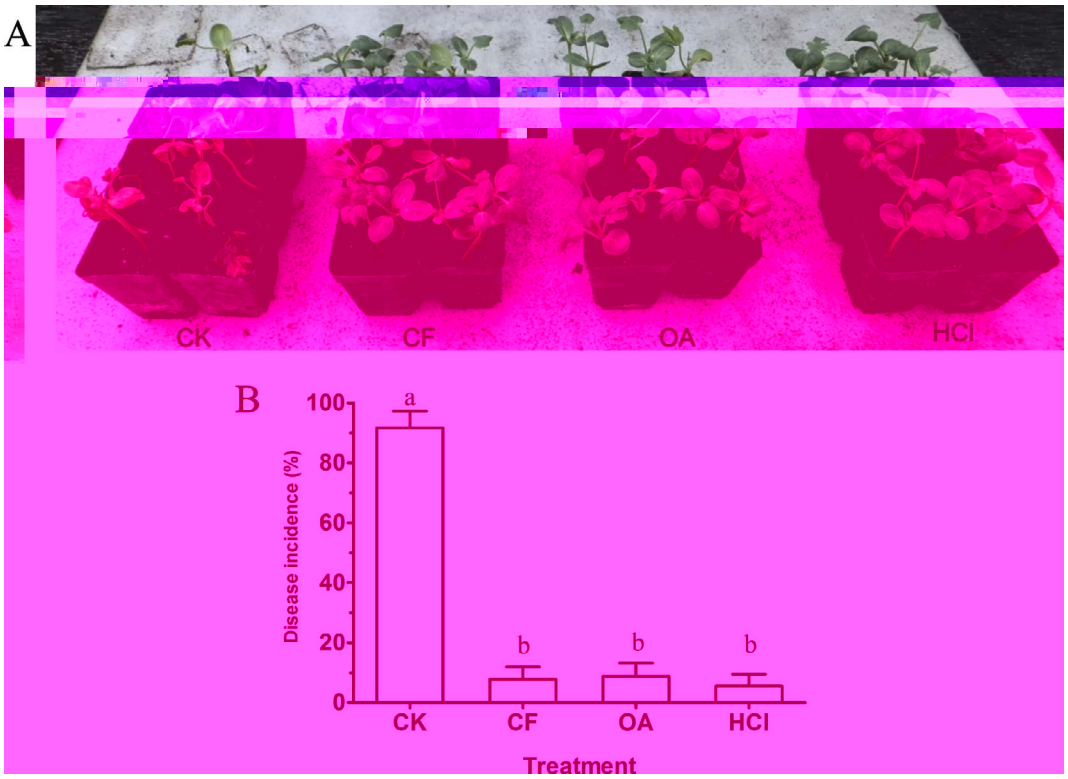

Fig. 5. Efficacy of the cultural filtrates of *Aspergillus niger* Y-1 (CF), oxalic acid (OA) and hydrochloric acid (HCl) in suppression of seedborne infection of by *Acidovorax citrulli.* (A) Watermelon seedlings for the treatments of control (CK), CF, OA and HCl showing difference in disease severity; (B) A histogram showing disease incidence values for the treatments of CK, CF, OA and HCl. Means with different letters indicated the significant difference ($P < 0.05$) among the treatments according to Fisher's LSD test. Bars indicate the standard deviations of the means ($n = 9$) from three independent assays.

There are two possible approaches for using *A. niger* Y-1 to control seedborne BFB caused by *A. citrulli*. First, the CF of *A. niger* Y-1 or pure OA can be used as disinfectants of watermelon seeds. The results of this study showed that this approach is practical. Second, *A. niger* Y-1 can be used amended into soil or seeding mix (substrate). It well recognized that *A. niger* is a saprophyte (Yang et al., 2017). It can colonize the soil or the seed mix for sowing watermelon seeds, and the colonization may produce the suppressive effect on infection of *A. citrulli*. Additional studies to validate the practicality of this approach are warranted.

In the seed germination assay of this study, both the CF of *A. niger* and OA showed inhibitory effect on germination of watermelon seeds. Therefore, both the CF and OA are toxic to watermelon seeds. This result is similar to that observed by Hopkins et al. (2003), who found that watermelon seeds treated HCl were inhibited for germination. Therefore, water washing is required for elimination of the toxicity of *A. niger* CF, OA and HCl before sowing.

The present study found that treatments of *A. citrulli*-contaminated seeds of watermelon with the CF of *A. niger* and OA (60 mmol/L) were effective in suppression of seedborne infection of watermelon seedlings by *A. citrulli*. We also found that the efficacy of the two treatments was comparable to that of the HCl treatment. Previous studies showed that the metabolites of *A. niger* and OA produced by *A. niger* have toxicity against plants such as oilseed rape (*Brassica napus*) (Kabbage et al., 2013.), and nematodes such as *Caenorhabditis elegans*, *Meloidogyne hapla*, *M. incognita*, and *Bursaphelenchus xylophilus* (Zuckerman et al., 1994; Jang et al., 2016). OA was found capable of inhibiting growth of the mycoparasitic fungus *Coniothyrium* (Wei et al., 2004). To our knowledge, this is the first report about the antibacterial activity of *A. niger* CF and OA against *A. citrulli*. The OA-containing cultural filtrate of *A. niger* and the pure OA have potential to be exploited as disinfectants of seeds of watermelon for elimination of seedborne infection by *A. citrulli*. Further studies on determination of the suppressive efficacy of the CF of *A. niger* Y-1 against *A. citrulli* in large scale commercial nurseries are warranted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2017.06.001.

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